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université de BORDEAUX

THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE Sciences de la Vie et de la Santé

SPÉCIALITÉ Neurosciences

Par Julie JEZEQUEL

Impact of psychotomimetic molecules on glutamatergic N-Methyl-D-Aspartate receptors surface trafficking

Sous la direction de Laurent GROC

Soutenue le 18 Novembre 2016

Membres du jury

M. OLIET Stéphane	Directeur de recherche CNRS	Président
Mme LENNOX Belinda	Professeur associé, Université d'Oxford	Rapporteur
M. PAOLETTI Pierre	Directeur de recherche INSERM	Rapporteur
Mme PERROY Julie	Directeur de recherche CNRS	Examinateur
Mme CONSTANTINE-PATON Martha	Professeur, MIT	Examinateur
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M. GROC Laurent	Directeur de recherche CNRS	Directeur

La culture de la bêtise

De ça oui je suis raciste

Stromae (Humain à l'eau)

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Pause. Qu'est-ce que c'est éprouvant d'écrire des remerciements… Trop de bons sentiments d'un coup !

On reprend. En écrivant tous ces mercis, je me rends compte à quel point j'ai toujours été bien entourée, et ça commence évidemment à la maison. Mais que dire à part que je suis plutôt chanceuse ~~pour une bouseuse~~. J'ai une famille de ouf, que voulez-vous. Papi, Mamie, Tata, Patrick, Cyndie, Jordan, Typhaine, et bien sûr ma petite sœur/maman Maëva, Vincent, Iléana, Papa & Maman, je ne sais pas quoi dire à part que je vous aime (même si je ne le dis pas assez souvent) et que je m'excuse pour ne pas avoir été aussi présente que je l'aurais voulu durant ces quelques années, mais j'espère que ça en valait la peine. Et la déclaration d'amour est bien évidemment valable pour la famille Geffroy qui est tout simplement une extension de ma famille : Lucie, Harry, petit Mahe, Maelle, Yannick, petit Hugo, Edith & Yannick :)

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Abstract

Glutamatergic N-Methyl-D-Aspartate receptors (NMDAR) play a key role in many physiological processes, and their implication in the pathophysiology of several neuropsychiatric disorders is now well established. Multiple lines of evidence converge towards a dysregulation of the NMDAR in psychotic disorders such as schizophrenia (SCZ). However, the molecular and cellular deficits underlying NMDAR dysfunction remain misunderstood. By tightly controlling NMDAR synaptic localization, surface trafficking represents a powerful regulator of synaptic transmission. *Could an alteration of NMDAR surface trafficking underlie NMDAR dysfunction and contribute to the emergence of psychotic disorders?* To tackle this question, my PhD project aimed at investigating the impact of different psychotomimetic molecules on NMDAR surface trafficking. In the first part of my project, I explored the impact of NMDAR autoantibodies (NMDAR-Ab) from SCZ and healthy subjects. My results revealed that NMDAR-Ab from SCZ patients rapidly disturb NMDAR synaptic trafficking and distribution, through a loss of NMDAR-EphrinB2 receptor interaction, eventually preventing the induction of synaptic plasticity. In the second part of my PhD project, I showed that psychotomimetic NMDAR antagonists also alter NMDAR synaptic mobility and localization. Downregulation of PSD proteins expression prevented NMDAR antagonists-induced deficits, suggesting that such alterations ensue from modifications of NMDAR intracellular interactions. Taken together, these results demonstrate that psychotomimetic molecules profoundly impact NMDAR surface trafficking, supporting a pathogenic role of this unsuspected process *in* the emergence of psychotic symptoms.

Key words: N-Methyl-D-Aspartate receptor, glutamatergic synapse, surface trafficking, nanoparticle tracking, quantum dots, schizophrenia, psychosis, autoantibodies, NMDAR antagonists

Résumé

Les récepteurs glutamatergiques de type N-Méthyl-D-Aspartate (RNMDA) jouent un rôle majeur dans de nombreux processus physiologiques, et leur implication dans la physiopathologie de certains troubles neuropsychiatriques tels que la schizophrénie est suggérée par un robuste faisceau de données cliniques et précliniques. Cependant, les mécanismes cellulaires et moléculaires conduisant à une telle dérégulation des RNMDA restent inexpliqués. La diffusion membranaire, mécanisme de contrôle spatial et temporel de la distribution des RNMDA à la surface des neurones, constitue un puissant régulateur de la transmission synaptique. Mon projet de thèse repose ainsi sur l'hypothèse originale qu'une altération de la diffusion de surface des RNMDA jouerait un rôle central dans l'émergence de troubles psychotiques. Afin d'explorer cette piste, j'ai étudié l'impact de molécules aux propriétés psychomimétiques (*i.e* induisant un état psychotique) sur la diffusion de surface des RNMDA. Les résultats obtenus au cours de ma thèse démontrent que des molécules psychomimétiques, aux modes d'action distincts (antagonistes du RNMDA et autoanticorps anti-RNMDA), perturbent la diffusion membranaire ainsi que la localisation synaptique des RNMDA, conduisant à terme à des défauts de transmission glutamatergique. Mon travail de thèse propose donc qu'un défaut de diffusion membranaire des RNMDA conduirait à des altérations fonctionnelles pouvant contribuer à l'émergence de troubles psychotiques. L'ensemble de mon travail apporte ainsi un regard nouveau sur la mécanistique des troubles psychotiques et ouvre la voie à de nouvelles pistes thérapeutiques.

Mots-clés: Récepteurs N-Méthyl-D-Aspartate, synapse glutamatergique, diffusion de surface, suivi de particules uniques, quantum dots, schizophrénie, psychose, autoanticorps, antagonistes NMDA

Résumé

Les récepteurs glutamatergiques excitateurs N-Méthyl-D-Aspartate (RNMDA) jouent un rôle essentiel dans de nombreux processus physiologiques, et leur implication dans la physiopathologie de plusieurs troubles neuropsychiatriques tels que la schizophrénie a été établie au cours des dernières décennies (Olney et al. 1999). La schizophrénie est une maladie psychiatrique complexe affectant 4-7‰ de la population mondiale (Saha et al. 2005). Ce trouble est caractérisé par l'expression d'une trilogie de symptômes répertoriés dans le DSM (Diagnostic and Statistical Manual of Mental Disorders) et catégorisés en symptômes positifs (hallucinations, délusions…), négatifs (retrait social, anhédonie…) et cognitifs (déficits de l'attention et de la mémoire). Bien que les antipsychotiques aient permis d'améliorer significativement le quotidien des patients, ils restent palliatifs et ne traitent pas efficacement l'ensemble de ces symptômes. De plus, environ 30% des patients schizophrènes s'avèrent résistants à la pharmacopée actuelle (Mouchlianitis et al. 2016). La découverte fortuite des propriétés antipsychotiques de la chlorpromazine, un antagoniste des récepteurs dopaminergiques D2, fut à l'origine de l'hypothèse « dopaminergique » de la schizophrénie. Cette théorie n'expliquant que partiellement les altérations observées dans la pathologie, elle fut largement remise en cause au profit de l'hypothèse « glutamatergique ». En effet, l'implication des RNMDA dans la physiopathologie de la schizophrénie est fortement suggérée par un faisceau de données cliniques et pré-cliniques (Snyder & Gao 2013) : 1) des anomalies fonctionnelles des RNMDA ont été observées *postmortem* dans le cerveau de patients schizophrènes ; 2) certains antagonistes des RNMDA tels que la kétamine ou la phencyclidine, sont capables d'induire un état mimant la schizophrénie chez des sujets sains, et d'exacerber les symptômes déjà présents chez des patients schizophrènes ; 3) de tels effets ont été confirmés dans des modèles animaux expérimentaux ; 4) de nombreuses mutations génétiques affectant des gènes codant pour le RNMDA ou pour des protéines appartenant à son interactome ont été identifiées chez des sujets atteints de schizophrénie ; 5) la découverte de l'encéphalite anti-RNMDA a permis de corréler la présence d'immunoglobulines de type G dirigées contre le RNMDA à l'apparition de symptômes psychotiques sévères.

Ainsi, depuis les années 50 et l'avènement des neuroleptiques, la stratégie thérapeutique fut de normaliser un défaut de transmission synaptique en réduisant la signalisation dopaminergique, et/ou en potentialisant la transmission glutamatergique. Mais force est de constater que cette stratégie s'est brisée sur les écueils de la biologie, intervenant possiblement trop en aval dans le processus de régulation de l'information neuronale. Ainsi, *quels sont les mécanismes primaires responsables de la modulation de la neurotransmission ?* De récentes données basées sur l'imagerie à haute résolution ont permis de montrer que les RNMDA sont hautement mobiles à la surface des neurones (Groc et al. 2004a). La diffusion membranaire, en tant que fin mécanisme de contrôle spatial et temporel de la distribution des RNMDA à la surface des neurones, constitue un puissant levier de régulation de la transmission synaptique. Mon travail de thèse s'articule ainsi autour de la question suivante : *l'altération de la diffusion membranaire des RNMDA contribuerait-elle à l'émergence de troubles psychotiques ?* L'objectif de mon projet de thèse vise donc à comprendre, à l'aide d'approches multidisciplinaires, comment la signalisation du RNMDA est altérée dans ce contexte pathologique, et, si un fil d'Ariane se dégage entre différents modèles de psychoses. Pour répondre à cette question, j'ai choisi de confronter les effets de 2 familles de molécules psychomimétiques (*i.e* qui induisent un état psychotique) et d'observer si elles conduisent à une même dérégulation des RNMDA, dans un modèle *in vitro* de cultures de neurones hippocampiques de rat.

La première partie de mon projet s'est focalisée sur l'étude du mécanisme d'action d'anticorps anti-RNMDA (Ac α-RNMDA). Découverte en 2007, l'encéphalite anti-RNMDA est un trouble auto-immun à spectre psychotique, dont les symptômes s'expliquent par la production d'autoanticorps dirigés contre le RNMDA (Dalmau et al. 2007). De manière surprenante, plusieurs études ont depuis confirmé la présence d'Ac α-RNMDA dans le sérum de patients schizophrènes et d'individus sains (Pearlman & Najjar 2014; Beck et al. 2015; Heresco-Levy et al. 2015; Pathmanandavel et al. 2015; Castillo-Gomez et al. 2016). Cette partie de mon travail de thèse a donc consisté à explorer la pathogénicité moléculaire d'Ac α-RNMDA détectés chez une fraction d'individus sains et schizophrènes. En utilisant des techniques d'imagerie à haute résolution permettant d'observer en temps réel les déplacements de

récepteurs individuels, nous avons étudié l'impact de ces anticorps sur la diffusion membranaire des RNMDA. De manière très intéressante, des divergences entre les Ac α-RNMDA issus de sujets sains et de patients schizophrènes ont émergé. En effet, seule la présence d'Ac α-RNMDA issus de sujets schizophrènes perturbe la diffusion membranaire des RNMDA au sein des synapses glutamatergiques, conduisant à une désorganisation complète de leur distribution synaptique. Ces données ont été confirmées par des marquages immunocytochimiques révélant une réduction du contenu synaptique en RNMDA, prémice d'un défaut de transmission glutamatergique, et de plasticité synaptique. Dans un second temps, nous avons exploré les mécanismes à l'origine de cette "vidange" synaptique des RNMDA. Nous avons ainsi observé des altérations du récepteur EphrinB2 (EphB2R), un partenaire direct du RNMDA impliqué dans sa rétention synaptique. De nouveau, en présence d'Ac α-RNMDA de patients schizophrènes, et non de sujets sains, la diffusion membranaire ainsi que le contenu synaptique de l'EphB2R sont altérés, sous-tendant les perturbations du RNMDA préalablement reportées. Dans un dernier temps, nous avons étudié l'hypothèse que de telles dérégulations de la diffusion et de la distribution membranaires du RNMDA s'accompagneraient de conséquences fonctionnelles. Nous avons ainsi montré que la présence d'Ac α-RNMDA de patients schizophrènes conduit à une occlusion de la plasticité synaptique à long-terme. En revanche, aucune modification de l'activité calcique des RNMDA n'a été observée en présence de ces mêmes anticorps, suggérant que l'effet délétère des Ac α-RNMDA n'est vraisemblablement pas dû à une action antagoniste sur le récepteur (*i.e.* à un blocage du flux d'ions à travers le récepteur) mais concorde davantage avec un défaut de localisation synaptique des RNMDA dû à une perte d'ancrage synaptique avec EphB2R. Ainsi, ces données témoignent de l'hétérogénéité des Ac α-RNMDA et montre que leur impact moléculaire varie en fonction de leur origine. En démontrant la pathogénicité moléculaire des Ac α-RNMDA détectés chez une partie des sujets schizophrènes, ce travail questionne les choix thérapeutiques actuellement proposés aux patients résistants ou répondant partiellement aux antipsychotiques. Une récente étude clinique a démontré l'efficacité de l'immunothérapie chez des patients séropositifs pour les Ac α-RNMDA (Zandi et al. 2014), confirmant l'utilité d'évaluer la présence

d'autoanticorps chez de tels sujets. L'ensemble de ce travail est actuellement en cours de soumission pour publication.

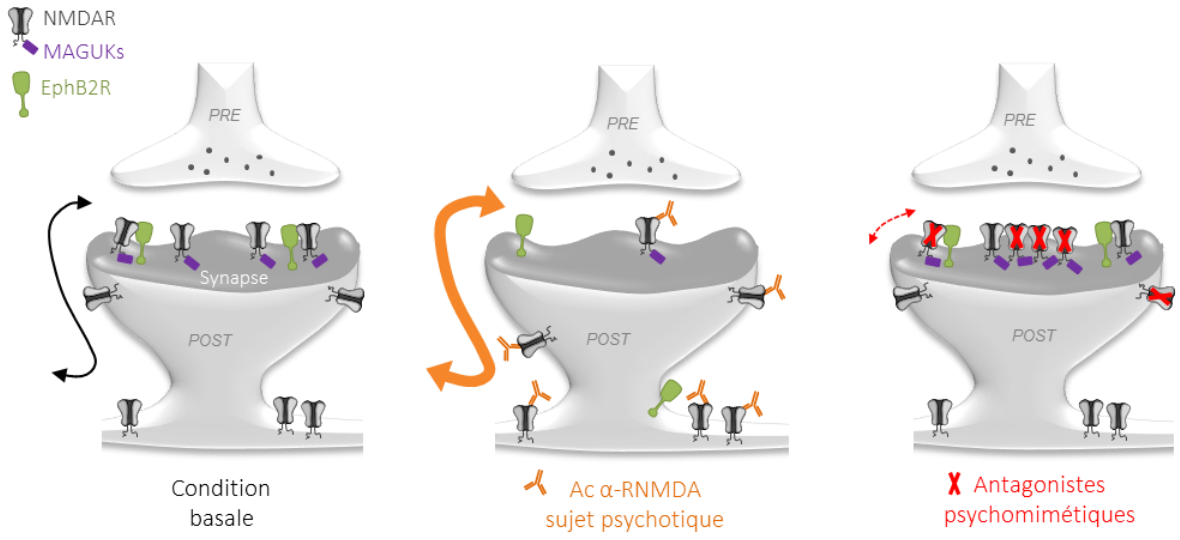
Cette première étude nous a amené à questionner les méthodes de détection des Ac α-RNMDA. Malgré que de nombreuses études aient identifié la présence d'anticorps ciblant les RNMDA chez des sujets schizophrènes, d'autres laboratoires n'ont pas confirmé ces résultats, alimentant un vif débat depuis plusieurs années (Sinmaz et al. 2015). Ces différences de détection peuvent s'expliquer par l'hétérogénéité des méthodes utilisées, notamment en termes de sensibilité et de spécificité. Classiquement, la détection d'anticorps circulants repose sur un test *in vitro* réalisé sur culture de cellules hétérologues, vivantes ou fixées, exprimant la cible voulue, *i.e.* le RNMDA. Afin de clarifier ce débat, cette seconde étude avait pour but de comparer, à partir de mêmes échantillons sanguins, les résultats issus de ces différentes méthodes de détection, réalisées dans différents laboratoires indépendants. Nous avons donc déterminé la présence ou l'absence d'Ac α-RNMDA dans le sérum d'une large cohorte de patients souffrant d'un premier épisode psychotique, en combinant des méthodes de détection classiques à une approche de suivi de nanoparticules uniques. Un premier laboratoire a identifié 14 sujets séropositifs sur l'ensemble de la cohorte (n=298) en utilisant la détection sur cellules hétérologues vivantes. 9 échantillons ont été confirmés comme positifs en utilisant la même méthode dans un second laboratoire indépendant. En revanche, la détection sur cellules hétérologues fixées n'a permis de détecter la présence d'Ac α-RNMDA dans seulement 2 sérums, confirmant l'existence d'une forte disparité en termes de sensibilité entre les méthodes de détection. Grâce à la technique de suivi de nanoparticules uniques, offrant une grande spécificité ainsi qu'une grande sensibilité de détection, nous avons confirmé la nature des anticorps détectés chez les patients psychotiques. En effet, en couplant directement des nanoparticules fluorescentes aux anticorps de patients, nous avons pu observer que les paramètres de diffusion de la cible des anticorps étaient identiques à ceux d'un RNMDA endogène. Par ailleurs, nous avons de nouveau mis en évidence le caractère pathogène des Ac α-RNMDA issus de sujets psychotiques. En comparaison aux Ac α-RNMDA issus de sujets sains, la diffusion membranaire des RNMDA est augmentée en présence d'Ac α-

RNMDA issus de patients souffrant d'un premier épisode psychotique, faisant écho aux résultats de notre première étude. Ainsi, cette seconde étude soulève l'existence de fortes différences de sensibilité et de spécificité entre les méthodes de détection, pouvant expliquer les résultats contradictoires rapportés par différents laboratoires. En combinant différentes techniques de détection, ce travail a permis de confirmer la présence d'Ac α-RNMDA dans une fraction de patients psychotiques, et suggère le potentiel pathogène de ces anticorps.

Dans la seconde partie de mon projet de thèse, je me suis intéressée à une seconde famille de molécules psychomimétiques : les antagonistes du RNMDA. Les propriétés psychomimétiques de certains antagonistes du RNMDA tels que la kétamine et du MK-801, ont été largement décrites tant chez l'homme que chez l'animal (Moghaddam & Krystal 2012). Cependant, les mécanismes moléculaires et cellulaires sous-tendant l'effet psychomimétique de ces drogues restent mal connus. Dans cette troisième étude, nous avons donc étudié l'impact d'antagonistes du RNMDA sur la diffusion de surface des RNMDA. De manière surprenante, des différences entre l'AP5, un antagoniste compétitif des RNMDA sans potentiel psychogénique, et le MK-801 et la kétamine, ont émergé. Nous avons observé une diminution majeure de la diffusion de surface des RNMDA induite par le MK-801 et la kétamine, alors que l'AP5 ne provoque aucune modification. De manière consistante, le contenu synaptique des RNMDA est également augmenté après exposition à la kétamine et au MK-801 tandis que l'AP5 n'induit lui aucune modification de l'expression des RNMDA. Ces données semblent indiquer qu'en présence d'antagonistes psychomimétiques, les RNMDA sont « bloqués » dans le compartiment synaptique. Nous avons donc exploré les mécanismes pouvant conduire à une telle rétention synaptique. En premier lieu, nous nous sommes intéressés au rôle potentiel de l'EphrinB2, partenaire privilégié du RNMDA, régulant notamment son expression à la synapse. Contrairement aux Ac α-RNMDA, aucun des antagonistes du RNMDA ne perturbe la diffusion ou la localisation de surface de l'EphrinB2, suggérant l'implication d'un autre mécanisme de régulation. En second lieu, nous avons testé l'hypothèse qu'un blocage du RNMDA pouvait conduire à une modification des interactions intracellulaires du RNMDA. Nos données indiquent qu'une extinction de l'expression des protéines

d'échafaudage PSD-95 et SAP102 *via* l'utilisation d'ARN interférent, abolit les altérations du RNMDA induites par la kétamine. Ces données semblent donc indiquer que les antagonistes psychomimétiques pourraient modifier les interactions entre le RNMDA et les protéines d'échafaudage PSD-95 et SAP102, conduisant à des défauts de localisation du récepteur. Bien qu'inachevé, ce travail souligne déjà l'existence d'une dichotomie entre antagonistes psychomimétiques et non-psychomimétiques.

L'ensemble des résultats obtenus au cours de ma thèse soutiennent donc l'idée que la diffusion membranaire des RNMDA serait une cible préférentielle des molécules psychomimétiques, conduisant à des altérations fonctionnelles pouvant contribuer à l'émergence de troubles psychotiques. Ces données ouvrent ainsi la voie à une nouvelle piste thérapeutique prometteuse et jusqu'alors jamais considérée.



Impact de différentes molécules psychomimétiques sur la diffusion membranaire des RNMDA
En condition basale, les RNMDA diffusent continuellement entre la synapse et ses abords (flèche noire). Les RNMDA synaptiques sont activement ancrés à la fois grâce à des intéractions avec des protéines d'échaffaudage telles que les MAGUKs, et des protéines membranaires comme le récepteur à l'EphrinB2. En présence d'Ac α-RNMDA issus de patients psychotiques, la diffusion membranaire des RNMDA est augmentée (flèche orange), due à une perte d'intéraction avec le récepteur EphrinB2, conduisant à une désorganisation complète de la distribution de surface des RNMDA. A l'inverse, les antagonistes psychomimétiques provoquent une forte réduction de la mobilité synaptique des RNMDA (flèche rouge), induisant une augmentation de leur contenu dans la synapse. Ce défaut de diffusion membranaire est vraisemblablement causé par des modifications de l'intéraction du RNMDA avec les protéines MAGUKs.

Mots-clés : Récepteur N-Méthyl-D-Aspartate, synapse glutamatergique, diffusion de surface, suivi de particules uniques, quantum dots, schizophrénie, psychose, autoanticorps, antagonistes NMDA

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Introduction

The glutamatergic synapse

The human brain contains over 100 billion neurons, and forms the foundation of all the cognitive processes that define us as self-conscious and social individuals. These processes rely on the operation of a functional unit: the synapse, which enables rapid signal transmission between neurons, and allows an organized flux of information in the brain. Chemical synapses are composed of two highly specialized compartments: the pre- (transmitting) and the post-synaptic (receiving) elements separated by the synaptic cleft, and connected through discrete trans-synaptic protein-based nanocolumns (Tang et al. 2016). The glutamatergic synapse represents the major type of synapses in the brain, and also one of the best understood. In the vertebrate central nervous system (CNS), excitatory neurotransmission is mainly driven by glutamate. The first targets of glutamate release from pre-synaptic terminals are the glutamate receptors on the post-synaptic element. Glutamate receptors are transmembrane proteins that specifically bind to glutamate on the extracellular side of the post-synaptic membrane. Upon binding of glutamate, glutamate receptors are activated and transduce this signal into intracellular responses. Glutamate receptors form two broad groups: metabotropic receptors (mGluR) which are glutamate-activated G protein-coupled receptors, and ionotropic receptors (iGluR), which are glutamate-gated ion channels. Ionotropic glutamate receptors can be further categorized into N-Methyl-D-Aspartate (NMDA) receptors, α-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid (AMPA) receptors and Kainate receptors (Hollmann & Heinemann 1994; Dingledine et al. 1999). Excitatory glutamatergic synapses are defined as “asymmetric synapses” because of their characteristically thick post-synaptic density (PSD) facing the pre-synaptic active zone, and typically localized on dendritic protrusions (Gray 1959). The PSD is a specific electron microscopic (EM) electron-dense organelle formed by a protein complex that adheres to the post-synaptic membrane. Morphologically, the PSD is a disc-like structure with a highly heterogeneous size, measuring in average 30-60nm thick and a few hundred nanometers wide (Carlin et al. 1980). The size of the PSD likely correlates with the size of the dendritic spine and with the abundance of postsynaptic glutamate receptors (Kasai et al. 2003).

Recent electron and super-resolution light microscopic imaging studies further dissected the anatomy of the PSD and provide a better understanding of its three-dimensional organization (Gold 2012; Maglione & Sigrist 2013). Proteins within the PSD form distinct layers along the axo-dendritic axis of synapses, with a sequential order of 1) membrane receptors and cell adhesion molecules, 2) MAGUK, 3) SAPAP, SH3 and SHANK scaffolds, and 4) the actin cytoskeleton contacting the interior face of the PSD. This very dense and compact number of proteins not only provides structural and molecular support to this complex molecular architecture, but also facilitates rapid and efficient synaptic transmission by bringing together various signaling components and pathways (Kennedy 1997; Sheng & Kim 2002; Boeckers 2006). Modulation of synapse activity constitutes a major strategy to control brain homeostasis, and slight but persistent perturbations in synapse physiology can result in major defects that may manifest as brain disorders.

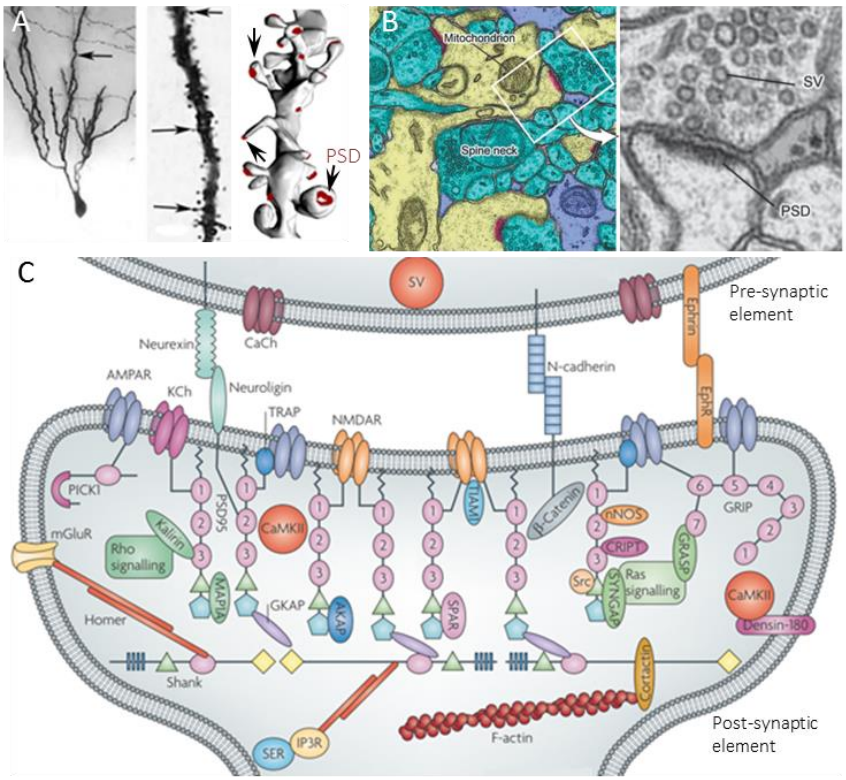

Figure 1. The glutamatergic synapse

(A) Golgi impregnated hippocampal neuron and a representative dendrite covered by dendritic spines (arrows). 3D reconstruction materializes the presence of post-synaptic densities (PSD) on dendritic protusions and spines (arrows). (B) EM morphology of a glutamatergic synapse. Dendritic elements appear in yellow, axons in turquoise, astrocytes in blue and PSD in pink. The pre-synaptic element containing synaptic vesicles loaded with glutamate (SV) faces the post-synaptic element with the typical electron-dense PSD. (C) Schematic protein organization of the glutamatergic synapse. Cell-adhesions proteins, transmembrane proteins, scaffold proteins and components of the cytoskeleton helps to maintain the synaptic architecture. Synaptic transmission is mediated through receptors and ion channels expressed at the cell surface, but also *via* intracellular scaffold and signaling proteins. (Adapted from Stewart et al., 2014; Sheng & Hoogenraad 2007; Feng & Zhang 2009)

I- The NMDAR

A. The NMDAR : composition, expression, function and regulation

1. NMDAR composition

NMDAR belong to the family of ionotropic glutamate receptors, which play a critical role in excitatory neurotransmission in the central nervous system of vertebrates. NMDAR are cationic channels permeable to sodium, potassium and calcium. The calcium influx through NMDAR is a critical factor that mediates many of the NMDAR-specific physiological and pathogenic conditions.

a. NMDAR subunits and genes

Cloning studies revealed the remarkable diversity of NMDAR subunits and isoforms. Seven different subunits divided in three families, which share sequence identity with other ionotropic glutamate receptor subunits, have been identified (Moriyoshi et al. 1991; Monyer et al. 1992): the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) encoded by four different genes, and a pair of GluN3 subunits (GluN3A and GluN3B) arising from two distinct genes. The GluN1 subunit is encoded by a single gene GRIN1 that bears three alternatively spliced exons: the exon 5 encoding the N1 cassette in the N-terminal domain, and exons 21 and 22 in the C-terminal domain encoding the C1 and C2 cassettes respectively. The alternative splicing of these cassettes gives rise to eight splice isoforms (GluN1-1a–GluN1-4a and GluN1-1b–GluN1-4b) (Moriyoshi et al. 1991). When the splice variants contain the N1 cassette, they are called "-b" isoforms. When they lack this domain they are designated as "-a" isoforms. The C-terminal splice variants account for differential subunit trafficking properties and variations in the

Glossary for basic knowledge in protein production

Gene: a region of DNA coding for either messenger RNA or functional RNA molecules.

Exons: coding sections of an RNA transcript that are translated into protein. Exons can be separated by non-coding sequences, known as introns. Following transcription, immature mRNA (or pre-mRNA) may contain both exons and introns. They will then go through a splicing step during which the introns are removed and exons are connected to produce mature mRNA.

Alternative splicing: refers to the process by which a single gene is spliced into more than one type of mRNA molecule, and will thus code for several proteins.

Cassette (or cassette exon): a type of exon that will either be included or skipped of the transcript during alternative splicing, giving rise to different protein isoforms.

length of the intracellular C-terminal tail (Paoletti 2011). It has been reported that GluN2 and GluN3 subunits also exist in several alternatively spliced forms, although the functional differences between them are far from clear. Alternative splicing of the NMDAR subunits provides additional layers of heterogeneity and complexity, shaping subtle modulations of pH sensitivity or intracellular interactions for instance (Traynelis et al. 2010). Functional NMDAR are heterotetrameric assemblies, usually associating two GluN1 and two GluN2 subunits (GluN1/GluN2 complexes). NMDAR incorporating GluN3 subunits form complexes either diheteromeric (GluN1/GluN3) or triheteromeric complexes (GluN1/GluN2/GluN3) (Traynelis et al. 2010). The diverse subunits combinations confer distinct characteristics of channel properties, expression pattern and function on the receptor.

b. Molecular architecture of the NMDAR

NMDAR are membrane proteins composed of four large subunits that form a central ion channel pore selective for cations (Na^+, K^+ and Ca^{2+}). NMDAR share with all other ionotropic glutamate receptors a typical modular design, in which each subunit consists of four domains (Dingledine et al. 1999; Paoletti & Neyton 2007; Traynelis et al. 2010): 1) a large extracellular N-terminus domain, 2) a membrane region composed of three transmembrane segments (TM1, 3 and 4) and a re-entrant pore loop (M2), 3) an extracellular loop between TM3 and TM4, and 4) a cytoplasmic C-terminus domain (Dingledine et al. 1999; Cull-Candy & Leszkiewicz 2004; Paoletti & Neyton 2007). The extracellular region is organized as a tandem of two clamshell-like domains. It comprises the N-terminal domain (NTD) which is involved in subunit assembly and allosteric regulation, and the agonist-binding domain (ABD) which binds two co-

Glossary for basic knowledge of receptor properties

Allosteric regulation: a control mechanism that regulates receptor activity through the binding of an effector molecule at one site, distinct from the agonist binding site, and which causes a change in the receptor conformation in such a way that its activity is either enhanced (positive modulator) or reduced (negative modulator).

Agonist: a molecule that is able to act as a ligand and to bind and activate a receptor to produce a biological response.

Co-agonist: works in conjunction with other co-agonists to produce the effect.

Conductance: the degree of permeability to certain ions.

Ion selectivity: acts as a filter which allows only ions of a certain size and/or a certain charge to pass through the receptor channel. For instance, the NMDAR is selective for the cations Na^+, K^+ and Ca^{2+}.

Affinity for the Mg^{2+} block: Mg^{2+} blocks NMDAR by binding to the channel pore with an apparent affinity that depends on the membrane potential.

Membrane potential: the difference in electrical potential between the intracellular and extracellular compartments of a cell. Typical values range from -80mV to +40mV.

agonists: glycine (or D-serine) in GluN1 and GluN3 subunits and glutamate in GluN2 subunits (Furukawa et al. 2005), which controls channel opening. The transmembrane domains (TM1, 3 & 4) and the pore loop (M2) form the ion channel and define the receptor conductance, ion selectivity and affinity for the Mg^{2+} block. The sequences of the regions lining the pore are highly conserved in GluN2 subunits with little variation among different receptor subtypes. The insertion of the ion channel actually separates the ABD in two S1 and S2 segments. Finally, the cytoplasmic C-terminal domain (CTD) shows a variable length depending on the subunit and provides multiple sites of interaction with intracellular proteins for implicated in receptor anchoring, trafficking and signaling. Even if NMDAR and iGluR share a high sequence homology and receptor topology, they have clear differences in basic ion channel physiology and pharmacology. In non-NMDAR, the ATD does not regulate ion channel activity, the LBD binds only one agonist, L-glutamate, and the TMD forms an ion channel pore with no voltage-sensing capacity and with substantially less calcium permeability than NMDAR. Moreover, the CTD, due to its shorter size, interacts with postsynaptic proteins that are distinct from the NMDAR (Dingledine et al. 1999).

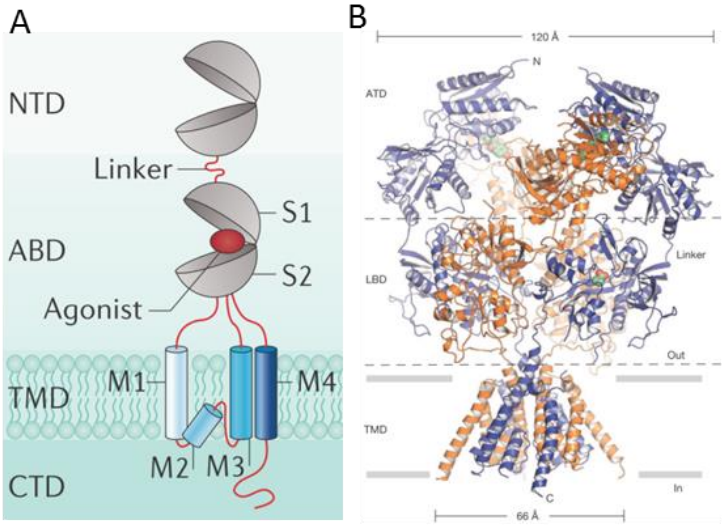

Figure 2. Molecular architecture of the NMDAR
(A) Topology of the NMDAR. Each GluN subunit isorganized into four layers with 1) at the top, the extracellular N-terminus domain (NTD or ABD for amino-terminal domain), 2) the agonist-binding domain (ABD) that binds to glutamate and the co-agonists D-serine and glycine, 3) the transmembrane domain (TMD) composed of three transmembrane segments (TM1, 3 and 4) and a re-entrant pore loop (M2) and 4) at the bottom, the intracellular C-terminus domain (CTD). (From Paoletti et al., 2013). (B) Crystallographic studies revealed that the structure of the GluN1-GluN2B NMDAR ressembles a mushroom, with a height of ≈150Å and a width of 120Å. (From Lee et al., 2014)

Crystallographic studies have revealed the structures of several isolated domains of NMDAR (Furukawa et al. 2005; Karakas et al. 2011). The GluN2B NTD has revealed a twisted clam-shell conformation (Karakas et al. 2009), which was similarly described in GluN2A NTD (Stroebel et al. 2011). More recently, Karakas & Furukawa (2014) showed the crystal structure of the intact heterotetrameric GluN1/GluN2B ion channel. Similar to other iGluR, GluN1 and GluN2B subunits are arranged in a classical GluN1/GluN2/GluN1/GluN2 (1-2-1-2) orientation. But contrary to AMPAR, ATD and LBD in GluN1/GluN2B receptors are tightly packed. In the ATD, the two GluN1/GluN2B heterodimers interact with each other at two interfaces involving upper lobes of the two GluN1 subunits and lower lobes of the two GluN2B subunits (Karakas & Furukawa 2014). Thus, NMDAR architecture shows clear differences with other iGluR and offers a multitude of motions and sites for drug action.

c. Triheteromers

NMDAR can also combine two types of GluN2 subunits to form triheteromeric NMDAR (Chazot et al. 1994; Hatton & Paoletti 2005). GluN1/2A/2B complexes have been proposed to be present in native tissues (Hatton & Paoletti 2005; Al-Hallaq et al. 2007; Rauner & Kohr 2011; Tovar et al. 2013; Frank et al. 2016). GluN1 and GluN2 subunits might also assemble with GluN3 subunits to form ternary complexes (Dingledine et al. 1999; Mayer 2006). NMDAR containing GluN2 and GluN3 subunits exhibit different properties in comparison with GluN1/GluN2 receptors (Sanz-clemente et al. 2013). Co-expression of GluN3A subunit with GluN1 and GluN2A subunits causes a reduction in whole-cell currents, single-channel conductance, and a lower Ca^{2+} permeability (Dingledine et al. 1999). Accordingly, NMDA-induced currents in cortical neurons are increased about 3-fold in GluN3A knockout mice (Dingledine et al. 1999). GluN1/GluN2B/GluN3A or GluN1/GluN2B/GluN2D complexes are likely expressed at early stages of development, and GluN1/GluN2A/GluN2B or GluN1/GluN2A/GluN2C in adulthood (Al-Hallaq et al. 2007). Quantitative Western blot analysis of rat

hippocampus has estimated that GluN1/2A/2B triheteromers would constitute approximately one-third of the total NMDAR population (Al-Hallaq et al. 2007). Using an electrophysiologically-based approach, Rauner & Köhr (2011) estimated that triheteromeric GluN1/2A/2B receptors represent a prominent population in CA1 synapses of adult mice. This estimation was based on the observation that the voltage dependence of NMDA EPSC decay is distinct for GluN1/2B and GluN1/2A receptors in the absence and presence of Mg^{2+}. Tovar et al. (2013) used GluN2A and GluN2B subunit knock-out mice to measure EPSC and define the properties of diheteromeric GluN1/2B or GluN1/2A receptors, respectively. By using the differences in opening probabilities between receptor subtypes and their relative contribution to the excitatory postsynaptic current (EPSC) amplitude, they estimated that triheteromers contribute to 2/3 of the signal. Several studies tried to further characterize the pharmacological properties of NMDAR triheteromers using heterologous expression systems. Hatton & Paoletti (2005) combined mutagenesis and pharmacology to isolate recombinant triheteromeric receptors. Contrary to heterodimers, GluN1/GluN2A/GluN2B receptors possess two different NTD, not allowing full inhibition. Based on this intrinsic property, GluN1/GluN2A/GluN2B receptors bind both Zn^{2+} and ifenprodil with high-affinity but show greatly reduced inhibition maximal inhibition (≈ 20%), which confers them a unique pharmacological signature (Hatton & Paoletti 2005). Another group took advantage of the subunit selectivity of two negative allosteric modulators families, conantokins and spermine, to further study NMDAR triheteromers properties (Cheriyan et al. 2016). GluN2A-specific inhibitors more pronouncedly inhibited recombinant GluN1/2A/2B receptors compared to GluN2B inhibitors, suggesting a decreased contribution of GluN2B subunit in GluN1/2A/2B complexes compared to heterodimers (Cheriyan et al. 2016). However, the fact that triheteromeric receptors show an “intermediate” sensitivity to subunit-selective modulators does not allow to fully eliminate diheteromers participation. To overcome this critical limitation, Traynelis’ group engineered GluN2 subunits with C-terminal peptide tags composed of leucine zipper motifs that can interact to form a heterodimeric coiled-coil structure, allowing selective surface expression of triheteromeric NMDAR (Hansen et al. 2014). In line with previous studies, the authors reported

strong similarities between GluN1/GluN2A/GluN2B receptors and GluN1/GluN2A diheteromers, raising the possibility that the contribution of GluN1/GluN2A diheteromers is overestimated in some studies (Hansen et al. 2014). These peptide tags constitute a very promising tool, and could be useful to study the contribution of NMDAR triheteromers in synaptic transmission and plasticity. However, an effective method to isolate this subgroup of receptors from NMDAR diheteromers *in vivo* still needs to be developed.

d. Activation

A hallmark of NMDAR, by contrast with AMPAR and kainate receptors, is that binding of two co-agonists is required for receptor activation. Thus, NMDAR composed of GluN1 and GluN2 subunits require the binding of the agonist glutamate, concomitant with the binding of the co-agonist glycine or D-Serine. GluN1/GluN3 receptors require only glycine for activation (Traynelis et al. 2010). At resting membrane potential, the pore of the NMDAR channel is blocked by Mg^{2+}. This blockade is voltage-dependent, defining the unique role of NMDAR as molecular coincidence detectors. Ion influx only occurs when both pre-synaptic and post-synaptic neurons are stimulated at the same time. Therefore, NMDAR activation requires postsynaptic depolarization (to relieve the Mg^{2+} block) that coincides with pre-synaptic release of glutamate that binds to the agonist binding site on GluN2 subunits, in presence of a co-agonist, glycine or D-serine. Investigation of iGluR crystal structures, allowed to understand the structural mechanisms underlying channel activation, and was initially performed on AMPAR and kainate receptors (Mayer 2006). Mechanistically, NMDAR channel opening is triggered by the following sequence of events: 1) agonists bind the central cleft of the clamshell-like ABD, each ABD containing a single agonist site (NMDAR activation requires occupation of the four agonist binding sites), 2) agonist binding promotes cleft-closure of the ABD, increasing the space between two adjacent ABD, 3) this separation in turn exerts tension on the linkers connecting the ABD to the transmembrane segments, eventually leading to reorientation of the transmembrane domains and channel gate opening (Paoletti 2011). This activation sequence seems to be conserved

in NMDAR even though differences exist between receptor subclasses (Furukawa et al. 2005; Mayer 2006; Paoletti & Neyton 2007; Traynelis et al. 2010).

e. Subunit composition determines NMDAR functional properties

NMDAR exhibit a number of characteristic properties that distinguish them from other ionotropic receptors (Dingledine et al. 1999). As previously shown, NMDAR activation requires the presence of both agonists and co-agonists, together with the removal of the Mg^{2+} block dependent on voltage variation. NMDAR are highly permeable to Ca^{2+} and display slow activation and deactivation kinetics compared to AMPAR and kainate receptors. Another hallmark of NMDAR activation is that it can be modulated by allosteric modulators such as protons and Zn^{2+}. All these properties are unique to NMDAR in the iGluR family. However, NMDAR subunits influence the biophysical, pharmacological and signaling properties of the receptor, thus creating a variety of NMDAR subtypes with unique functional features. First, GluN1 is a ubiquitous and obligatory subunit of the NMDAR. Its genetic elimination is lethal in neonatal stages, and studies using conditional GluN1 knock-out mice revealed an absence of functional NMDAR as the GluN2 subunits are retained in the endoplasmic reticulum (Fukaya et al. 2003). Then, one of the major properties affected by NMDAR subunits is the receptor ion permeation. “Classical” GluN1/GluN2A or GluN1/GluN2B receptors generate “high-conductance” channel openings with high sensitivity to Mg^{2+} blockade and Ca^{2+} permeability (Traynelis et al. 2010). GluN2C subunit shows relatively unique channel properties, including low conductance and low sensitivity to Mg^{2+} (Farrant et al. 1994). GluN2D is characterized by extremely slow decay time. As so, GluN1/GluN2C or GluN1/GluN2D receptors show lower conductances, lower sensitivity to Mg^{2+} and lower Ca^{2+} permeability compared to GluN2A/2B receptors (Dingledine et al. 1999). Incorporation of a GluN3 subunit results in an even more dramatic decrease in Mg^{2+} blockade. Unlike GluN2 subunits, GluN3 binds to glycine and not to glutamate. NMDAR containing exclusively GluN1/GluN3 subunits can act as excitatory glycine receptors, which are impermeable to calcium (Pérez-Otaño et al. 2016).

GluN1 splice variants and GluN2 subunits also determine NMDAR sensitivity to agonists, activation and deactivation kinetics and channel open probability and duration (Traynelis et al. 2010; Paoletti 2011). GluN1/GluN2A receptors have a higher open probability than GluN1/GluN2B or GluN1/GluN2C and GluN1/GluN2D receptors; these two latter subtypes having a surprisingly low open probability. However, GluN1/GluN2A receptors have the lowest sensitivity to both glutamate and glycine. Glutamate deactivation kinetics governs the EPSC decay, with GluN1/GluN2A receptors having the fastest decay and GluN1/GluN2D receptors the slowest. Glutamate deactivation kinetics are also influenced by GluN1 isoforms (Paoletti et al. 2013). Finally, NMDAR activity is subject to allosteric modulation, which also varies according to the receptor subunit composition. Protons and Zn^{2+} represent endogenous NMDAR allosteric modulators. Protons preferentially inhibit GluN1/GluN2B or GluN1/GluN2D receptors whereas Zn^{2+} ions act as highly specific antagonists of GluN1/GluN2A receptors (Paoletti 2011). Many pharmacological compounds can also discriminate between NMDAR subtypes. Ifenprodil and its derivatives (such as Ro 25-6981), which is a synthetic compound that selectively inhibits GluN1/GluN2B receptors by acting at the GluN1-GluN2B NTD interface (Karakas et al. 2011).

2. NMDAR assembly and transport to the cell membrane

Before NMDAR reach the synapse, they are synthesized, assembled, and trafficked to the plasma membrane. NMDAR are synthesized as monomers in the endoplasmic reticulum (ER), where they assemble into tetramers (Bard & Groc 2011). NMDAR contain different ER-retention/export signals ensuring that only correctly folded and assembled heterotetramers reach the cell surface. The C-terminal region of the GluN1 subunit was shown to have a major role on the cell surface expression of NMDAR (Okabe et al. 1999). Indeed, the GluN1 splice variants have distinct properties in the early trafficking stage because of the different C-terminal cassette combinations they contain. The C1 cassette has been shown to contain the RRR and KKK ER-retention signals that are masked upon

assembly of functional tetramers (Carroll & Zukin 2002). These ER retention signals function as a quality control mechanism which retains unassembled receptors in the ER. During receptor assembly, binding of the GluN2 subunit masks the GluN1 retention signal and promotes trafficking of the heterodimeric receptors through the secretory pathway to the cell surface. Accordingly, unassembled GluN1 subunits show little or no surface expression and are retained in the ER. Once assembled with GluN2 subunits, they are efficiently delivered to the cell surface (McIlhinney et al. 1998). GluN3 subunit assembly with other NMDAR subunits is not yet fully understood although GluN3 subunits control NMDAR synaptic signaling during development (Pérez-Otaño & Ehlers 2004). After the quality-control machinery in the ER, NMDAR enter the Golgi apparatus and the trans-Golgi network where they are processed into mobile transport packets which are composed of NMDAR subunits, the microtubule-dependent motor protein KIF17, and the postsynaptic adaptor proteins CASK and SAP-97. They associate with different MAGUKs including SAP102 and PSD-95 along the secretory pathway (Horak et al. 2014), which also promotes trafficking to the postsynaptic membrane. Intracellular transport of NMDAR along microtubules requires kinesin KIF17 together with the adaptor protein LIN10, which binds through intermediate adaptor proteins (LIN2 and LIN7) to the GluN2B subunit (Lau & Zukin 2007).

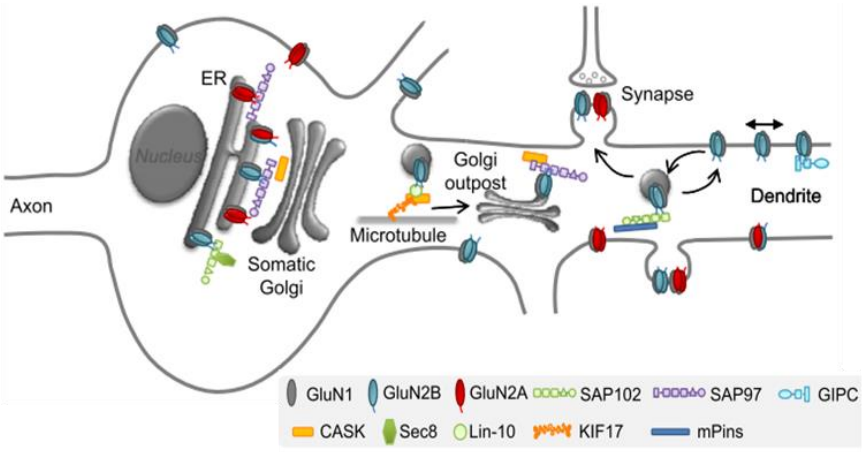

Figure 3. NMDAR intracellular trafficking and transport to the cell membrane
Functional NMDAR subunits assembly occurs in the ER. ER retention signals work as a quality control mechanism, allowing only NMDAR tetramers containing both GluN1 and GluN2 subunits to reach the « Golgi step ». In the ER, NMDAR also binds to SAP102 before being trafficked through the somatic Golgi and along microtubules thanks to the interaction with KIF17 and LIN adaptor proteins. Association of NMDAR with protein complexes such as SAP97/CASK will target NMDAR to a non-conventional pathway via the dendritic Golgi. NMDAR are then expressed at the cell surface via exocytosis, and eventually reach the synaptic compartment thanks to lateral diffusion. The number and distribution of NMDAR thus results from the dynamic equilibrium between exocytosis/endocytosis cycles and lateral diffusion.
(From Bard & Groc 2011)

The conventional hypothesis states that proteins synthesis and assembly takes place in the soma. However, GluN1 subunit mRNA have been found in dendrites (Steward & Schuman 2003), suggesting that NMDAR can be synthesized both in the cell body and in the dendrites. Hence, in mature neurons (>12*div*), NMDAR bypass the somatic Golgi and are transported within the ER until they reach the dendritic Golgi outposts (Zhang & Luo 2013). GluN2A subunit which is expressed later in development might then undergo non-canonical trafficking.

3. NMDAR localization

a. NMDAR distribution throughout the body

There is a significant body of evidence reporting the expression of NMDAR in non-neuronal cells of the CNS and peripheral tissues. NMDAR subunit expression was identified in a variety of non-neuronal cells of the CNS, including multiple glial cell types and brain endothelial cells (reviewed in Skerry & Genever 2001; Hogan-Cann & Anderson 2016). Non-neuronal NMDAR are regulated by endogenous agonists, glutamate, quinolinic acid, and L-homocysteic acid. NMDAR subunit expression has been detected in nascent and activated microglial cells *in vitro* (Murugan et al. 2011; Kaindl et al. 2012), even though their presence on microglia remains controversial. NMDAR are also expressed by oligodendrocyte cells and may play a role in white matter damage and myelination (Li et al. 2013). Aside from nervous system tissues and brain endothelial cells, NMDAR have been identified across a wide array of tissues in many systems (Skerry & Genever 2001; Hogan-Cann & Anderson 2016). Emerging data suggest that peripheral NMDAR maybe responsible for diverse physiological roles, including myelination, pain sensitivity, and inflammation. NMDAR in bone, kidney, pancreas, and other tissues are promising therapeutic targets for disorders such as osteoporosis, acute renal injury, diabetes, and cancer (Hogan-Cann & Anderson 2016).

b. NMDAR distribution throughout the brain

Regional brain distribution

NMDAR are widely distributed throughout the central nervous system (CNS), although the expression of individual subunits is highly dependent on brain areas and developmental stages. The obligatory GluN1 subunit, which is ubiquitously expressed in the CNS, displays isoform-specific differences in expression (R Dingledine et al. 1999). However, the functional significance of the differential expression of GluN1 isoforms is not clear. Regarding GluN2 subunit expression, there are four genes encoding GluN2 subunits and each has a unique spatiotemporal profile (Monyer et al. 1994). The expression patterns of the GluN2 subunits change drastically during the first 2 weeks following birth. The GluN2A subunit gradually increases after birth and is abundantly expressed in the entire CNS at adulthood. On the other hand, GluN2B subunit is widely expressed during prenatal development, peaks around postnatal day (P) 7-10, and progressively becomes restricted to forebrain areas (cortex, hippocampus, striatum, olfactory bulb) where it remains expressed at quite high levels (Monyer et al. 1994). GluN2C subunit expression appears late in development (≈P10) and is highly enriched in the adult cerebellum and olfactory bulb. GluN2D subunit is present early in development and is strongest in the diencephalon, brainstem and spinal cord in adulthood (Monyer et al. 1994). GluN3 subunits also show differential expression patterns (Henson et al. 2008). GluN3A subunit expression is low before birth, peaks during early postnatal life and then decreases to low levels in adulthood. On the opposite, GluN3B subunit expression level increases throughout development until reaching a maximum in adult animals. Different combinations of subunit assembly can occur and give rise to various NMDAR subtypes (Paoletti et al. 2013). A single neuron can express different GluN1 isoforms and GluN2 subunits (Cull-Candy & Leszkiewicz 2004). The subunit composition also differs between different types of neurons (Monyer et al. 1994). For instance, hippocampal pyramidal cells express GluN2A and GluN2B mRNA while GluN2C and GluN2D transcripts are found in interneurons. GluN2C mRNA were also found in glial cells (Monyer et al. 1994). Therefore, NMDAR subunit content is

subject to spatiotemporal regulation allowing for functional differences following NMDAR activation at different synapses throughout the brain.

Pre-synaptic NMDAR

NMDAR were first discovered as postsynaptic receptors at glutamatergic synapses, but physiological and anatomical evidence suggest the existence and function of pre-synaptic NMDAR (pre-NMDAR). Electron microscopy (EM) revealed the expression of GluN1 and GluN2B subunits of the NMDAR on the presynaptic membrane (Aoki et al. 1994; DeBiasi et al. 1996; Charton et al. 1999). Brasier and Feldman (2008) demonstrated that pre-NMDAR are expressed in only a subset of pyramidal neurons terminals in the developing somatosensory cortex. Evidence for a presynaptic location of NMDAR also emerged from electrophysiological approaches. Bath application of AP5, an NMDAR antagonist, decreased the frequency of spontaneous mEPSCs in the entorhinal cortex even when postsynaptic NMDAR were previously blocked intracellularly, suggesting that pre-NMDAR enhance spontaneous neurotransmitter release (Berretta & Jones 1996). Similar data were further collected at excitatory synapses in the primary visual cortex (Sjöström et al. 2003; Corlew et al. 2008; Li & Han 2007), the CA1 region of the hippocampus (Mameli et al. 2005), the entorhinal cortex (Yang 2006), and the somatosensory cortex. In addition to affecting spontaneous release, pre-NMDAR can modulate evoked transmitter release (Sjöström et al. 2003; Brasier & Feldman 2008), acting as auto-receptors which may help to maintain a high release probability in a context of continuous firing. Pre-NMDAR also play a critical role in long-term synaptic plasticity (Corlew et al. 2008; Pinheiro & Mulle 2008; Duguid 2013). Future studies should help in elucidating pre-NMDAR subunit composition and function, as it seems that the role and existence of pre-NMDAR is still challenged (Duguid 2013; Carter & Jahr 2016).

Synaptic and extrasynaptic NMDAR

In glutamatergic neurons, neurotransmitter receptors have long been known to be concentrated in the postsynaptic density, but it is well established now that NMDAR occupy both synaptic and extrasynaptic locations. Subcellular localization of NMDAR was first assessed using classical immunohistochemical techniques, which revealed that glutamate receptors are widely distributed throughout the dendritic arborization, and are present both at synaptic and extrasynaptic sites (Aoki et al. 1994; Siegel et al. 1994). Subcellular immunogold labeling of NMDAR subunits provided high-resolution EM visualization of receptors at synaptic and extrasynaptic sites on spines, dendrites, somata, and within intracellular compartments (Petralia et al. 2010). Electrophysiological approaches were also used to explore the surface distribution of NMDAR. The irreversible blocking of synaptic NMDAR by the open channel blocker MK-801 unveiled a "remaining" extrasynaptic pool of NMDAR sensitive to ifenprodil (Tovar & Westbrook 1999), suggesting that GluN2B subunit is present at extrasynaptic sites. In addition, glutamate spillover (from astrocytes or neighboring neurons) activates mainly ifenprodil-sensitive NMDAR (Scimemi et al. 2004). Many studies indeed took advantage of the subunit selectivity of ifenprodil to examine the subcellular distribution of the GluN2B subunit. From that work emerged the idea that extrasynaptic NMDAR mainly contain GluN2B subunit while synaptic GluN2A subunit become more important as the neuron matures (Groc et al. 2004; Groc et al. 2006; Groc et al. 2009; Bard & Groc 2011; Tovar & Westbrook 1999; Li et al. 2002), following the expression pattern change of NMDAR subunits during development (Monyer et al. 1994). As so, 75% of NMDAR were found to be extrasynaptic during the first week *in vitro* (Tovar & Westbrook 1999; Tovar & Westbrook 2002) and would then progressively decrease to 20-50% by 2 weeks *in vitro* (Ivanov et al. 2006). Similar proportions are found in hippocampal slices, where about 36% of NMDAR are extrasynaptic at P14-21 (Harris & Pettit 2007). However, some others failed to observe such a differential subunit distribution (Petralia et al. 2010). Like synaptic receptors, extrasynaptic NMDAR can be activated by synaptically released glutamate (Clark & Cull-Candy 2002; Chen & Diamond 2002).

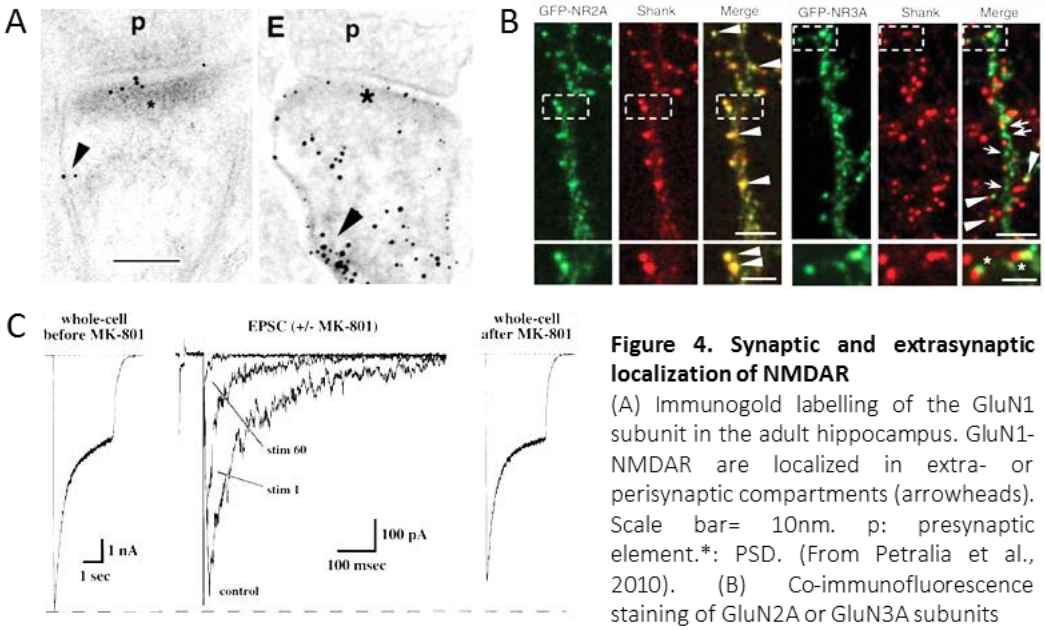

Figure 4. Synaptic and extrasynaptic localization of NMDAR

(A) Immunogold labelling of the GluN1 subunit in the adult hippocampus. GluN1-NMDAR are localized in extra- or perisynaptic compartments (arrowheads). Scale bar= 10nm. p: presynaptic element.*: PSD. (From Petralia et al., 2010). (B) Co-immunofluorescence staining of GluN2A or GluN3A subunits the synaptic marker hank in hippocampal neurons. Scale bar= 5µm, insets= 2µm. While the GluN2A subunit shows a clear synaptic localization (white arrowheads), the GluN3A subunit shows limited overlap with Shank indicating its preferential expression at extra- or perisynaptic sites (asterisks). (From Perez-Otano et al.,2006). (C) Electrophysiological approach used to determine the ratio of extrasynaptic to synaptic receptors. The peak current of NMDAR EPSC from whole-cell recordings is measured in response to 1mM NMDA before and after irreversibly blocking the EPSC (60 stimulations of 0.1Hz) with the use-dependent antagonist MK-801. Using this protocol, the contribution of synaptic receptors is eliminated from the whole-cell response. The difference between the peak in the first current (left panel) and the last current (right panel) reflects the proportion of synaptic NMDAR, and by substraction, the proportion of extrasynaptic NMDAR. (From Tovar & Westbrook, 1999)

Uncaged glutamate experiments suggest a similar sensitivity for ifenprodil between synaptic and extrasynaptic sites (Harris & Pettit 2007), supporting similar proportions of GluN2B subunit at both localizations. Then, the proposed dichotomy of synaptic GluN2A subunit (Dingledine et al. 1999; Cull-Candy et al. 2001) and extrasynaptic GluN2B subunit occuring during postnatal development may not be so definitive. Apart from the GluN2A and GluN2B subunits, little attention has been given to other NMDAR subunits. For instance, Perez-Otano and colleagues (2006) have reported the prevalent presence of GluN3A subunit at peri- and extrasynaptic sites, while synaptic GluN3A subunit tends to be concentrated in the outer portion of the synapse (Pérez-Otaño et al. 2006). GluN2D subunit, which for long was thought to be exclusively extrasynaptic, can also be found at synaptic sites (Brothwell et al. 2008; Harney et al. 2008). Overall, there seems to be no simple rule than would relate a particular NMDAR subunit to a given subcellular region (Cull-Candy & Leszkiewicz 2004; Köhr 2006).

4. NMDAR regulation

a. Endocytosis/exocytosis cycling

NMDAR endocytosis

Roche et al (2001) observed robust internalization in young neurons *in vitro*, a feature that is lost as the neurons mature. They also identified a consensus internalization motif in the C-terminus of GluN2B subunit (Roche et al. 2001), which regulates NMDAR endocytosis. Indeed, this motif is a strong consensus sequence for binding to the clathrin adaptor protein AP-2, which links internalized cargo to clathrin. PSD-95 significantly inhibited internalization and stabilized GluN2B subunit at the surface (Roche et al. 2001). The reduction in NMDAR internalization during neuronal maturation might involve the downregulation of GluN2B subunit and the upregulation of GluN2A subunit. A preferential interaction of the C-terminus of GluN2B subunit, but not of GluN2A subunit with AP-2 might underlie the higher rate of internalization of GluN1/GluN2B receptors. In addition to the role of NMDAR subunits in endocytosis, activity can also modulate internalization (Vissel et al. 2001; Prybylowski et al. 2005), through a ligand-induced conformational change that would promote receptor internalization. Stimulation of the NMDAR glycine site "primes" receptors for clathrin-dependent endocytosis (Nong et al. 2003). The presence of specialized endocytic zones in regions lateral to the PSD (Blanpied et al. 2002; Petralia et al. 2003) suggests that NMDAR might be required to move laterally away from the synaptic area into this zone to be internalized.

NMDAR exocytosis

The insertion of NMDAR at the cell surface is tightly regulated during development and in response to synaptic activity and sensory experience. Phosphorylation is a well-known mechanism for regulating receptor trafficking. Phosphorylation of GluN1 subunit near the RRR ER retention signal by PKC and PKA promotes NMDAR trafficking to the plasma membrane through the secretory pathway (Scott et al. 2001). Activation of PKC enhances NMDAR channel opening and rapidly delivers new channels to the surface of hippocampal neurons, through SNARE-dependent exocytosis (Lan et al.

2001). Association of NMDAR with PSD-95 also enhances the surface expression of NMDAR but occludes PKC potentiation of channel activity (Lin et al. 2007). In addition, the activation of group I mGluR (Lan et al. 2001), dopamine receptors (Dunah & Standaert 2001), and TNFα (Wheeler et al. 2009) promote the insertion of NMDAR in cell membrane. Palmitoylation can also dramatically regulate the trafficking of NMDAR, and notably GluN2A and GluN2B subunits synaptic expression *via* two palmitoylation sites in their C-terminal region (Mattison et al. 2012).

b. Lateral diffusion

NMDAR not only cycle in and out of synaptic sites through endocytosis/exocytosis cycles, they also move laterally in the plasma membrane between synaptic and extrasynaptic sites (Bard & Groc 2011). This initial concept of receptors lateral diffusion was first established at the neuromuscular junction. Experiments showing that extra-junctional acetylcholine receptors rapidly diffuse in the muscle membrane (Young & Poo 1983) led to the "diffusion-trap" model according to which the nerve contact region is a trap for rapidly diffusing receptors in the membrane, concentrating receptors during innervation. The concept was forgotten for a couple of decades until a series of studies established that neuronal activity could lead to a rapid redistribution of receptors from synaptic to non-synaptic sites (reviewed in (Choquet & Triller 2003).

B. NMDAR surface trafficking

1. How to measure NMDAR surface mobility?

As a result of thermal agitation, all membrane receptors naturally move within the lipid plasma membrane and undergo random Brownian motion. However, in a typical cell membrane, receptor mobility is strongly influenced by physical obstacles and reversible biochemical interactions (Choquet & Triller 2013). A variety of methods have been developed to optically track receptor movement (Triller & Choquet 2005; Groc, Lafourcade, et al. 2007). They can be separated in 2 major groups: 1) ensemble methods which measure the average surface diffusion of a population of receptors and 2) single-molecule detection methods which retrieve the diffusion properties of individual receptors over time.

a. Ensemble methods

Electrophysiology: first hints of NMDAR mobility

NMDAR lateral mobility was first demonstrated using an electrophysiological tag approach. This technique relies on the pharmacological inactivation of synaptic receptors with use-dependent blockers, allowing for temporal measurements of endogenous receptor exchange at synapses (Tovar & Westbrook 2002; Adesnik et al. 2005; Harris & Pettit 2007). Schematically, a subpopulation of surface receptors is irreversibly blocked, and the receptor surface diffusion is estimated from time-dependent functional recovery after receptor blockade. For instance, a substantial recovery of NMDAR-mediated EPSC was observed after blockade of synaptic NMDAR by MK-801 and subsequent washout (Tovar & Westbrook 2002). Indeed, 65% of NMDAR activated by synaptic stimulation were exchanged within 7 min (Tovar & Westbrook 2002). The recovery from MK-801 block did not result from new receptor insertion as blocking all surface receptors with whole-cell co-application of NMDA and MK-801 prevented synaptic recovery (Tovar & Westbrook 2002). A similar approach found no recovery of synaptic NMDA current after MK-801 synaptic blockade and subsequent washout in hippocampal slices, claiming that a stable pool of extrasynaptic NMDAR does not rapidly exchange

with synaptic receptors (Harris & Pettit 2007). Such discrepancy is likely due to the different protocols used to stimulate (electrical stimulation *versus* glutamate uncaging) and record NMDAR-mediated currents (whole-cell *versus* field recording).

Fluorescence Recovery after Photobleaching (FRAP)

Fluorescence labeling techniques coupled to live microscopy also provide a way to measure surface receptor trafficking. It is possible to quantify the proportion of receptors that diffuse in a given area by measuring fluorescence recovery after photobleaching (FRAP), which in turn provides important information on the bulk dynamics of a receptor population. Two main parameters can be extracted from the recovery curves: the time constant of recovery and the amount of recovery at the end of the experiment, also called mobile fraction. Initial FRAP studies mainly focused on AMPAR trafficking. Still, one group assessed NMDAR surface trafficking using FRAP after labeling cortical cultured neurons with conotoxin (NMDAR antagonist) bound to tetramethylrhodamine (Benke et al. 1993). Although the contribution of internalized NMDAR is unclear, approximately 30% of surface NMDAR were mobile with an average diffusion coefficient of 0.05μm^2/s. Another study used the pH-sensitive GFP variant superecliptic pHluorin (SEP), which fluoresces at the neutral pH of the extracellular space but is quenched within acidic internal compartments (Ashby et al. 2004; Ashby et al. 2006) to specifically track the surface population of NMDAR (Bard et al. 2010). A 50% fluorescence recovery of SEP-tagged NMDAR was observed in dendrites and 20% in synapses, indicating that about 80% of synaptic receptors are immobile (Bard et al. 2010), consistent with previous reports (Sharma et al. 2006).

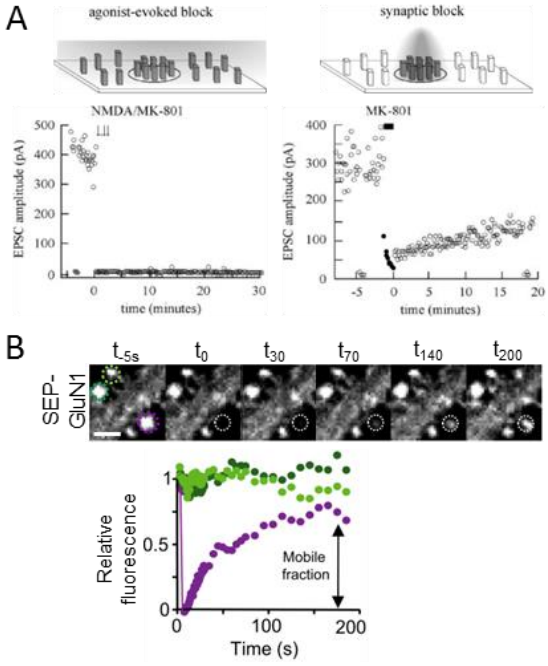

Figure 5. NMDAR lateral diffusion was first assessed using electrophysiological and bulk fluorescence imaging approaches

(A) Application of the use-dependent channel blocker MK-801 in presence of NMDA leads to a complete and irreversible (persistent after MK-801 wash) suppression of NMDAR-mediated EPSC, indicating that all surface NMDAR are inhibited. By contrast, MK-801 application alone allows the selective block of synaptic NMDAR, activated by spontaneous glutamate release. In this case, EPSC are also suppressed but recover after a few minutes, indicating that non-inhibited NMDAR replace inhibited ones in the synaptic area. (From Tovar & Westbrook 2002).

(B) Time-lapse imaging of SEP-GluN1 in hippocampal neurons in basal condition (green circles) or FRAP condition (purple circle). Scale bar= 1µm. The principal of this bulk imaging method is conceptually similar to the electrophysiological approach. The fluorescence signal of SEP-GluN1 is photobleached by a laser hit restricted to a determined area (purple spine). Few seconds after photobleaching, the fluorescence reappears in the « FRAP spine » to reach back a steady-state, indicating that photobleached receptors have been replaced by naive SEP-GluN1 receptors. (Adapted from Dupuis et al., 2014)

b. Single-molecule detection methods

Single-Particle Tracking (SPT)

Single-particle tracking is a powerful technique to track the movement of individual receptors in real time with high temporal and spatial resolutions (Cognet et al. 2006; Groc, Lafourcade, et al. 2007). Unlike FRAP and other ensemble methods which measure the bulk exchange of a population of molecules, SPT can be used to measure the diffusion of individual receptors. This method is based on the coupling between a molecule (*e.g.* organic dye) or a particle (*e.g.* quantum dot) and an antibody directed against an extracellular epitope, allowing the visualization and mapping of receptor trajectories. The typical outcome is the complete distribution of the behavior of surface proteins, which have revealed non-Gaussian shapes and a variety of diffusion characteristics at a given time. SPT confirmed that NMDAR are indeed mobile at the surface of hippocampal neurons and exchange between synaptic and extrasynaptic areas through lateral diffusion (Groc et al. 2004a; Groc et al. 2006; Bard et al. 2010; Mikasova et al. 2012; Dupuis et al. 2014). NMDAR surface trafficking relies on a continuous and dynamic equilibrium between stabilized and diffusive states. Once entered in the synaptic area, NMDAR diffuse and explore the PSD membrane for only several seconds (Groc et al.

2006). About 30% of NMDAR exchange between the PSD and the extrasynaptic compartment, consistent with previous observations (Tovar & Westbrook 2002; Harris & Pettit 2007). NMDAR lateral mobility varies depending on the GluN2 subunit composition (Groc et al. 2006; Bard et al. 2010). To date, this approach has unraveled the surface trafficking of numerous neurotransmitter receptors and channels, including glutamatergic ionotropic AMPA (Borgdorff & Choquet 2002; Tardin et al. 2003; Groc et al. 2004b; Groc & Choquet 2006; Groc et al. 2008) and metabotropic mGluR5 (Sergé et al. 2003), glycine receptors (Dahan et al. 2003), GABA receptors (Bouzigues & Dahan 2007), Kv potassium channels (O'Connell et al. 2006), CB1 receptors (Mikasova et al. 2008), EphrinB2 receptors (Mikasova et al. 2012), dopamine D1 receptors (Ladépêche, Julien P Dupuis, et al. 2013; Ladépêche, Yang, et al. 2013), KCC2 transporter (Chamma et al. 2013), GLT-1 transporter (Murphy-Royal et al. 2015; Al Awabdh et al. 2016).

High-resolution technique: Quantum Dots (QD) tracking

QD are passive semiconductor nanocrystals with large cross section absorption, which are water soluble and functionalized for biological applications (Groc, Lafourcade, et al. 2007). In addition to their superior brightness compared with single dyes, QD are much more photostable than single dyes, allowing tracking of labeled receptors for minutes (Michalet et al. 2005; Groc, Lafourcade, et al. 2007). They are subject to blinking, which actually provides a criterion to identify individual QD because fluorescence changes between "on" and "off" states for a single Dot alternate only in two levels (Michalet et al. 2005). In addition, the high signal-to-noise ratio allows one to fit the fluorescent signal to a two-dimensional Gaussian function to identify the centroid of the object with a pointing accuracy typically between 5 and 30 nm. This pointing accuracy is below the diffraction limited resolution of the light microscope, which makes possible high-resolution spatial tracking of receptors within a compartment. QD tracking allows to follow the diffusion of a single receptor across different surface areas over time. Several parameters can be extracted from each receptor trajectory (Triller & Choquet 2008) (cf figure):

- *the Mean Square Displacement (MSD) over time* is a measure of the area explored by the receptor over time. The MSD reflects the diffusion behavior of the receptor. In the case of Brownian or free diffusion, the MSD plot over time appears linear whereas for confined receptor movement (for example in the PSD, a dense protein area), the MSD plot will curve to a plateau.

- *the instantaneous diffusion coefficient ($\mu m^2/s$)*, is related to the apparent speed of displacement of the molecule, and which more or less reflects the speed of the receptor within the plasma membrane. It is calculated by plotting the first 4 points of the MSD curve such as:

$$\mathrm{MSD}\,(\tau) = < \mathrm{r}^2(\tau) > = \; 4\mathrm{D}.\tau \qquad with\ \mathrm{D}: instantaneous\ diffusion\ coefficient;\ \tau: time$$

- *the exchange frequency (Hz)* quantifies the exchange rate between distinct compartments, *i.e.* the number of receptor exits and entries between the synapse and its vicinity.

- *the residency time (s)* is a measure of the time spent by the receptor within a given compartment.

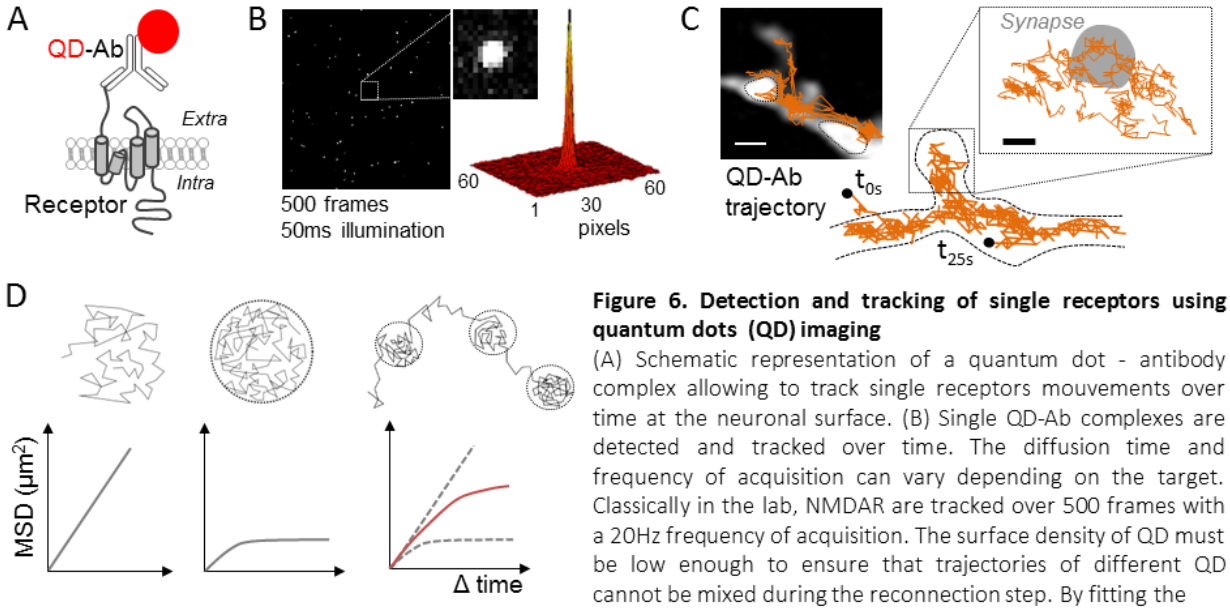

Figure 6. Detection and tracking of single receptors using quantum dots (QD) imaging

(A) Schematic representation of a quantum dot - antibody complex allowing to track single receptors mouvements over time at the neuronal surface. (B) Single QD-Ab complexes are detected and tracked over time. The diffusion time and frequency of acquisition can vary depending on the target. Classically in the lab, NMDAR are tracked over 500 frames with a 20Hz frequency of acquisition. The surface density of QD must be low enough to ensure that trajectories of different QD cannot be mixed during the reconnection step. By fitting the fluorescence QD-Ab signal to a 2D Gaussian function, nanoparticles can be localized with a pointing accuracy of 10-30nm. (C) The different trajectories collected over the 500 frames are the reconnected. To account for the blinking of QD, an algorithm was further used to reconnect iteratively portions of trajectories originating from the same QD. Full trajectories can be superimposed to a synaptic labelling which allows to study the behavior of single receptors in the different neuronal compartments (synapse versus extrasynaptic compartment). Diffusion parameters of QD-Ab trajectories can further be analyzed for quantification. (D) Examples of MSD as a function of time and the corresponding trajectories. Left panel: Brownian diffusion (free diffusion) with full correlation between the time and the area explored; Middle panel: restricted diffusion in a given area is characterized by a confined behavior quickly reaching a plateau. This behavior is typical of a synaptic receptor; Right panel: partially confined diffusion which is typical of a receptor exploring different compartments. The resulting MSD curve is intermediate between free and restricted behaviors.

Despite the high temporal and spatial resolution provided by the technique, there are several limitations to SPT which lies with the labeling method itself. First, the amount of antibodies needs to be tightly controlled in order to find a good equilibrium between a low amount of receptors labelled (thus enough statistical elements to analyze) and avoid cross-link that could alter receptor diffusion properties and the endocytosis rate. For such reasons, SPT studies have been largely restricted to cultured brain cells. But recent optimization allowed neurotransmitter receptors tracking in an integrated environment such as organotypic slices (Biermann et al. 2014; Varela, Ferreira, et al. 2016) and acute brain slices (Varela, Dupuis, et al. 2016). The total size of the QD-antibody-receptor complex (can reach in principle 30 nm) may influence diffusive properties or restrict access to sterically confined membrane domains. Such factors are especially important when tracking receptors within restricted synaptic compartments. Indeed, QD nanoparticles are still bigger than organic dyes and rather bulky (10-30 nm). As a consequence, synaptic receptors tracked using organic-dye-conjugated antibodies have faster mobility than QD-conjugated antibodies (Groc et al. 2004a). Nonetheless, QD do enter into and exchange at synapses (Dahan et al. 2003; Groc, Lafourcade, et al. 2007). Recent advances in reducing the size of the probes and engineering small monovalent affinity ligands allow for better access to the synapse (Leduc et al. 2015; Chamma et al. 2016).

Super-resolution techniques

A number of approaches have emerged that generate reconstructed images of single-molecule localizations at high density. Photoactivatable localization microscopy (PALM) and related methods that use photoactivatable fluorescent proteins (Betzig et al. 2006; Hess et al. 2006) are directly suitable for super-resolution imaging on living cells (Hess et al. 2007; Shroff et al. 2008). Using evanescent wave illumination, single-particle tracking can be obtained by sptPALM at high densities in the basal membrane of the cells. Another single-molecule-based super-resolution imaging approach, named points-accumulation-for-imaging-in-nanoscale-topography (PAINT), was introduced a few years ago (Sharonov & Hochstrasser 2006). An optimized version named universal PAINT

(uPAINT) has been developed a few years ago (Giannone et al. 2010) provides super-resolved images as well as long single-molecule trajectories (up to tens of seconds) at high densities (up to 120µm^{-2}). uPAINT and sptPALM are incontestably very powerful methods allowing to combine super-resolution and real time single molecule detection and tracking tend to be more and more used (Nair et al. 2013; Chamma et al. 2016; Li & Blanpied 2016).

Thus, a variety of experimental approaches gives access to diffusional properties of surface receptors, and more generally to surface proteins. No single technique can reveal the entire complex behavior of a dynamic receptor population. It is only when combining several techniques and interpreting the data as a whole that a clear and more complete picture of receptor dynamics begins to emerge.

2. Activity-dependent modulators of NMDAR surface trafficking

a. Developmental switch, synapse maturation and NMDAR surface trafficking

NMDAR subunit composition is not static but changes during development in response to neuronal activity or sensory experiences. GluN2A subunit replaces GluN2B subunit at synaptic sites during the second postnatal week (Monyer et al. 1994). At early developmental stages, mainly GluN1/GluN2B diheteromers are expressed at synapses as indicated by the high sensitivity of EPSCs to the selective GluN2B inhibitor ifenprodil. At mature synapses, the ifenprodil sensitivity is much reduced and the kinetic decay of EPSCs is faster than at younger synapses, consistent with a switch in the synaptic content from predominantly GluN1/GluN2B to predominantly GluN1/GluN2A receptors (Bellone & Nicoll 2007; Rauner & Kohr 2011). This shift in synaptic GluN2 subunit composition requires synaptic activity or sensory experience to occur, and is often associated with the refinement of neuronal connections within cortical areas (van Zundert et al. 2004). Dark reared rats show an impaired GluN2 subunit switch, with a reduced GluN2A/2B ratio at synapses, higher sensitivity for ifenprodil, and

slower kinetics than control animals. This experience-dependent process is bidirectional since returning the animals to the dark reduces the GluN2A/2B ratio to the level observed in animals that have never been exposed to the light (Philpot et al. 2001). Similarly, an acute and bidirectional change in GluN2 subunit composition can be induced by synaptic activity in the hippocampus. The induction of LTP in young (P2-9) hippocampal slices results in a very rapid switch in synaptic composition from GluN2B to GluN2A subunit, as revealed by faster kinetics and a reduction of the ifenprodil sensitivity of NMDAR (Bellone & Nicoll 2007). LTP in older hippocampal slices failed to induce such a switch (Bellone & Nicoll 2007). Although it is well documented that the total expression of GluN2A subunit is dramatically increased during the critical period, and that GluN2A replaces GluN2B subunit in synaptic membranes in a NMDAR activity-dependent manner (Barria & Malinow 2002), the precise mechanisms underlying this subunit switch are unknown. Using single particle and molecule approaches, Groc et al. demonstrated that the developmental change in the synaptic content of GluN2A and GluN2B subunits was correlated with a developmental change in the time spent by the different receptor subtypes within the synapse (Groc et al. 2006). GluN2B subunit residency time (*i.e.* estimates the receptor stabilization within the PSD) was three-fold decreased from 8*div* to 15*div*, indicating a higher surface stabilization of GluN2B subunit in early synapses when compared with more mature ones. Consistently, GluN2A subunit showed a symmetric evolution with a better stabilization later in development (Groc et al. 2006). These data support a developmental model in which the regulation of synaptic NMDAR subtypes is dependent on the synaptic surface stabilization of the receptors. Mice lacking the scaffold protein PSD-95 and PSD-93 (Elias et al. 2008) show a deficit in the GluN2 subunit switch. Moreover, the developmental expression of PSD-95 family proteins follows that of GluN2 subunits; SAP102 and GluN2B subunit are expressed at early stages while GluN2A subunit and PSD-95 are expressed at more mature stages (Petralia et al. 2005; Sans et al. 2000). Additionally, SAP102 and PSD-95 have been proposed to play a role in the NMDAR subunit switch during development (van Zundert et al. 2004). Schematically, a preference of certain MAGUKs for different NMDAR subtypes suggests that different NMDAR scaffolding proteins could

affect the trafficking and synaptic localization of GluN2A and GluN2B subunits during synaptic development (Shi et al. 1997; Sans et al. 2000; Yoshii et al. 2003). In this model, GluN2A subunits are incorporated in the synapse, PSD-95 is inserted into the center of the PSD and displaces GluN2B-SAP102 complexes initially located at the PSD to the perisynaptic and extrasynaptic membranes (Shi et al. 2000; Yoshii et al. 2003; Townsend et al. 2003). Consistent with this hypothesis, the disruption of the GluN2A/PSD-95 interaction using a synthetic peptide rapidly increases GluN2A subunit surface diffusion hippocampal neuronal cultures (Bard et al. 2010). Intracellular dialysis with this peptide induces a slowdown of NMDAR mEPSCs and increases the ifenprodil sensitivity of NMDAR EPSCs, consistent with the increased GluN2B subunit synaptic content and parallel decrease of GluN2A subunits (Bard et al. 2010). This report therefore suggests that the synaptic localization of NMDAR subtypes is dynamically regulated by PSD-95 family proteins. It is noteworthy that the highest affinity of PSD-95 and SAP102 to GluN2A and GluN2B subunits has been challenged (Al-Hallaq et al. 2007). The current developmental data on surface diffusion of GluN2A and GluN2B subunits indicate that the change in synaptic retention results from differences in surface stabilization of the NMDAR subtypes. It is possible that the preferential localization of the GluN2 subunits with specific scaffold proteins implicate other partners, as NMDAR interact with many other proteins.

b. Synaptic plasticity and NMDAR surface trafficking

Long-lasting experience-dependent changes in the efficacy of synaptic transmission are believed to represent cellular correlates of learning and memory. These adaptive properties often require the activation of NMDAR and NMDAR-dependent calcium influx in the postsynaptic compartment. In addition to glutamate and co-agonists, activation of NMDAR requires membrane depolarization to remove the voltage-dependent magnesium block. The combined requirement for glutamate and postsynaptic depolarization enables NMDAR to detect coincident pre- and postsynaptic activity, a

prerequisite for the induction of Hebbian synaptic plasticity such as long-term potentiation (LTP) or depression (LTD) (Collingridge et al. 2004; 2010).

Contribution of NMDAR subunits to LTP and LTD

The determining role of NMDAR in various forms of LTP and LTD has been long established, but the specific role of NMDAR subunits and their surface localization (synaptic *versus* extrasynaptic) in the formation of synaptic plasticity remains controversial (reviewed in Yashiro & Philpot 2009; Paoletti et al. 2013; Shipton & Paulsen 2014; Volianskis et al. 2015). Still, many studies report a critical role for the GluN2B subunit in LTP induction. The GluN2B-specific antagonist ifenprodil blocks LTP (Barria & Malinow 2005; Lu et al. 2001; Zhao et al. 2005) whereas it is attenuated by the overexpression of the GluN2A subunit (Barria & Malinow 2005). LTP is impaired in the CA1 hippocampal region after GluN2B knockdown but remains unaffected by GluN2A knockdown (Foster et al. 2010). A chimeric GluN2B subunit in which the C-tail is replaced by that of GluN2A fails to rescue LTP after GluN2B knockdown, whereas the reverse chimera, a GluN2A channel with a GluN2B tail could restore LTP (Foster et al. 2010). These results strongly support the idea that GluN2B plays a critical role for LTP and more precisely that its C-terminal tail could provide anchoring sites for synaptic molecules crucial for LTP mechanism. In line with this, LTP is impaired when the interaction between the GluN2B subunit and PSD-95 (Gardoni et al. 2009) or the GluN2B/CaMKII complex (Zhou et al. 2007) are disrupted. Genetic studies also support a role for GluN2B in LTP since GluN2A-lacking mice are still able to show hippocampal LTP (Berberich et al. 2005; Weitlauf et al. 2005). Overexpression of the GluN2B subunit in mice enhances hippocampal LTP as well as learning and memory (Tang et al. 1999) whereas GluN2B subunit deletion in the frontal lobe or hippocampal region impairs behavior and short-term spatial working memory (von Engelhardt et al. 2008). However, another reported that specific blockade of GluN2A subunit suppresses LTP while blocking GluN2B subunit suppresses LTD, suggesting that GluN2A subunit is involved in LTP while GluN2B subunit participates in LTD (Liu et al. 2004). Papouin et al (2012) showed that the magnitude of LTP expression is attenuated when D-serine is degraded, suggesting a key role for synaptic NMDAR in LTP. By contrast, degradation of

glycine has no effect on LTP, indicating that extrasynaptic NMDAR activity would not be required for LTP induction. Although much less studied than LTP, few studies have explored the role of NMDAR subunits on the induction of LTD. After blockade of synaptic NMDAR using MK-801, a theta-burst simulation protocol that normally generates LTP can instead produce LTD, suggesting that selective activation of extrasynaptic NMDAR induces LTD (D. dan Liu et al. 2013). LTD induction is impaired in mice lacking the GluN2B subunit in the CA1 hippocampus region (Brigman et al. 2010). But several studies report that mice lacking GluN2A subunit also fail to induce LTD (Philpot et al. 2007; Zhao & Constantine-paton 2007). One study gathering the data from several independent laboratories has reported that ifenprodil failed to block LTD (Morishita et al. 2007), concluding that the GluN2B subunit is not required for this type of plasticity. Another study proposes that both synaptic and extrasynaptic NMDAR are required for full LTD expression, although the LTD deficit is more pronounced when extrasynaptic NMDAR are inhibited by glycine degradation (Papouin et al. 2012). Besides, the role of triheteromeric NMDAR is often undetermined and their contribution in plasticity processes is undoubtedly underestimated (Paoletti et al. 2013). It is also recognized that LTP and LTD can be generated via different mechanisms, and may be associated with different receptors (Bellone et al. 2008; Sihra et al. 2014; Chater & Goda 2014). Overall, no clear-cut conclusion can be drawn regarding the preferential role of GluN2A and GluN2B in the induction of LTP and/or LTD, and whether synaptic plasticity is associated with changes in the number and/or the composition of synaptic NMDAR remains an open question. Though, beyond the contribution of one or another subunit, it seems that it is the ratio between GluN2A and GluN2B subunits that matters (Yashiro & Philpot 2009; Xu et al. 2009).

Is NMDAR trafficking involved in synaptic plasticity?

Beyond changes in NMDAR surface expression and subunit composition, NMDAR lateral diffusion can also have profound influences on the functioning of synapses, and appears to be required for LTP expression. Dupuis and collaborators (Dupuis et al. 2014) provided the first evidence that LTP induction is indeed associated with a local and rapid lateral redistribution of surface GluN2B subunit.

Following LTP induction, GluN2B subunit surface diffusion is increased while GluN2A subunit diffusion remains unchanged. Contrary to GluN2A subunits that are stabilized at the PSD, synaptic GluN2B subunits are rapidly displaced toward peri and extrasynaptic areas (Dupuis et al. 2014). Strikingly, immobilization of surface GluN1/GluN2B receptors using a cross-link protocol prevents LTP induction (Dupuis et al. 2014). Noteworthy, NMDAR cross-link obtained via a double-layer of antibodies targeting extracellular epitopes of the receptor, efficiently immobilized NMDAR without affecting NMDAR-mediated currents. Thus, these data highlight the existence of a non-canonical and key role of GluN2B subunit surface dynamics in controlling synaptic plasticity in the young hippocampus (Dupuis et al. 2014). A recent study using a similar cross-linking procedure further confirmed the key role of NMDAR surface dynamics in plastic mechanisms (Potier et al. 2015). Through a combination of *in vitro*, *ex-vivo* and *in vivo* approaches, the authors demonstrated that reducing NMDAR surface trafficking in the hippocampus selectively impairs LTP and associated temporal fear memory (Potier et al. 2015). But one could argue that such an artifical cross-link of NMDAR might not have any physiological (or rather pathophysiological in this case) relevance, and that the alterations previously observed only result from bulk experimental interventions. Remarkably, NMDAR cross-link has also been observed in a naturally occurring autoimmune disorder (Mikasova et al. 2012). Autoimmune anti-NMDAR encephalitis is a neuropsychiatric disorder characterized by the presence of autoantibodies targeting the extracellular part of NMDAR, and associated with early psychiatric symptoms and cognitive impairment (Dalmau et al. 2007). The presence of NMDAR autoantibodies was shown to prevent GluN2B subunit diffusion in the extrasynaptic compartment, and consequently led to the abolishment of LTP (Mikasova et al. 2012; Zhang et al. 2012; Dupuis et al. 2014), further supporting a major role for NMDAR surface dynamics in LTP expression. Altogether, these results suggest that beyond the type of subunit and the location of the NMDAR, its surface trafficking may be responsible for LTP and LTD, supporting the idea that not only NMDAR channel activity *per se* regulates LTP and sustains the formation of associative long-term memory (Potier et al. 2015).

4. Extracellular regulators of NMDAR surface trafficking

a. NMDAR co-agonists

NMDAR activation not only requires the binding of glutamate, but also requires the concomitant binding of co-agonists glycine or D-serine. The regional preference for a co-agonist appears closely related to the molecular composition of synaptic NMDAR (Henneberger et al. 2013). Besides the regional specificity, a segregated role for D-serine and glycine has been reported at the level of the synapse. Papouin et al. (2012) demonstrated that D-serine and glycine differentially modulate the surface behavior of GluN2A and GluN2B subunits. GluN2A subunit surface diffusion is not affected in presence of exogenous D-serine but is slowed down by glycine. On the contrary, glycine does not affect GluN2B subunit trafficking while D-serine does. Such phenomenon persists in the presence of AP5, a NMDAR antagonist (Papouin et al. 2012). Of note, a switch from glycine to D-serine occurs during postnatal development (Le Bail et al. 2015), and parallels the progressive replacement of GluN2B by GluN2A subunits in NMDAR assemblies (Bellone & Nicoll 2007).

b. Extracellular matrix proteins (ECM)

Reelin

Reelin is a large secreted glycoprotein acting as a signaling molecule. Exogenous application of recombinant reelin has been reported to modulate glutamatergic synaptic plasticity in the hippocampus (Beffert et al. 2005; Qiu et al. 2006), which most likely goes with changes of NMDAR surface mobility (Groc 2007). Inhibition of reelin decreases the surface mobility of GluN2B subunit whereas reelin overexpression strongly alters GluN2B subunit localization and reduces GluN2B-mediated synaptic currents (Groc, Choquet, et al. 2007). Interestingly, this effect is mediated by the activation of β1-integrin (Groc, Choquet, et al. 2007). In addition, the decreased contribution of GluN1/2B receptors to NMDAR-mediated synaptic currents, which normally occurs during synaptic maturation, is concomitant with the accumulation of reelin at active synapses (Groc, Choquet, et al.

2007). Thus, by controlling NMDAR lateral mobility, reelin represents a mode of control of synaptic NMDAR assembly at postnatal hippocampal synapses.

Matrix metalloproteinases (MMP)

Matrix metalloproteinases are present in the extracellular space and act as endopeptidases capable of cleaving the extracellular matrix. MMP, and especially MMP-9 has been found to colocalize with NMDAR (Gawlak et al. 2009) and is involved in NMDAR-dependent synaptic plasticity, as well as learning and memory (Meighan et al. 2006; Bozdagi et al. 2007; Michaluk & Kaczmarek 2007). MMP-9, but not its inactive form E402A, markedly increases GluN1 subunit surface diffusion both in synaptic and extrasynaptic compartments, indicating that MMP-9 affects NMDAR surface diffusion through its enzymatic activity (Michaluk et al. 2009). This effect is not mediated by cleavage of the NMDAR subunits but involves β1-integrin. Indeed, in presence of a function-blocking antibody against the β1-integrin, the effect of MMP-9 on NMDAR surface mobility was completely abolished, suggesting that MMP-9 requires β1-integrin to regulate NMDAR surface trafficking. Interestingly, MMP-9 interacts with integrin (Wang et al. 2003) and can act via integrin signaling (Nagy et al. 2006). It thus emerges that the presence of specific extracellular matrix proteins, such as MMP-9 or reelin, impact on the surface trafficking and signaling of NMDAR, notably through the activation of integrins.

c. Hormones: the example of estradiol

One of the most potent physiological regulators of NMDAR-dependent plasticity and memory in the hippocampus is the sex hormone 17β-estradiol E2 (Smith et al. 2009). Potier et al. (2015) demonstrated that E2 modulates NMDAR surface content and trafficking, a necessary condition for E2-induced LTP in the hippocampus. E2 exposure leads GluN2A subunit to be strongly anchored in the synapse while synaptic GluN2B subunit is laterally displaced, thus increasing the synaptic GluN2A/GluN2B ratio (Potier et al. 2015). Reducing NMDAR surface trafficking by using a cross-linking protocol (which immobilizes membrane receptors) in the CA1 area selectively impaired LTP

and mnemonic retention of temporal associative fear memory (Potier et al. 2015). Therefore, NMDAR surface dynamics regulate LTP and sustain the formation of associative long-term memory (Potier et al. 2015).

4. Membrane regulators of NMDAR surface trafficking

a. Dopamine receptors

Dopamine is a powerful modulator of glutamatergic neurotransmission and NMDAR-dependent synaptic plasticity. Dopamine D1 receptors (D1R) and NMDAR interact both physically and functionally (Lee et al. 2002; Lee & Liu 2004; Scott et al. 2002). D1R are highly dynamic at the surface of hippocampal neurons and form clusters in the vicinity of glutamatergic synapses, providing a perisynaptic reservoir of NMDAR (Ladépêche, Julien P Dupuis, et al. 2013). Disrupting D1R-NMDAR interactions either through D1R activation or by using a competing peptide rapidly and reversibly increases D1R lateral diffusion within the synaptic area, indicating that the acute disruption of D1R-NMDAR interactions laterally displaces D1R out of the synaptic area (Ladépêche, Dupuis, et al. 2013; Ladépêche, Yang, et al. 2013). In parallel, releasing NMDAR from D1R interaction increases NMDAR mobile fraction, upregulates NMDAR synaptic content and enhances LTP. This is all consistent with a scenario in which disrupting perisynaptic D1R-NMDAR complexes would lead to the lateral dispersal of NMDAR, and their trapping within the PSD by PDZ proteins (Ladépêche, Julien P Dupuis, et al. 2013). Altogether, these data indicate that D1R-NMDAR interactions specifically and bidirectionally regulate the surface diffusion and distribution of both receptors in the synaptic area. Acctivating D1R laterally redistributes D1R and NMDAR, thereby specifically increasing the synaptic trapping of NMDAR.

b. EphrinB2 receptor

Another membrane partner directly interacting with NMDAR is the tyrosine kinase receptor of EphrinB2 (EphB2R), a postsynaptic protein involved in cell migration mechanisms. EphB2R directly interact with NMDAR through the extracellular domain of EphB2R binding to the extracellular N-terminus of GluN1 subunit (Dalva et al. 2000). Ligand binding of EphB2R potentiates EphB2R-NMDAR interaction and stabilizes NMDAR at the synapse (Dalva et al. 2000; Nolt et al. 2011). Indeed, activation of EphB2R triggers the synaptic accumulation of GluN2B subunit and potentiates NMDAR-mediated Ca^{2+} influx via the tyrosine phosphorylation of GluN2B subunit by Src (Takasu et al. 2002). Moreover, EphB2R-KO mice show a decreased NMDAR synaptic content and a reduction of the amplitude of NMDA-mediated EPSC associated with a reduced LTP in the hippocampus (Sheffler-Collins & Dalva 2012). Finally, autoantibodies associated with NMDAR autoimmune encephalitis have been shown to weaken the EphB2R-NMDAR interaction, to alter the NMDAR synaptic content and to impair the expression of synaptic plasticity (Mikasova et al. 2012), confirming that direct interactions with the EphB2R rule the dynamic retention of synaptic NMDAR and thereby modulate synaptic adaptation.

c. Metabotropic glutamate receptor 5 (mGluR5)

A direct surface crosstalk also exists between NMDAR and metabotropic glutamate receptor mGluR5 (Perroy et al. 2008). Both receptors physically interact at the plasma membrane via the C-terminal segment of mGluR5 which allows them to undergo a reciprocal constitutive inhibition (Perroy et al. 2008). In addition to this physical crosstalk, mGluR5 and NMDAR also functionally interact (Perroy et al. 2008). Activation of the mGluR5 upregulates NMDAR surface expression in striatal neurons (Jin et al. 2015), and enhances NMDAR and CaMKII phosphorylation in striatal and hippocampal neurons (Takagi et al. 2012; Jin et al. 2015). In addition, mGluR5 activation is required for the activity-dependent GluN2B/2A subunit switch (Matta et al. 2011). Beyond modulating NMDAR expression

and function, mGluR activation also regulates NMDAR surface trafficking. In that way, Dupuis et al. demonstrated that blocking mGluR5 activity during LTP prevents the increase in lateral diffusion of GluN2B subunit normally triggered by an LTP stimulus, further supporting a role of mGluR5 in this process (Dupuis et al. 2014).

5. Intracellular regulators of NMDAR surface trafficking

a. MAGUKs (membrane-associated guanylate kinases)

The C-terminus tail of NMDAR plays a role in determining the precise location of GluN2 subunits. A genetically-modified mouse line expressing GluN2A subunit lacking its C-terminus (GluN2A ΔC/ΔC) shows a reduced synaptic GluN2A subunit expression (Steigerwald et al. 2000). Consistently, GluN2A subunit ΔC/ΔC mice display slower NMDAR kinetics, indicating a decrease in synaptic NMDAR containing GluN2A subunits (Steigerwald et al. 2000). GluN2A and GluN2B subunits C-tails contain PDZ binding motifs which both bind to a PDZ domain of MAGUK proteins. Disruption of GluN2/PSD-MAGUK interaction has been shown to decrease the localization of GluN2 subunits at the synaptic membrane (Gardoni et al. 2006; 2012). Members of this MAGUK family (PSD-93, PSD-95, SAP97 and SAP102) show differential subcellular localization, with PSD-95 predominantly expressed at the PSD and SAP102 being distributed more evenly between synaptic and extrasynaptic sites (van Zundert et al. 2004). Several studies suggest that synaptic and extrasynaptic NMDAR are likely to be associated with different MAGUK scaffolding molecules. Specifically, at mature synapses, GluN1/GluN2A receptors are linked to PSD-95 and located principally at the PSD, whereas GluN1/GluN2B receptors coupled to SAP102 are predominantly located at peri- or extrasynaptic sites (Sans et al. 2000; van Zundert et al. 2004; Zhang & Diamond 2009; Groc et al. 2009). In line with this, PSD-95 knockdown reduces NMDAR EPSC amplitude in hippocampal slice cultures (Ehrlich et al. 2007). However, similar associations between GluN2A or GluN2B subunits with MAGUK proteins have also been reported in isolated brain fractions of rats (Al-Hallaq et al. 2007). RNA silencing of PSD-95, PSD-93 or SAP102 has

no effect on NMDAR EPSC amplitude whereas double knockdown of PSD-95 and PSD-93 reduces NMDAR transmission (Elias et al. 2006). Similarly, a complete deletion of PSD-95 does not affect NMDAR EPSC amplitude but NMDAR EPSCs display slower kinetics and a higher sensitivity to the GluN2B-specific antagonist ifenprodil, suggesting a redistribution of NMDAR subtypes at the synapse (Béïque et al. 2006). A recent study confirmed this hypothesis using a competing peptide disrupting GluN2A/PDZ interaction (Bard et al. 2010). Disruption of GluN2A-PDZ interaction specifically affects GluN2A/PSD-95 interaction and eventually leads to a decrease of synaptic GluN2A subunit content. A rapid rearrangement of GluN2B subunit localization accompanies GluN2A subunit-specific alterations, as evidenced by the increase of Ro 25-6981 (GluN2B subunit specific antagonist)-induced inhibition of NMDAR currents (Bard et al. 2010). Consistent with a reduced synaptic retention, GluN2A synaptic trafficking is increased and GluN1/GluN2A receptors are displaced out of synapses. In parallel, GluN2B subunit are redistributed to the synapse in order to compensate GluN2A subunit synaptic loss, and are stabilized in the PSD (Bard et al. 2010). Besides, PDZ-independent binding sites between GluN2 subunits and MAGUKs have also been identified (Chen et al. 2011), supporting the idea that other protein-protein interactions could modulate NMDAR subunit localization.

b. CaMKII (calcium/calmodulin-dependent protein kinase II)

CaMKII is a large holoenzyme consisting of 12 subunits playing an essential role in LTP (Lisman et al. 2002). Briefly, activation of NMDAR leads to an increase of intracellular Ca^{2+} that activates calmoduline, which in turn activates CaMKII through autophosphorylation processes (Lisman et al. 2012). Once activated, CaMKII moves from the cytoplasm to the synapse (Shen & Meyer 1999), 1999), which increases its binding to NMDAR and thereby locks CaMKII in an active conformation (Bayer et al. 2006). Several binding sites are involved in this interaction, and CaMKII can directly bind GluN1, GluN2A and GluN2B subunits *via* binding sites located in the C-terminus tail of both subunits (Leonard et al. 1999; Gardoni et al. 1999; Strack et al. 2000; Bayer et al. 2001; 2006; Lisman et al. 2012). The formation of CaMKII-NMDAR complexes plays a key role in synaptic plasticity and learning

(reviewed in (Lisman et al. 2012)). Interestingly, CaMKII also participates in the regulation of NMDAR surface trafficking (Dupuis et al. 2014). CaMKII inhibition strongly reduces the dynamics of synaptic GluN2B subunit, with no effect on GluN2A subunit (Dupuis et al. 2014). The GluN2B point mutation R1300Q/S1303D, known to block the binding of CaMKII to GluN2B (Strack et al. 2000) prevents the activity-dependent upregulation of GluN2B subunit surface dynamics, indicating that CaMKII contributes to the lateral redistribution of GluN2B subunit during LTP through their physical interaction (Dupuis et al. 2014). In addition, both GluN2B double mutant R1300Q/S1303D and NMDAR cross-link prevents CaMKII synaptic translocation after LTP induction, indicating that the interaction between GluN2B and CaMKII plays a key role in CaMKII trafficking during the onset of plasticity (Dupuis et al. 2014).

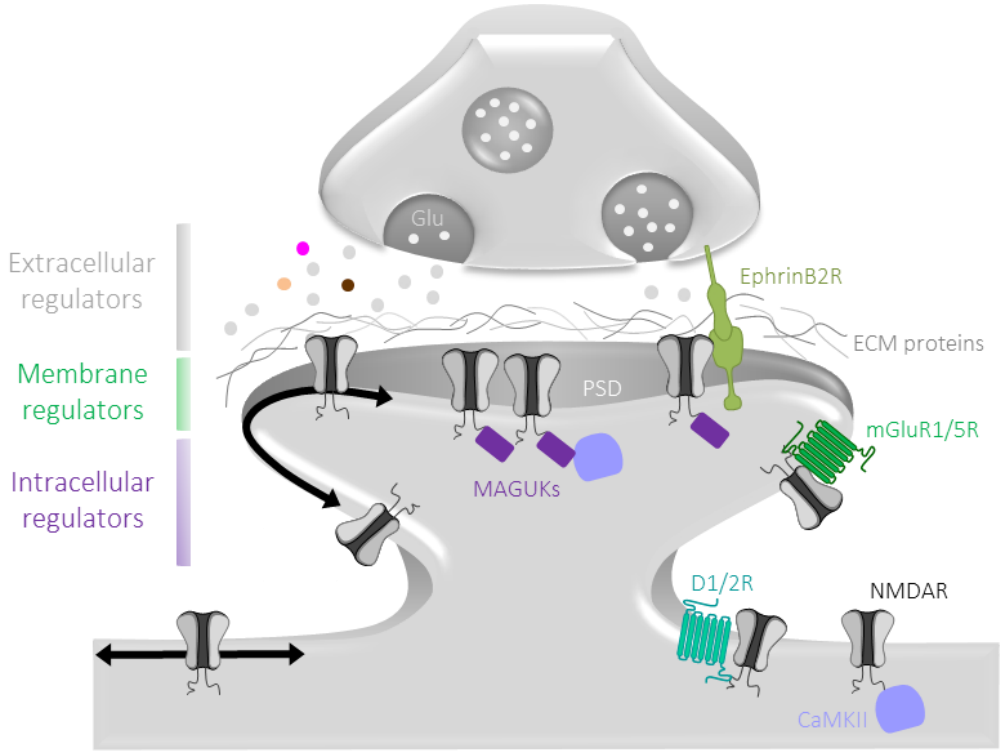

Figure 7. Regulation of NMDAR surface trafficking occurs at different cellular levels through multiple protein interactions

NMDAR do not freely diffuse at the neuronal surface but interact with many proteins modulating its exchanges between the PSD and the extrasynaptic compartments. Hence, NMDAR lateral diffusion is subjected to a multiple regulatory process occuring at: 1) The extracellular level via the action of neurotransmitters or hormones. The extracellular matrix also plays a role in the regulation of NMDAR surface trafficking and function; 2) At the membrane level, where the NMDAR directly and functionally interacts with many other membrane receptors and proteins; 3) At the intracellular level with scaffold (MAGUKs) and signalling proteins contributing to NMDAR regulation.

NMDAR surface trafficking plays a major role in synaptic plasticity, which contributes to the maintenance of many physiological functions like memory or reward. Thus, NMDAR abnormal trafficking and alteration of receptors surface distribution and/or activity can contribute to the emergence of neurological and psychiatric disorders (Lau & Zukin 2007; Paoletti et al. 2013). Neuropsychiatric disorders are characterized by an unbalance between NMDAR synaptic and extrasynaptic populations, most likely resulting from aberrant NMDAR trafficking. The main challenge that lies ahead is to understand the relationship between the different dynamic levels and how they eventually integrate to control neural network activity and, hence, brain function and dysfunction.

II - The NMDAR and its role in the emergence of schizophrenia

A. NMDAR hypofunction and schizophrenia

The term schizophrenia, from the german *schizophrenie,* literally meaning "a splitting of the mind", was first used by the psychiatrist Eugen Bleuler more than hundred years ago (Heckers 2011). Schizophrenia is a severe mental illness with a neurodevelopmental aspect, implicating genetic susceptibility and environmental determinants (Owen et al. 2016). It is typically characterized by a triad of core features: 1) positive symptoms (*e.g.* delusions, hallucinations, thought disorganization), reflecting a loss of contact with reality, 2) negative symptoms associated with social withdrawal, impaired motivation or anhedonia, and 3) cognitive symptoms (*e.g.* attention deficit, working memory impairment) which constitute the major source of disability for patients. Positive symptoms tend to be episodic and are pretty well attenuated by antipsychotics, whereas negative and cognitive symptoms are tend to be chronic and associated with long-term effects on social function. Although major progress has been done since Bleuler's era, schizophrenia remains a major challenge for psychiatry. Resistance to antipsychotic treatment is indeed far to be erratic (Mouchlianitis et al. 2016) and reminds us that much is still to be learnt about the cause of schizophrenia.

1. <u>The etiology of schizophrenia: a history of diverse hypotheses</u>

The dopaminergic hypothesis of schizophrenia

The dopaminergic hypothesis of schizophrenia (SCZ) initially arose from the search of anti-malarial treatment during World War II. Laborit was the first to appreciate the neuroleptic potential of a "cocktail" of anti-histaminergic drugs containing chlorpromazine, a derivative of phenothiazine, initially used to reduce shock in injured soldiers (Laborit and Huegenard 1951). Soon after, Delay and Deniker (1952) made the fortuitous discovery that chlorpromazine alleviates hallucinations and extinguishes internal "voices" in agitated and aggressive psychotic patients, giving birth to the first

antipsychotic treatment. The mechanism of action of chlorpromazine and derivative compounds was further explored in animals, and first suggested that neuroleptics block monoaminergic receptors and/or interfere with the release of monoamines (Carlsson & Lindqvist 1963). Conversely, several studies observed that amphetamine, a psychostimulant which increases monoamine levels, could induce psychotic symptoms. Positron Emission Tomography (PET) imaging studies later detected overactivation of dopamine synthesis as well as elevated dopamine release in SCZ patients, supporting the role of a dopaminergic alteration in the etiology of SCZ (Carlsson et al. 2001; Howes & Kapur 2009). Van Rossum was the first to explicitly propose a "dopamine hypothesis of schizophrenia", claiming that neuroleptics attenuate psychosis by blocking dopamine receptors (Van Rossum 1966). It is only a decade after that receptor-binding studies provided the direct evidence that neuroleptics indeed interfere with the dopaminergic transmission by selectively inhibiting dopamine D2 receptors (Seeman & Lee 1975). However, the dopaminergic hypothesis of SCZ does not appear to be sufficient to fully explain the pathology as an alteration of the dopaminergic pathway only triggers positive symptoms.

The glutamatergic hypothesis of schizophrenia

The concept of NMDAR hypofunction in SCZ, first formulated by Javitt in 1987 (Hillside J. Clinical Psych), takes its origin from the observation that recreational drugs such as phencyclidine, ketamine were able to induce SCZ-like psychotic effects in patients (Luby et al. 1959). Indeed, in the late 1950s, investigations on the potential of phencyclidine (PCP) as a human anesthetic rapidly revealed the psychogenic trait of the molecule (Luby et al. 1959; 1961). Ketamine, a less potent derivative of PCP, was also characterized as "dissociative" (Domino et al. 1965). In the meantime, brain imaging studies reported that phencyclidine could inhibit dopamine uptake and enhance dopamine release (Poels et al. 2015). The dopaminergic hypothesis then progressively stepped aside for the glutamatergic hypothesis of SCZ, stating that NMDAR hypofunction constitutes a primary alteration, followed by dopamine and other neurotransmitter systems dysfunction (Javitt et al. 2012). Twenty years later, the NMDAR was identified as the common target of these molecules (Zukin & Zukin 1979), where

they act as antagonists (Anis et al. 1983). MK-801, an anticonvulsant and anxiolytic molecule (Clineschmidt 1982), was also described as a highly potent NMDAR antagonist, binding to the same site as ketamine and PCP on the NMDAR (Wong et al. 1986). Contrary to psychogenic molecules like LSD, NMDAR antagonists are not only able to induce psychosis (taking part of the positive symptoms of SCZ), but can also produce negative and cognitive symptoms, suggesting an alternative model for SCZ pathogenesis (Javitt & Zukin 1991).

2. NMDAR hypofunction, a convergent point in schizophrenia

a. NMDAR alterations observed in schizophrenia

Postmortem studies

Multiple lines of evidence support NMDAR dysregulation as a key pathogenic mechanism of SCZ. Although mixed results emerged from human *postmortem* studies, abnormal NMDAR expression appears as a consistent alteration in SCZ patients brains (see table). Several studies have reported decreased mRNA expression of GRIN1 in the hippocampus (Gao et al. 2000; Law & Deakin 2001) while GluN2B subunit mRNA was upregulated (Gao et al. 2000). Of note, Law & Deakin (2001) observed that NMDAR alterations are asymmetrical and left-lateralized in individuals with SCZ. Downregulation of GRIN1 expression (Sokolov 1998; Beneyto & Meador-Woodruff 2008; Weickert et al. 2013; Catts et al. 2015), GRIN2A and GRIN2C mRNA (Akbarian et al. 1996; Beneyto & Meador-Woodruff 2008; Weickert et al. 2013) were also observed in the prefrontal cortex. In contrast, increased GRIN1 and GRIN2 expression in the occipital cortex (Dracheva et al. 2001), and GRIN2B in the hippocampus (Gao et al. 2000) have been reported. Abnormal NMDAR expression in the thalamus of SCZ patients, always accompanied by changes in PSD proteins levels was also observed (Clinton et al. 2003; Clinton & Meador-Woodruff 2004). Other studies found no change of NMDAR subunits gene levels in frontal and hippocampal areas (Woo et al. 2004, 2008; McCullumsmith et al. 2007; Vrajová et al. 2010; Rao et al. 2013; Catts et al. 2015; but see Akbarian et al. 1996).

Interestingly, a recent meta-analysis estimated that more than 30 subjects are required to reliably detect significant differences in GluN1 expression when using *postmortem* brain tissue from medicated and chronic SCZ patients compared to controls (Catts et al. 2016). It is thus noteworthy to mention that very few studies reach this threshold (see table). Including 95 patients and 95 controls, the same meta-analysis found a significant decrease of GluN1 subunit mRNA expression in the PFC of SCZ subjects compared with controls (Catts 2015), whereas cortical GluN2 or GluN3A subunits mRNA levels were unchanged (Weickert et al. 2013; Catts et al. 2015).

Compared to mRNA, fewer studies explored NMDAR protein levels in *postmortem* tissues, reaching similar outcomes. Older studies mainly used receptor autoradiography to estimate NMDAR density. Korhuber et al. (1989) reported an increase of MK-801 binding only in the putamen while Ishimaru and colleagues (1992) found increased glycine binding in the parietal cortex, suggesting a postsynaptic compensation for the impaired glutamatergic neurotransmission. Increased GluN2B subunit expression associated with upregulated PSD-95 levels were also found in the thalamus of SCZ patients (Clinton et al. 2006). No change of NMDAR expression has been found in the hippocampus (Kerwin et al. 1990; Gao et al. 2000; Toro & Deakin 2005; McCullumsmith et al. 2007; Geddes et al. 2014) or the PFC of SCZ patients (Henson et al. 2008). More recently, Banerjee et al. (2014) also used autoradiography to assess NMDAR density and found increased GluN1 subunit levels in the DLPFC of patients with SCZ compared to control individuals. Left-lateralization was also reported in the hippocampus of SCZ patients with lower GluN2B subunit levels in the left hippocampus compared to the right side (Geddes et al. 2014). In the prefrontal cortex, lower GluN1 (Errico et al. 2013; Weickert et al. 2013), GluN2A and GluN2B subunits (Errico et al. 2013) protein levels were detected in SCZ. Emamian et al. (2004) also reported decreased phosphorylation levels of the GluN1 subunit of the NMDAR in the frontal cortex and hippocampus of patients affected with SCZ. No changes in expression of NMDA receptor subunits (GluN1, GluN2A-D) in either the DLPFC (Kristiansen et al. 2006; Weickert et al. 2013; Catts et al. 2015) or the anterior cingulate cortex (ACC) were found, except the C2' isoform of GRIN1 that was higher in the ACC of SCZ subjects (Kristiansen et al. 2006).

Similarly, Rao et al. (2013) found that GluN1 and GluN2B subunits were unchanged in the DLPFC. In PSD-enriched fractions of *postmortem* DLPFC, there is a marked reduction in the activity of signaling cascades downstream of the NMDAR in SCZ, despite an apparent increase in NMDAR density and GluN1 expression (Banerjee et al. 2014). These results confirm previous work showing decreased GluN2A tyrosine phosphorylation, while NMDAR/PSD-95 complexes were upregulated in the PFC of patients with SCZ (Hahn et al. 2006, but see Catts et al. 2015). Another study reported concomitant increase of GluN2B subunit and PSD-95 in the CA3 region of SCZ patients (Li et al. 2015). Overall, no consensus emerges from these works, with NMDAR gene and protein levels varying in function of the brain region examined and methodology used. Indeed, receptor autoradiography might not be as accurate as recent techniques to measure protein density. Beyond these technical issues, patients' medications might also impact gene and protein expression. Sokolov et al. (1998) reported differences between SCZ patients with or without neuroleptics treatment. While GluN1 mRNA was reduced in the DLPFC of drug-free patients, GRIN1 expression was normalized in patients under neuroleptics (administered within 72h prior to death) (Sokolov 1998). However, several studies did not report any significant effect of chronic antipsychotic on NMDAR expression (Gao et al. 2000; Dracheva et al. 2001; Banerjee et al. 2014; Li et al. 2015 but see Zink et al. 2014). In view of these contradictory results, it seems difficult to give any clear-cut conclusion except that a consistent NMDAR alteration is observed in *postmortem* tissues from patients with SCZ.

Table 1. Non-exhaustive list of *post-mortem* NMDAR alterations in schizophrenic patients

↘ Decrease ↗Increase = No change

Study	Cohort details	Brain area	Technics	Outcome SCZ group
mRNA expression				
(Akbarian et al. 1996)	SCZ n= 15 Mixed psychiatric disorders neuroleptics-treated n= 8 Controls n= 15	Frontal, parietal, temporal, cerebellar cortices	In situ hybridization	↗ GluN2D mRNA in PFC ↘ GluN2C mRNA in PFC Difference between SCZ and neuroleptics-treated groups in PFC
(Sokolov 1998)	SCZ n= 21 (5 cases later excluded) Controls n= 9	DLPFC	Quantitative RT-PCR	↘ GluN1 mRNA in neuroleptic-free patients = GluN1 mRNA in neuroleptic-treated patients (within 72h prior to death) compared to controls
(Gao et al. 2000)	2 data banks Bank 1 SCZ n= 16/ Control=12 Bank 2 SCZ n= 19/ Control n=19	HPC (Entorhinal cortex, DG, CA1, CA2, CA3, subiculum)	In situ hybridization	↘ GluN1 mRNA in DG ↗ GluN2B mRNA in CA2
(Dracheva et al. 2001)	SCZ n= 26 Control n= 13 (elderly people) Alzheimer's disease n= 10	DLPFC Occipital cortex	Quantitative RT-PCR	↗ GluN1 mRNA ↗GluN2A mRNA in occipital cx = GluN2B mRNA ↗ PSD-95 mRNA
(Law & W Deakin 2001)	SCZ n= 14-16 BP n= 14-16 MD n= 14-16 Controls n= 14-16	HPC	In situ hybridization	↘ GluN1 mRNA in left DG but not right DG ↘ GluN1 mRNA in left CA3
(Clinton et al. 2003)	SCZ n= 13 Controls n= 8 (mean age 70 years old)	Thalamus	In situ hybridization	↘ GluN1 exon 22 isoform mRNA = GluN1 exons 5 & 21 mRNA ↗ NL-F, PSD-95 & SAP102 mRNA
(Clinton & Meador-Woodruff 2004)	SCZ n= 15 BP n= 15 MD n= 15 Controls n= 15 (mean age 43 years old)	Thalamus	In situ hybridization	↗ GluN2B mRNA = GluN1, GluN2A, GluN2C & GluN2D ↘ NF-L, PSD-95 & SAP102 mRNA
(Woo et al. 2004)	SCZ n= 17 BP n= 17 Controls n= 17	ACC	Double in situ hybridization	GluN2A mRNA in GAD_{67} mRNA-containing cells = between SCZ and controls
(McCullumsmith et al. 2007)	SCZ n= 8 BP n= 8 Controls n= 8	HPC	In situ hybridization	= GluN1, GluN2A, 2B, 2C, 2D mRNA = NF-L, PSD-95, PSD-93, SAP102 mRNA
(Woo et al. 2008)	SCZ n= 20 BP n= 20 Controls n= 20	ACC	Double in situ hybridization	GluN2A mRNA in CB mRNA-containing cells = between SCZ and controls
(Beneyto & Meador-Woodruff 2008)	SCZ n= 15 MD n= 15 BP n= 15 Controls n= 15	DLPFC	In situ hybridization	↘ GluN1, GluN2A & GluN2C mRNA
(Vrajová et al. 2010)	SCZ n= 13 Controls n= 8 (elderly people)	HPC	Quantitative RT-PCR WB	= GluN1 mRNA
(Weickert et al. 2013)	SCZ/Schizo affective n= 37 Controls n= 37	DLPFC	Quantitative RT-PCR WB	↘ GluN1 mRNA ↘ GluN2C mRNA
(Rao et al. 2013)	SCZ= 10 Controls= 10	DLPFC	RT-PCR	= GluN1 & GluN2B mRNA
(Catts et al. 2015)	SCZ n= 94 Controls n= 82	PFC	Meta-analysis	↘ GluN1 mRNA = GluN2A, 2B, 2D & GluN3A mRNA

Protein expression

(Kornhuber et al. 1989)	N.A Only number of brain areas/condition	Frontal cortex, HPC, amygdala, putamen	Receptor autoradiography (^{3}H-MK-801)	↗ MK-801 binding only in the putamen (tendency to ↗ in all regions)
(Kerwin et al. 1990)	SCZ n= 7 Controls n= 8	HPC (DG, CA1, CA2, CA3, CA4, paraHPC gyrus)	Receptor autoradiography (^{3}H-Glutamate)	= ^{3}H-Glu binding But ^{3}H-Glu full saturation not always reached so not really conclusive
(Ishimaru et al. 1992)	SCZ n= 13 Controls n= 10	Cerebral cortex and HPC	Receptor autoradiography (^{3}H-g|ycine)	↗ Glycine binding in the parietal cx (supra-marginal cx and angular gyrus)
(Gao et al. 2000)	2 data banks Bank 1 SCZ n= 16/ Control n=12 Bank 2 SCZ n= 19/ Control n=19	HPC (Entorhinal cortex, DG, CA1, CA2, CA3, subiculum)	Receptor autoradiography (^{3}H-NMDA)	= NMDAR binding
(Emamian et al. 2004)	N.A Tissues from MRC and Stanley Consortium Brain Bank (London, UK)	Frontal cortex HPC	WB	= total GluN1 ↘ pGluN1^{Ser897}/total GluN1 in HPC and frontal cortex
(Toro & Deakin 2005)	SCZ n= 15 MD n= 15 BP n=15 Controls n= 15	HPC Orbitofrontal cortex	Receptor autoradiography WB	= GluN1 ↘ PSD-95 in HPC (DG molecular layer) in SCZ and BP
(Hahn et al. 2006)	SCZ n= 14 Controls n= 14	PFC	IP WB	↗ erbB4-GluN1 complexes ↗ GluN1-PSD & GluN2A-PSD ↘ Tyr phosphorylation of GluN2A
(Kristiansen et al. 2006)	SCZ n= 24 Controls n= 16	DLPFC ACC	WB	↗ GluN1$^{C2'}$ in ACC = GluN1^{C2}, GluN2A, 2B, 2C, 2D ↘ PSD-95, PSD-93 proteins in ACC ↘ NF-L protein in DLPFC
(Clinton et al. 2006)	SCZ n= 15 Controls n= 8 (mean age > 70 years old)	Thalamus	WB	↗ GluN2B in dorso-medial thalamus = GluN1 & GluN2A ↗ PSD-95 in dorso-medial thalamus = NF-L & SAP102
(McCullumsmith et al. 2007)	SCZ n= 8 BP n= 8 Controls n= 8	HPC	Receptor autoradiography (^{3}H-MK801)	= MK-801 binding
(Henson et al. 2008)	SCZ n= 15 Controls n= 20	PFC	WB	= GluN1 = GluN3A
(Vrajová et al. 2010)	SCZ n= 13 Controls n= 8 (elderly people)	HPC	WB	↘ GluN1 (GluN1-4b and GluN1-2b isoforms)
(Errico et al. 2013)	SCZ n= 15 Controls n= 15	PFC Striatum	HPLC WB	↘ GluN1, GluN2A, 2B in PFC
(Weickert et al. 2013)	SCZ/Schizo affective n= 37 Controls n= 37	DLPFC	WB	↘ GluN1 = GluN2A, 2B & GluN3A
(Rao et al. 2013)	SCZ n= 10 Controls n= 10	DLPFC	WB	= GluN1 & GluN2B
(Banerjee et al. 2014)	SCZ n= 17 Controls n= 17	DLPFC PSD-enriched fractions	Receptor autoradiography (^{3}H-MK-801) IP Src kinase assay	↗ GluN1 in SCZ ↗ GluN1-PSD-95 complexes ↘ Tyr phosphorylation of GluN2
(Geddes et al. 2014)	SCZ n= 20 Controls n= 20	HPC	In situ radioligand binding (^{3}H-ifenprodil) WB	GluN2B left HPC SCZ < left HPC controls GluN2B left HPC SCZ < right HPC SCZ = GluN2B binding

(Catts et al. 2015)	SCZ n= 37 Controls n= 37 (*cf*. cohort Weickert 2013)	PFC PSD-enriched fractions	WB	↘ GluN1 and PSD-95
(Catts et al. 2016)	SCZ n= 95 Controls n= 95	PFC	Meta-analysis	↘ GluN1 = GluN2A, 2B, 2D & GluN3A
(Wei et al. 2015)	SCZ n= 21 (10 on medication, 11 off medication at death) Controls n= 21	HPC (CA1, CA3)	WB	↗ GluN2B and PSD-95 in CA3 = GluN1 in CA3 = GluN1, GluN2B and PSD-95 in CA1

N.A: Not Available; SCZ: Schizophrenia; BP: Bipolar Disorder; MD: Major Depression; HPC: Hippocampus; DG: Dentate Gyrus; CA1: Cornus Ammonis 1; PFC: Pre Frontal Cortex; DLPFC: Dorso-Lateral Pre Frontal Cortex; ACC: Anterior Cingulate Cortex; RT-PCR: Reverse transcription polymerase chain reaction; IP: Immunoprecipitation; WB: Western Blot

Brain imaging

Brain imaging studies also explored the putative relationship between SCZ and the function of brain regions. A decreased brain volume in SCZ was found in cortical areas, as well as in the hippocampus (Shepherd et al. 2012). In addition to anatomical modifications, abnormal basal activity of the hippocampus is a robust feature of SCZ, identified in early PET studies and most recently with high-resolution magnetic resonance (MR) methods. Several studies indicate that perfusion is elevated in the hippocampus in SCZ, particularly in medication-free individuals, suggesting that antipsychotic treatment partially normalize brain perfusion (Tamminga & Zukin 2015). Notable differences exist between antipsychotic-responsive and non-responsive patients, the latter showing more pronounced anatomical and functional alterations (Mouchlianitis et al. 2016). However, a recent MR study described reduced glutamate levels in the hippocampus of patients with SCZ, regardless of whether they were medicated (Stan et al. 2015). However, to date, no anatomical or functional abnormalities specific to SCZ have been identified. This could be partially due to the heterogeneity of cohorts, which compare data using different techniques with unpowered sample sizes including patients with different illness duration, previous drug treatment, etc. But the strong overlap existing between SCZ and other psychiatric disorders also complicates the identification of specific biomarkers.

b. NMDAR mutations in schizophrenia

SCZ is a polygenic disorder, resulting from variants in more than one gene. Several variants in multiple genes have been identified and are thought to act in combination with environmental risk factors to favor the emergence of psychiatric disorders. Of particular interest, genetic variants of the NMDAR have been associated with SCZ. A meta-analysis indeed reported a significant association between the GRIN2B gene, coding for the GluN2B subunit of the NMDAR, and SCZ (Li & He 2007). Another meta-analysis identified 24 genetic variants in 16 different genes including GRIN2B, APOE, COMT, DAO, DRD1, DRD2, DRD4, DTNBP1, GABRB2, GRIN2B, HP, IL1B, MTHFR, PLXNA2, SLC6A4, TP53 and TPH1, that showed significant association with SCZ (Allen et al. 2008). Of note, several of these variants encode proteins involved in dopamine and GABA transmission, 2 main neurotransmitter systems also affected in SCZ. The significant excess of rare GRIN2B missense mutations found in large cohorts of patients with either autism or SCZ (Myers et al. 2011) further emphasizes the influence of GRIN2B in psychiatric disorders. In addition, Tarabeux et al. (2011) reported 2 *de novo* mutations in GRIN2A in patients with SCZ. More recently, a large GWAS (36,989 SCZ cases and 113,075 controls) identified 108 independent loci of genome-wide significance. Of these 108 loci, notable associations include GRIN2A, dopamine D2R and many genes (*e.g.* GRM3, SRR, GRIA1) involved in glutamatergic neurotransmission and synaptic plasticity (Ripke et al. 2014). In line with this, a large exome-sequencing-based study of *de novo* mutations in SCZ revealed that small *de novo* mutations are overrepresented among NMDAR complexes and glutamatergic PSD proteins (Fromer, Andrew J. Pocklington, et al. 2014). But to date, the most significant association from GWAS of SCZ is with the major histocompatibility complex (Stefansson et al. 2009), further supporting the link between immune dysregulation and SCZ. Mutated genes found in SCZ overlap with other psychiatric disorders like bipolar disorder, and to a lesser extent with autism spectrum disorder (Lee et al. 2013; Fromer, Andrew J. Pocklington, et al. 2014). Hence, mutations in the same gene could lead to different phenotypes in different individuals while mutations in many genes affecting neuronal development or function could give rise to the same clinical phenotype (Sebat et al. 2009).

How to identify SCZ risk genes?

4 major types of genetic analyses helped to identify risk genes in SCZ over the last years:
Linkage analysis studies: detect the chromosomal location of disease genes. Linkage analysis studies led to many regions of the human genome being identified as harboring predisposing genes for SCZ rather than one or several genes of major effect (Owen 2004).

Candidate gene association studies: type of statistical test that compares the frequency of common (>1%) s*ingle-nucleotide polymorphism (*SNPs) allele markers in unrelated cohorts of SCZ patients and healthy controls.

Genome wide association studies (GWAS): "optimized" version of candidate gene association studies, using microarray chip genotyping platforms to screen up to 1 million genetic variants (SNPs) across the entire genome.

Copy number variant analyses (CNVs): these structural variants are submicroscopic deletions or duplications of DNA, ranging in length from 1 kb to several megabases. They occur in healthy individuals, but there is growing evidence that large rare CNVs are also pathogenic and increase the risk of developing SCZ.

c. NMDAR autoantibodies

The autoimmune basis of SCZ is an old concept that has been brought up to date with recent studies reporting the presence of autoantibodies in neuropsychiatric disorders (Diamond et al. 2009b; Kayser & Dalmau 2011; Coutinho et al. 2014). Among them, the most frequent and well described is the NMDAR encephalitis, an autoimmune disorder associated with autoantibodies directed against the extracellular part of the GluN1 subunit of NMDAR (Dalmau et al. 2007). NMDAR encephalitis patients express prominent psychotic symptoms (but also negative and cognitive symptoms) directly correlated with the antibody titers (Dalmau et al. 2007, 2008; 2011). Binding of such antibodies rapidly alters NMDAR surface trafficking, and trigger massive internalization of NMDAR (Masdeu et al. 2016), eventually leading to synaptic plasticity impairment in the hippocampus (Zhang et al. 2012; Planagumà et al. 2016). Recent studies have reported the presence of NMDAR autoantibodies in the serum of SCZ patients (Ezeoke et al. 2013a; Pollak et al. 2013; Pearlman & Najjar 2014) and even observed a beneficial impact of immunotherapy treatment in those individuals (Zandi et al. 2011; Gungor et al. 2016; Senda et al. 2016), strongly supporting the idea that such antibodies might also have a pathogenic action. Yet, whether NMDAR autoantibodies found in patients with SCZ share the same properties as the ones produced in NMDAR encephalitis is still unknown. Thus, it is crucial to better explore this question in order to understand their possible implication in the etiology of psychotic disorders (see article 1).

d. Psychotomimetic NMDAR antagonists

Pharmacological properties of psychotomimetic NMDAR antagonists

Psychotomimetic NMDAR antagonists, which are typically channel blockers, target the most highly conserved portion of the NMDA receptor. The binding site for uncompetitive NMDAR antagonists, also called the "PCP binding site", is located deep within the channel and thus requires the receptor to be in an active state to allow the drugs to gain access to the pore. Such uncompetitive antagonists include MK-801, PCP, and ketamine. The incidence of psychotomimetic effects after administration of these compounds is rather variable but appears to correlate with the affinity of the drug for the PCP binding site of the NMDAR (Kornhuber & Weller 1997). Different behavioral profiles of psychotomimetic NMDAR antagonists are likely influenced by potency differences between NMDAR subtypes, even if NMDAR channel blockers show little selectivity across NMDAR subunits (Dravid et al. 2007). MK-801 is more potent at inhibiting GluN1/GluN2A and GluN1/GluN2B receptors than GluN1/GluN2C and GluN1/GluN2D ones (Ogden & Traynelis 2011). (-)-ketamine and (+)-ketamine, two enantiomers of ketamine, have different pharmacological properties as well as different psychotomimetic effects, (+)-ketamine showing a higher affinity for the PCP binding site and being able to induce positive symptoms contrary to (-)-ketamine (Dravid et al. 2007). Interestingly, (+)-ketamine is less potent than the racemate at inhibiting GluN1/GluN2A and GluN1/GluN2B receptors, but about 1.5 times more potent at inhibiting GluN1/GluN2C and GluN1/GluN2D receptors. (+)-ketamine, memantine and PCP are less potent on GluN1/GluN2A than on GluN1/GluN2B-D receptors (Dravid et al. 2007). Although structurally diverse, NMDAR channel blockers are all positively charged and act in a voltage-dependent manner. Ketamine and memantine are less use- and more voltage-dependent than MK-801, with PCP being intermediate (Macdonald et al. 1991). Memantine and ketamine inhibit NMDAR with similar affinities and kinetics. Though, ketamine shows psychotomimetic properties while memantine does not. This divergence in effect could actually be explained by a mechanistic difference between both compounds in their off-rate or "trapping block" (Sleigh et al. 2014). Compounds with a slow off-rate such as ketamine (86% trapping) and MK-801

(almost 100% trapping) are high-trapping antagonists (Sleigh et al. 2014), meaning that when the glutamate has dissociated from its binding site, these drugs remain trapped in the ion channel, causing a prolonged tonic blockade. In contrast, fast off-rate antagonists like memantine are able to escape from the channel before it closes, and is thus associated with minimal psychotomimetic effects (Kotermanski et al. 2009). In addition, memantine would bind to a superficial non-trapping site, distinct from the deep site where other psychotomimetic compounds are trapped (Kotermanski et al. 2009). Depending on the concentration, uncompetitive NMDAR antagonists can also target other membrane proteins. Kapur and Seeman (2002) proposed that the affinity of ketamine for D2R (Ki= 0.5 ± 0.2µM) is equal to its affinity for NMDAR. Yet, Jordan et al. (2006) demonstrated using *in vitro* assays that PCP, ketamine and MK-801 lack D2R full agonist, partial agonist and antagonist activity. Ketamine is also able to bind α7nAChR, serotonin 5-HT_{2A} and $GABA_A$ receptors (Lodge & Mercier 2015; Hevers et al. 2008). Potassium channels and monoaminergic systems constitute additional targets for PCP and ketamine, but require dissociative concentrations in the tens of micromolar (Lodge & Mercier 2015). In any case, it is likely that at clinically relevant doses, these non-selective effects of NMDAR antagonists are not a major component of their molecular action. Although the pharmacological properties of psychotomimetic NMDAR channel blockers have been extensively studied, the cellular and molecular mechanisms underlying their psychogenic effect remain unclear.

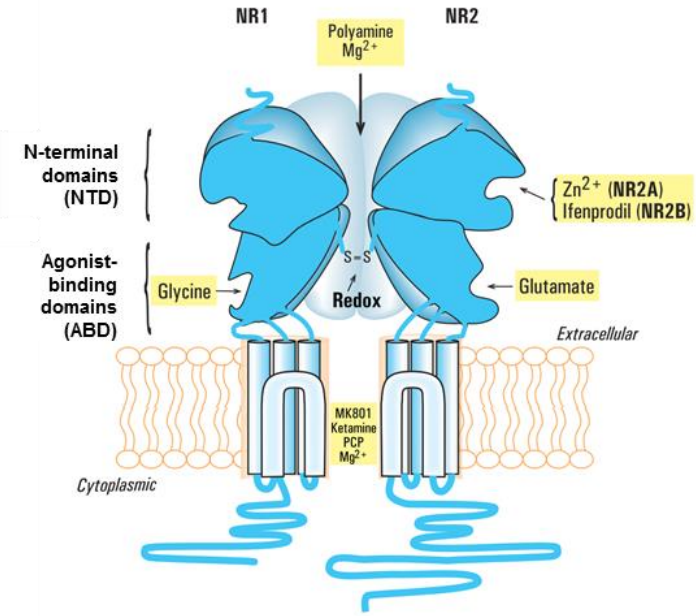

Figure 8. NMDAR binding sites for agonists, antagonists and allosteric modulators.
NMDAR are assembled as tetramers, organize as « dimers of dimers » composed of 2 GluN1 subunits and 2 GluN2/3 subunits. Competitive agonists and antagonists binds to the glutamate binding site located on the GluN2 subunit, and to the co-agonist binding site on the GluN1 subunit. The ion-channel contains a binding site also called the « PCP site » for pore blockers such as endogenous Mg^{2+} or synthetic compounds such as PCP, MK-801 and ketamine. Binding of channel blockers necessitates the receptor to be activated so that the channel is open and the binding site accessible. The NMDAR also contains modulatory sites for positive and negative allosteric modulators such as Zn^{2+} or ifenprodil.
(Adapted from Kemp & McKernan, 2002)

Effect on human individuals

Psychotomimetic NMDAR antagonists have been widely used to model SCZ, with the advantage of presenting robust face validity. Unlike psychostimulants dopamine compounds (*e.g*. amphetamine), NMDAR antagonists reproduce a large range of symptoms expressed by patients with SCZ, and do not necessitate a prolonged use to induce psychosis. In humans, a single dose of PCP and ketamine induce psychosis in healthy volunteers (Javitt & Zukin 1991; Krystal et al. 1994; Malhotra et al. 1996; Lahti et al. 2001) and worsen mental status of SCZ patients (Luby et al. 1959; Javitt & Zukin 1991; Krystal et al. 1994; Lahti et al. 1995, 2001; Malhotra et al. 1997). The similarities between SCZ and the effect of NMDAR antagonists are remarkable in mimicking disorganization symptoms, *i.e.* formal thought disorders and behavioral disorganization (Adler et al. 1999; Krystal et al. 1999). Administration of PCP and ketamine in healthy volunteers also results in cognitive dysfunction, such as a decrease in learning and memory performances similar to findings in SCZ (Morgan & Curran 2006). Brain imaging studies further supported this "NMDAR antagonists" model, mainly focusing on the effect of ketamine. In the absence of PET tracers for glutamate receptors, initial imaging research took an indirect approach by administering ketamine and measuring changes in blood flow, metabolism or dopamine receptor binding. Ketamine administration increases blood flow and metabolism in frontal areas of the brain, suggesting an increased glutamate release similarly observed in SCZ patients and rodent models (Stone 2009). The acute administration of ketamine to healthy volunteers also decreased dopamine D2 receptor binding in striatum (Stone 2009). MRS studies found that ketamine led to increased glutamine levels in medial prefrontal cortex, reflecting increased glutamate release, whereas SPECT imaging showed that ketamine led to a global reduction in [^{123}I]CNS-1261 binding, which binds to the pore of the NMDAR (Stone 2009). Overall, the SCZ-like phenotype induced by psychotomimetic NMDAR antagonists in healthy volunteers suggests that SCZ relies on a dysfunction of NMDAR.

3. Animal models to further understand schizophrenia

Animal models are an important tool, and still necessary, for understanding pathological mechanisms and for testing hypotheses that cannot be addressed in human studies. Obviously, investigating SCZ as any psychiatric illness in animals is challenging, as these illnesses disturb "high" functions often assigned to humans only (*e.g.* delusions and thought disorders). Three main validation criteria have been proposed to score the relevance of an animal model (van der Staay 2006; Adell et al. 2012):

- *face validity,* refers to the fact that a model should resemble the condition to be modeled and should recapitulate important anatomical, biochemical, neuropathological or behavioral features of a human disease. In most psychiatric disorders, the etiology is unknown and face validity is thus restricted to the similarity of symptomatology.

- *predictive validity,* refers to the potential of a model to make adequate predictions in humans. In psychiatry, predictive validity is often limited to predict the efficacy of new therapeutics in human subjects.

- *construct (or etiological) validity,* refers to the disease relevance of the methods by which a model is constructed. In theory, the model should recreate in an animal the etiological processes that cause a disease in humans, and thus should replicate cellular and behavioral features of the illness. Construct validity is, theoretically speaking, impossible to reach for mental illnesses.

Table 2. Comparison of clinical manifestations of schizophrenia with schizophrenia-like behaviors induced by NMDA antagonist (modified from Bubenikova-Valesova 2008)

Clinical manifestations of schizophrenia	Behavioral changes in animal models of schizophrenia
Psychotic symptoms	Increased locomotor activity, hyperactivity
	Increased sensitivity to psychostimulant drugs
Stereotypic behaviors	Stereotypic behaviors
Vulnerability to stress	Change of locomotor activity induced by stress
Information processing deficits	Deficits of pre-pulse inhibition
Attentional deficits	Deficits of latent inhibition
Cognitive deficits	Working and spatial memory impairments
Social withdrawal	Reduced social interaction

No animal model is able to fully mimic a complex psychiatric disease like SCZ but certain symptoms have been modelled (see table). Positive symptoms include hallucinations and delusions, and are believed to reflect aberrant dopaminergic transmission as classical antipsychotics blocking D2R partly attenuate such symptoms (Howes & Kapur 2009). In animals, dopaminergic alterations can be assessed by locomotor activity, which is dependent on dopaminergic innervation. Exacerbated response to psychostimulants is also often used as a sign of positive-like behavior. Negative symptoms are characterized by blunted affect, poor speech, asocial behavior, lack of motivation, self-negligence. In animal models, these symptoms are often modelled by deficits in PPI, in latent inhibition, lack of sucrose preference, reduced social interaction with peers. Finally, in SCZ patients, cognitive symptoms include deficits in working memory and conscious control of behavior. Hence, a good animal model for SCZ should reproduce all these deficits to show good face validity.

a. Pharmacological model of NMDAR hypofunction (see table 3)

The pharmacological model of NMDAR hypofunction consists in acute or chronic administration of NMDAR antagonists. Basically, PCP, ketamine and MK-801 are the three psychotomimetic molecules almost exclusively used to induce pharmacological NMDAR hypofunction. The ability of NMDAR antagonists to induce a large range of symptoms similar to SCZ has been extensively described over the last decades, and shows robust face validity. However, the molecular mechanisms underlying such behavioral alterations remain underexplored and misunderstood although several studies examined NMDAR expression after acute or chronic treatment with NMDAR antagonists (see table). Anastasio and Johnson (2008) described an increase of GluN1 and GluN2B subunits in the frontal cortex of rats injected 8h or 24h ahead with PCP. Increased GluN1 subunit levels are also found in cortical cultures incubated for 24h with ketamine (F. Liu et al. 2013). By contrast, MK-801 rapidly (by 10 min) reduces GluN2B phosphorylation levels both in cortical cultures and in the frontal cortex of injected animals (Carty et al. 2012). Decreased levels of GluN2B and GluN2C subunits mRNA are also observed in the entorhinal and parietal cortex of animals exposed to MK-801 for a few hours (Linden

et al. 1997; 2001). Such modifications of NMDAR gene and protein expression have an impact on NMDAR-dependent synaptic plasticity. Indeed, LTP is impaired in the hippocampus of animals injected with NMDAR antagonists. Synaptic plasticity (LTP and LTD) is abolished in hippocampal slices treated 30 min with ketamine (Izumi & Zorumski 2014). Strikingly, a single systemic injection of MK-801 is sufficient to induce long-lasting impairments of synaptic plasticity (Manahan-vaughan et al. 2008). LTP is profoundly impaired in freely moving rats 7 days after MK-801 injection, and marked deficits are still evident up to 4 weeks after the initial administration (Wöhrl et al. 2007; Manahan-vaughan et al. 2008; Wiescholleck & Manahan-Vaughan 2013). Similarly, long-lasting LTD deficits are induced by MK-801 treatment (Manahan-Vaughan et al. 2008).

Chronic administration of MK-801 decreases hippocampal expression of GRIN2A (Oh et al. 2001; Rujescu et al. 2006) while increasing GRIN1, and especially GluN1 exon 5 inclusion splice variant (Rujescu et al. 2006). Oh et al. (2001) reported increased GRIN2B expression with no change of GRIN2C, whereas Rujescu et al (2006) found that both GRIN2B and 2C expression are reduced. Another study reported that systemic injections of MK-801 decrease GRIN1 expression in the thalamus and the dorsal hippocampus (Wiseman Harris et al. 2003; but see Matthews et al. 2000). Such discrepancy might result from different protocols and administration routes. Indeed, one group used continuous intra-cerebro-ventricular infusion during 7 days (Oh et al. 2001) whereas the others opted for chronic intraperitoneal injections for 14 days (Matthews et al. 2000; Rujescu et al. 2006), which might differently target NMDAR and affect their expression. Chronic PCP injections upregulate GluN1 and GluN2A cortical levels (Anastasio & Johnson 2008), and are associated with increased GluN2A/PSD-95 interactions (Anastasio et al. 2009). This upregulation is still observed in the prefrontal cortex, but not the hippocampus, of P80 rats that received injections 1 week after birth (Owczarek et al. 2011a). Elevated GluN1 and GluN2B subunits are also detected in the cortex of mice chronically treated with ketamine (Chatterjee et al. 2012). While increasing total GluN1 expression, chronic PCP treatment strongly decreases cortical GluN1 phosphorylation levels (Mouri et al. 2007). Of note, dopamine D1R agonist is able to restore normal levels of phosphorylated GluN1 as well as

learning deficits, consistent with the well-established functional interplay between glutamatergic and dopaminergic systems (Mouri et al. 2007). These observations are consistent with the alterations of NMDAR phosphorylation described in *postmortem* tissues from SCZ patients (Emamian et al. 2004; Hahn et al. 2006b; Banerjee et al. 2014b). The GABAergic neurotransmission is also compromised following acute and chronic administration of NMDAR antagonists. Reversible loss of GAD67 and parvalbumin (PV) GABAergic interneurons is observed in primary cortical cultures exposed to ketamine (Behrens et al. 2008). Similarly, chronic MK-801 injections decrease GAD67 mRNA expression and PV-positive interneurons (Rujescu et al. 2006). By contrast, acute PCP treatment does not affect the expression or phosphorylation of the GluN1 subunit of the NMDAR (Mouri et al. 2007). In conclusion, NMDAR inhibition tends to downregulate NMDAR in cortical and hippocampal areas. However, many parameters can influence NMDAR expression (*e.g* the type of antagonist, the dose, the treatment duration, the administration route, the animals species, the brain region examined, the detection technique used, etc.). Therefore, data comparison should be done with caution and carefully consider all these elements.

Table 3. NMDAR-related molecular changes induced by acute or chronic treatment with psychotomimetic NMDAR antagonists

↘ Decrease ↗Increase = No change

Study	Dose	Duration	Animals	Region	mRNA levels	Protein levels	Plasticity
Acute injection							
MK-801							
(Abraham & Mason 1988)	0.1, 0.5, & 1mg/kg i.p. CPP 0.45, 1 & 10mg/kg i.p.	Single i.p. injection	Sprague-Dawley 300-500g	HPC			Ø LTP after 150min MK-801 5mg/kg Ø LTP after 6-8h CPP 10mg/kg ↘ LTP 80% after 150min MK801 1mg/kg
(Linden et al. 1997)	5 mg/kg i.p.	Single injection Killed 4h after	Wister rats	HPC	↘ GluN2C only in EC layer III = GluN2A/2B/2C/2D		
(Lindén et al. 2001)	0.5, 2.5, 5mg/kg i.p.	Single injection Killed 4h or 8h after	Wistar rats 200-250g	Cortex	↘ GluN2B 4h after MK-801 5mg/kg in parietal cortex ↘ GluN2C 8h after MK-801 2,5 & 5mg/kg in EC layer III		

(Wiseman Harris et al. 2003)	0.5mg/kg s.c.	2 injections, 8h apart	Sprague-Dawley rats P7	LDT dHPC vHPC	↘GluN1 in LDT and dHPC ↗ GluN1 in vHPC		
(Wöhrl et al. 2007)	5mg/kg i.p.	Single injection	Wistar rats P90-120	HPC			Ø LTP after 24h LTP still altered for up to 7 days
(Manahan-vaughan et al. 2008)	5mg/kg i.p.	Single injection 7days or 4weeks before experiments	Wistar rats (260–280g) 7-8 weeks	HPC (DG)			Ø LTP 7days after MK-801 Weak LTP 4weeks after MK-801
(Manahan-Vaughan et al. 2008)	5mg/kg i.p.	Single injection 7days or 4weeks before experiments	Wistar rats 7-8 weeks	HPC (DG)			= LTD 7days after MK-801 LTP restored by GlyT inhibitors
(Carty et al. 2012)	50mM 0.6mg/kg i.p.	14-21div 10, 30, 60min 10, 30, 60min	Rat C57BL/6 mice (6–8 months)	Cortical cultures Frontal cortex		↘ pGluN2B 10, 30 & 60min after incubation ↘ GluN1 & GluN2B after 2h incubation ↘ pGluN2B/STEP interaction ↘ pGluN2B 10 & 30min after injection	
(Wieschollek & Manahan-Vaughan 2013)	5mg/kg i.p	Single injection 7days before experiments	Wistar rats (7-8 weeks)	HPC			↘ LTP 7days after MK-801 LTP restored by PDE4 inhibitor (rolipram)
PCP							
(Anastasio & Johnson 2008)	10mg/kg s.c.	0h, 4h, 8h, 24h Killed 24h after last injection	Sprague-Dawley rats P7	Frontal cortex		↗ GluN1 after 8h & 24h ↗ GluN2B after 8h & 24h = GluN2A ↘ GluN1 & GluN2B in ER fractions	
Ketamine							
(Liu et al. 2013)	10µM	5div 24h incubation	Rat	Forebrain cultures		↗ GluN1	
(Izumi & Zorumski 2014)	1-100µM	30min incubation and washout	Sprague-Dawley rats P30-32	HPC slices			Ø LTP 100µM Ø LTD 1-10µM
(Iafrati et al. 2014)	30 or 100mg/kg i.p.	Single injection Killed 24h after injection	Heterozygous reeler mouse (HRM) P22–28	PFC			↗ LTP with KET100 (rescues HRM mouse deficits)
Subchronic/Chronic injections							
MK-801							
(Matthews et al. 2000)	0.4mg/kg i.p	i.p. injection every 12h for 14 days	Sprague-Dawley rats P15	HPC & cerebral Cortex		= GluN1, GluN2A & 2B	
(Oh et al. 2001)	1pmol/10ml/h i.c.v.	Continuous infusion for 7days	N.A	HPC & cortex	↗ GluN1 & GluN2B ↘ GluN2A = GluN2C		
(Rujescu et al. 2006)	0.02mg/kg i.p.	Daily injections for 14 days	Long Evans rat P32-40	HPC	↘GluN2B ↗ GluN1 exon 5 inclusion splice variant ↘ GluN2C/GluN2A	↘ PV+ interneurons	
(Baier et al. 2009)	0.25 mg/kg i.p	Injections from P6-P21 Tested at P30, P60, P90, P120,	Wistar rats P6-P21	Cortex		↗ GluN1 at P120	

		P150 & P180					
PCP							
(Sircar et al. 1996)	5mg/kg i.p	Daily injections between P5-P15 + withdrawal 6 days	Sprague-Dawley rat P21	Cortex Cerebellum	= GluN1, 2A & 2C ↘ GluN2B in cortex = NMDAR in cerebellum	↘ GluN1 (↘^{3}H-MK-801 binding)	
(Mouri et al. 2007)	10mg/kg s.c.	Daily injections during 14days + Withdrawal 4days	Male mice 30g	PFC		↗ GluN1 in total tissue ↘ p(Ser^{897})-GluN1 in total tissue extracts, rescued by D1R agonist ↘ p(Ser^{897})-GluN1 in membrane-enriched extracts	
(Anastasio & Johnson 2008)	10mg/kg s.c.	P7 + P9 + 11 Killed 24h after last injection	Sprague-Dawley rats P7	Cortex		↗ GluN1 & GluN2A ↘ GluN1 &GluN2A in ER fraction	
(Anastasio et al. 2009)	10mg/kg s.c	P7, P9 & 11	Rat	Cortex		↗ GluN2A bound to PSD-95 ↗ GluN2A & 2B bound to SAP-102	
(du Bois et al. 2009)	10mg/kg s.c	P7, P9 & 11 Killed 24h, 7days, 3weeks or 12weeks after the last injection	Sprague-Dawley rats	ACC, PFC, HPC		↗ [3H]MK-801 binding in PFC, ACC ↗thalamus at P18-P96 ↗ [3H]MK-801 binding in HPC at P96	
(Owczarek et al. 2011)	10 mg/kg s.c	P7, P9 & P11	Rat <P80 (12 weeks)	PFC HPC		↗ GluN2A /2B in PFC = GluN1, GluN2A & 2B in HPC	
Ketamine							
(Chatterjee et al. 2012)	100mg/kg i.p.	10 days	Swiss mice P21	Cortex	↗ GluN1 & GluN2B = GluN2A		

N.A: Not Available; PCP: phencyclidine; MK-801: dizocilpine; i.p.: intraperitoneal; s.c.: subcutaneous; i.c.v.: intra-cerebro-vetricular; P7: Post-Natal day 7; HPC: Hippocampus; DG: Dentate Gyrus; EC : Entorhinal cortex ; dHPC: dorsal hippocampus; cHPC; ventral hippocampus; LDT: Latero-Dorsal Thalamus; PFC: Pre Frontal Cortex; LTP: Long-Term Potentiation; LTD: Long-Term Depression

b. Genetic models of NMDAR hypofunction

Genetic models represent an alternative tool to explore the role of NMDAR in the pathophysiology of SCZ. Numerous transgenic lines are available to manipulate NMDAR in either a constitutive or conditional manner. So far, nearly all NMDAR mutants have been designed to study the role of NMDAR signaling in learning and memory processes. A full gene deletion of the GluN1 subunit is not viable, and thus requires the use of either partial knock-out (KO) or heterozygous animals. Several studies used transgenic mice expressing low levels of NMDAR in the entire brain (Mohn et al. 1999; Duncan et al. 2004; Halene et al. 2009), and all report typical SCZ-like behavioral alterations such as

PPI deficits, impairment of spatial and working memory or decreased social interaction. Duncan et al. (2004) observed that partial NMDAR KO in adult animals do not induce any behavioral deficits contrary to a "developmental" KO, supporting the neurodevelopmental hypothesis of NMDAR-dependent deficits in SCZ. Based on *postmortem* findings from SCZ patients (Emamian et al. 2004), Li et al. designed a constitutive "phospho-mutant" NMDAR knock-in in which the GluN1 S897 is replaced with alanine avoiding its phosphorylation. Mutant animals exhibit impaired LTP, associated with impaired PPI and social behavior (Li et al. 2009). Specific deletion of NMDAR in the cortex also leads to cognitive deficits but fails to induce positive or negative-like behaviors (Rompala et al. 2013). Deletion of the GluN1 subunit in corticolimbic inhibitory neurons or in forebrain pyramidal neurons leads to behavioral and cellular changes reminiscent of SCZ (Belforte et al. 2010; Tatard-Leitman et al. 2015). Mice with targeted suppression of NMDAR on PV-positive interneurons exhibit reduced GAD67 and PV levels, accompanied by a disinhibition of cortical excitatory neurons and reduced neuronal synchrony (Belforte et al. 2010). However, a recent study using the same mouse model failed to reproduce such behavioral deficits (Bygrave et al. 2016), and temper the current view that some symptoms of SCZ result from hypofunction of NMDAR on cortical GABAergic interneurons (Homayoun & Moghaddam 2007; Belforte et al. 2010). Other transgenic models of SCZ which do not directly affect the NMDAR exist. All these models share the characteristic to target elements of the glutamatergic synapse, either directly (DISC-1, α7nAChR, SR) or indirectly (NRG1, dysbindin, synGAP) interacting with the NMDAR.

Table 4. Genetic models of NMDAR hypofunction to study schizophrenia

Study	Animals	Genetic manipulation	Brain region	Major findings
		NMDAR deletion		
(Mohn et al. 1999)	B6D2 mice 8 weeks	Constitutive KO *Nr1^{neo} -/-* Hypomorphic GluN1 allele	Entire brain	1- GluN1 mRNA levels reduced to 8.1% and protein levels to 7.3% of normal levels 2- *Nr1^{neo} -/-* mice exhibit hyperlocomotion, stereotypy and social deficits, which are reversed by antipychotics 3- PCP & MK-801 lack psychostimulant effects in *Nr1$^{neo\ -/-}$* mice
(Duncan et al. 2004)	129/SvEv, C57BL/6, and DBA/2 mice	Constitutive KO *GluN1 -/-* Hypomorphic GluN1 allele	Entire brain	1- *GluN1 -/-* mice show dramatic reduction of ^{3}H-MK-801 binding in HPC, somatosensory cortex and putamen 2- *GluN1 -/-* mice show altered social interaction and deficient PPI 3- *GluN1 -/-* mice show altered spatial working memory, and impaired PPI 4- Adult KO do not show any behavioral deficits observed in mice with developmental KO
(Halene et al. 2009)	> 2months	Constitutive KO *Nr1^{neo} -/-* *cf*. Mohn 1999	Entire brain	1- *Nr1$^{neo\ -/-}$* mice show dramatic reduction of ^{3}H-MK-801 binding in HPC, somatosensory cortex and putamen 2- *Nr1$^{neo\ -/-}$* mice show reduced sociability
(Li et al. 2009)	C57BL/6 mice 6-12weeks	Constitutive KI S897A NR1 phosphomutant	Entire brain	1- NMDAR/AMPAR ratio is decreased in the HPC of S897A NR1 mutant mice 2- LTP is impaired in mutant mice, and is associated with decreased synaptic levels of GluN1-NMDAR and GluR1-AMPAR 3- S897A NR1 mutant mice do not exhibit normal social behavior and show impaired PPI
(Belforte et al. 2010)	Mice >2 months	Spatial constitutive KO *Ppp1r2-Cre* (protein phosphatase1, regulatory subunit 2)	Cortex & HPC GABA neurons	1- GluN1 mRNA levels are reduced by 40-50% in GAD_{67} HPC and cortical neurons 2- GAD_{67} & PV expression are reduced in mutant neurons, associated with increased firing of excitatory neurons 3- *Ppp1r2-Cre* mice show altered spatial working memory, and impaired PPI 4- Adult KO do not show any behavioral deficits observed in mice with developmental KO
(Rompala et al. 2013)	C57BL/6	Spatial constitutive KO *CtxGluN1 KO*	Cortex	1- Only 50% of CA1 pyramidal neurons show NMDAR currents 2- *CtxGluN1 KO* mice show impaired PPI, reduced social interaction and spatial working memory deficits 3- *CtxGluN1 KO* mice do not display positive or negative-like behavior 4- MK-801 still show a psychostimulant effect in *CtxGluN1 KO* mice
(Tatard-Leitman et al. 2015)	?	Spatial constitutive KO *Camk2αCre-cKO;(td)TomatoFlox* Floxed GluN1 allele crossed with Camk2αCre	Forebrain pyramidal neuron	1- GluN1 expression is reduced by 57% in the cortex, 66% in the HPC and 34% in the striatum of *Camk2αCre-cKO* mice 2- No LTP induced in *Camk2αCre-cKO* mice 3- Pyramidal neurons in *Camk2αCre-cKO* mice fire more, and are more excitable 4- GluN1 KO is associated with decreased expression of GIRK2, D1R & D2R in the cortex, and 5HT2A in the HPC 5-*Camk2αCre-cKO* mice exhibit a schizophrenia-like phenotype (hyperactivity, reduced social behavior, spatial memory and working memory deficits) 6-Neuronal oscillations (gamma, beta, theta) are also increased in KO mice
(Bygrave et al. 2016)	Mice 2 months	Constitutive KO *Grin1$^{\Delta PV}$* PV-Cre-driver line crossed with the Grin1-2lox line	PV-interneurons	1- *Grin1$^{\Delta PV}$* mice do not show behavioral deficits at 2 months 2- *Grin1$^{\Delta PV}$* mice are still sensitive to psychostimulant MK-801 effect, and even show increased stereotypy and catalepsy after MK-801 injection 3- MK-801 induces cortical delta-oscillations in *Grin1$^{\Delta PV}$* mice

Non-NMDAR deletion

(Guo et al. 2009)	S129sv and c57/B6J mice	Constitutive KO *SynGAP* $^{+/-}$	Entire brain	1- *SynGAP* $^{+/-}$ mice exhibit elevated stereotypy and hyperactivity, which is reversed by clozapine treatment 2- *SynGAP* $^{+/-}$ mice also show impaired PPI and cognitive, social and working memory deficits
(Zhou et al. 2010)	Black Swiss and S129sv mice 6 months	Constitutive KO *Sp4* $^{neo\ -/-}$ Hypomorphic Sp4 allele	Entire brain	1- *Sp4* hypomorphic mice display deficits in PPI and contextual memory 2- *Sp4* hypomorphic mice have a specific deficit in spatial learning/memory 3- LTP induction is impaired in *Sp4* hypomorphic, while synaptic transmission is normal 4- GluN1 expression is reduced in the HPC and cortex of *Sp4* hypomorphic mice, while GluN2 subunits levels are unchanged 5- Human *Sp4* gene Is deleted in sporadic schizophrenia patients
(Karlsgodt et al. 2011)	C57Bl mice	Constitutive KO Dysbindin *dys-/-*	Entire brain	1- NMDAR currents amplitude from pyramidal neurons in PFC is reduced in *dys -/-* and *dys -/+* mice 2- GluN1 mRNA expression ↘ in the PFC of *dys-/-* and *dys -/+* mice (stronger alteration in *dys -/-*) 3- Strong correlation between NMDAR gene expression and working memory performance
(Yin et al. 2013)	C57BL/6N mice	Conditional *ctoNrg1* CamK2α-tTA;TRE-Nrg1	Forebrain	1- 50%–100% NRG1 ↗ in forebrain of *ctoNrg1* mice 2- *ctoNrg1* mice show hyperactivity, impaired PPI and short-term memory/attentional deficits, ameliorated by clozapine 3-NMDAR density or composition in *ctoNrg1* mice is comparable with WT mice *4- ctoNrg1* mice have reduced mEPSC frequency in HPC and PFC, due to impaired glutamate release 5- $GABA_A$ receptor density in *ctoNrg1* mice, affecting GABAergic transmission
(Balu et al. 2013)	Adult mice 3-5 months	Constitutive KO *SR-/-* Exon 1 of the SR gene constitutively deleted	Entire brain	1- Amplitude of NMDAR currents decreased in *SR-/-* mice, without any impairment of NMDAR functional properties 2- LTP induction is maintained but the magnitude of LTP is reduced in *SR-/-* mice 3- *SR-/-* mice have a smaller HPC volume and smaller spine in the DG granule neurons 4- BDNF mRNA and protein levels, as well as p-TrkB protein are reduced in the HPC of *SR-/-* mice 5- Chronic D-serine treatment restores D-serine levels and LTP, together with cognitive deficits in *SR-/-* mice
(Wei et al. 2014)	Sprague-Dawley rats	DISC-1 KD shRNA *In vitro* transfection A2-14div – 48h expression *In vivo* lentiviral injection	Frontal cortex neuronal culture PFC	1- DISC-1 KD increases NMDAR currents, and are mainly mediated by GluN2A-NMDAR 2- DISC-1 KD induces bigger evoked NMDAR-EPSC, associated with bigger GluN2A-EPSC 3- DISC-1 KD upregulated GluN2A expression, which is particularly increased in the synaptic area 4- DISC1 shRNA increases NMDAR currents via a mechanism at least partially dependent on elevated PKA/CREB activity
Lin 2014	C57BL/6 mice	Constitutive KO *α7nAChR -/-* (B6.129S7-Chrna7tm1Bay/J)	Primary cortical cultures	1- GluN1, GluN2A and GluN2B levels are reduced in the cortex of P1-P21 *α7nAChR -/-* mice 2- Deletion of *α7nAChR* affects the development of glutamatergic synapses in the PFC 3- *α7nAChR -/-* mice have a reduced expression of cortical NMDAR, especially in glutamatergic synapses 4- sEPSC decay time and frequency were reduced in *α7nAChR -/-* pyramidal neurons 5- SR levels are reduced in *α7nAChR -/-* neurons, and D-serine does not increase sEPSC in *α7nAChR -/-* neurons

N.A: Not Available; KO: Knock-Out; KI: Knock-In; KD: Knock-Down; HPC: Hippocampus; CA1: Cornus Ammonis 1; GIRK2: G-protein-regulated inward-rectifier potassium *channel* 2; D1R: Dopamine D1 receptor; 5HT2A: Serotonine 5-HT2A receptor; GAD67: Glutamate Decarboxylase 67; PV: Parvalbumin; NRG1: Neuregulin-1; PFC: Pre Frontal Cortex; SR: Serine Racemase; LTP: Long-Term Potentiation; LTD: Long-Term Depression; PPI: Pre-Pulse Inhibition

c. NMDAR hypofunction and dysregulation of other neurotransmitter systems

Dysfunction of the GABAergic transmission has been observed in SCZ patients (Lewis & Gonzalez-Burgos 2006). The possibility that GABAergic interneurons are a preferential target for NMDAR hypofunction (Olney & Farber 1995; Lisman et al. 2008) is supported by different lines of evidence. First, acute systemic administration of NMDAR antagonists results in hyperactivity of pyramidal neurons. Second, hippocampal GABAergic interneurons are more sensitive to NMDAR antagonists than pyramidal neurons (Grunze et al. 1996). Thus, NMDAR antagonists may preferentially reduce the firing of fast-spiking interneurons, resulting in the disinhibition of excitatory neurons (Homayoun & Moghaddam 2007). Removing this GABAergic brake may then lead to a disbalance of the excitation/inhibition transmission, which may underlie cognitive and negative symptoms of SCZ (Lisman et al. 2008). Third, repeated administration of NMDAR antagonists decreases the expression of GAD67 and PV in GABAergic neurons (Cochran et al. 2003; Keilhoff et al. 2004; Rujescu et al. 2006; Behrens et al. 2007). Besides, NMDAR hypofunction may be responsible for the abnormal dopaminergic activity associated with the symptoms of SCZ. The hyperactivity of excitatory neurons projecting to dopaminergic structures would produce an hyperactivation of the dopamine mesolimbic pathway, and ultimately the positive symptoms (Ellaithy et al. 2015). Disinhibition of the excitatory output from the hippocampus would also drive increased dopaminergic VTA neuronal activity and onset of psychosis (Lisman et al. 2008).

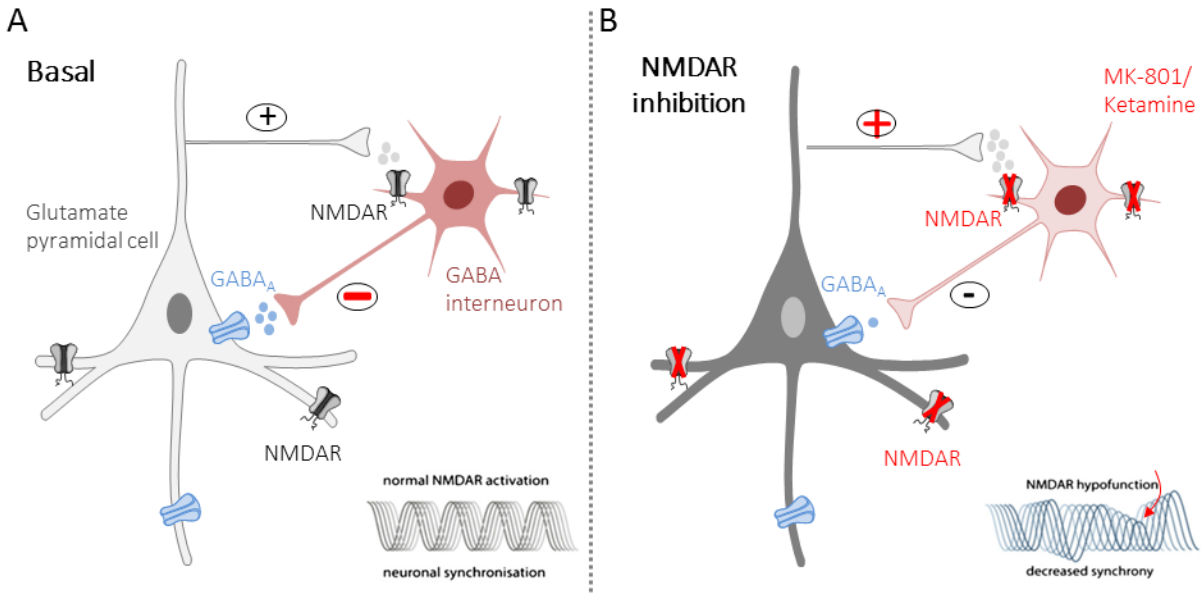

Figure 9. Current hypothetic model of NMDA hypofunction
(A) In basal condition, the reciprocal interactions between glutamate pyramidal cells and GABA interneurons is responsible for homeostasis of firing of pyramidal cells and generation of a neuronal oscillations underlying neuronal synchronization between brain areas. Activation of NMDAR on pyramidal cells in turn activates NMDAR present in GABA interneurons. Stimulation of GABA interneurons activates GABA release and generates a feedback inhibition which maintains the activity of pyramidal cells in a physiological range. (B) NMDAR hypofunction, for instance induced by the presence of NMDAR antagonists such as MK-801 or ketamine, could decrease GABA interneuron activation, and is known to cause a decrease in GAD65/67 levels (ref). Low activation of GABA interneurons leads to pyramidal cells dishinibition. Increased firing of pyramidal cells generates abnormal neuronal oscillations, thus causing neuronal asynchrony which might underlie cognitive and negative symptoms. (Adapted from Snyder & Gao, 2013; Moreau & Kullman, 2013)

d. Alternative ways to model schizophrenia

Beyond the NMDAR hypofunction model, other models have been developed in order to test various causative theories of SCZ, and notably to explore its neurodevelopmental aspect. Environmental insults during gestation or perinatal period, such as maternal stress, infection or immune activation, increase the risk for SCZ. Gestational administration of the anti-mitotic agent methylazoxymethanol acetate (MAM) to pregnant rats has been shown to selectively affect brain development, producing long-lasting anatomical and behavioral deficits in the offspring (Lodge & Grace 2012). Social deprivation of rat pups from the age of weaning leads to similar outcomes (Jones et al. 2011). While MAM and isolation rearing have been extensively used as animal models, maternal immune activation (MIA) is also known to cause neurodevelopmental alterations. The MIA model relies on exposure to either bacterial or viral infection during pregnancy, which elevates circulatory pro-inflammatory cytokines and other mediators of inflammation, affecting brain development and

increasing the risk of SCZ in the offspring (Jones et al. 2011). A number of lesion animal models have also been developed in an attempt to mirror anatomical changes such as a reduced hippocampal volume, seen in first-episode SCZ patients (Lipska & Weinberger 2000). One of the most famous of these lesion models is the neonatal lesion of the ventral hippocampus, induced by local injection of excitotoxin which causes behavioral abnormalities that emerge after puberty (Lipska et al. 1993). The main objective of this model is to disrupt the development of the hippocampus, a brain area consistently implicated in SCZ, and thus disrupt development of the cortical and subcortical circuitry in which the hippocampus participates. However, some of these models show poor face validity as they do not reproduce the physiopathology of SCZ, and also lack construct validity. Indeed, the brain of SCZ patients does not manifest a "lesion" analogous to any of these lesion models. On the other hand, the pharmacological model of NMDAR hypofunction although showing robust face validity, does not address the developmental component of SCZ. Genetic manipulation can approach this developmental aspect through the use of inducible transgenic animals, but does not model the polygenic effect of SCZ. Induced pluripotent stem cells (iPSC) technology is a very promising tool to generate new disease models. A major advantage of patient-derived iPSC studies is the possibility to follow neurodevelopment *in vitro* while carrying the complete genetic background of a SCZ individual. To data, several studies have used iPSCs to model SCZ and successfully identified differences in synaptic functions in iPSC-derived cells from SCZ patients (Falk et al. 2016; Habela et al. 2016). In conclusion, none of the existing models of SCZ fully reflect the pathophysiology of the disease, but they allowed great advances in the understanding of the pathology. Creating a "perfect" model represents an unreachable goal until we do not understand the precise molecular pathways and cellular structures involved in SCZ.

B. NMDAR trafficking alterations in schizophrenia

1. <u>NMDAR intracellular trafficking in schizophrenia</u>

As previously discussed, growing evidence implicates glutamate dysfunction in the pathophysiology of SCZ. Altered expression of NMDAR subunits has been reported in the brains of patients with SCZ as in rodent models. Abnormal N-glycosylation could contribute to alter NMDAR intracellular trafficking, as several studies already showed disturbances of intracellular AMPAR trafficking in SCZ (Tucholski, Simmons, Pinner, Haroutunian, et al. 2013). However, no differences in N-glycosylation of NMDAR subunits were detected between *postmortem* tissues of SCZ patients and healthy individuals (Tucholski, Simmons, Pinner, McMillan, et al. 2013). Endocytosis is an important process controlling NMDAR expression at the plasma membrane, and an increased NMDAR internalization could account for the NMDAR hypofunction hypothesis of SCZ. A significant decrease of GluN1 subunit phosphorylation at serine 897 (S897) has been reported in patients with SCZ (Emamian et al. 2004). Interestingly, mice injected chronically with the psychotomimetic drug PCP also showed decreased phosphorylation levels of Ser 897 (Mouri et al. 2007), and transgenic mice expressing the GluN1 S897A phospho-mutant exhibit a SCZ-like behavior (Li et al. 2009). Since several studies also reported reduced levels of GluN2A subunit tyrosine phosphorylation in *postmortem* tissues from SCZ patients (Hahn et al. 2006b; Banerjee et al. 2014), NMDAR dephosphorylation could produce NMDAR internalization through a clathrin-dependent mechanism. The SCZ candidate gene, PP2B γ-subunit (PPP3CC) could promote NMDAR endocytosis. PPP3CC (also called calcineurin) is a serine/threonine phosphatase, which dephosphorylates and activates striatal-enriched tyrosine phosphatase (STEP), which in turn dephosphorylates the GluN2B subunit and promotes NMDAR internalization (Braithwaite et al. 2006). Elevated $STEP_{61}$ levels have been described in cortical tissues from SCZ patients, as well in the frontal cortex of mice treated with PCP (Carty et al. 2012), suggesting that overactivation of phosphatases contribute to NMDAR hypofunction. The neuregulin NRG1-ErbB4 pathway could also favor abnormal NMDAR dephosphorylation. NRG1 and its receptor ErbB4 are overexpressed in the brains of SCZ individuals (Geddes et al. 2011), and the excessive binding of

neuregulin-1 (NRG1) to ErbB4 dephosphorylates the GluN2A subunit, leading to an altered downstream signaling of the NMDAR (Hahn et al. 2006b). This suggests that excessive signaling of NRG1 and ErbB4 proteins can also promote NMDAR dephosphorylation and enhance its internalization.

2. NMDAR surface trafficking and its multiple levels of regulation

a. Interactions with the extracellular environment

Extracellular matrix proteins

Extracellular matrix (ECM) molecules are secreted by neurons and glial cells, and accumulate in the extracellular space to regulate various aspects of synaptic maturation and plasticity (Wang & Fawcett 2012). Chondroitin sulfate proteoglycans (CSPG) are one of the main components of the brain ECM and of perineuronal nets (PNN). PNN form large stable aggregates that envelop the neuronal soma and proximal dendrites, and are typically associated with PV-positive interneurons (Berretta 2012), a neuronal type particularly affected in SCZ (Marín 2012). The PNN is an important regulator of CNS plasticity, both during development and into adulthood. PNN deposition around neurons helps to stabilize the established neuronal connections and restricts the plastic changes to novel experience within the CNS (Dityatev & Schachner 2003; 2006; Rhodes & Fawcett 2004; Wang & Fawcett 2012). Of interest, breakdown of ECM after hyaluronidase or chondroitine ABC treatment in dissociated hippocampal neurons increases AMPAR surface mobility and affects synaptic transmission (Frischknecht et al. 2009). Reelin and MMP have also been shown to efficiently modulate NMDAR surface trafficking, and as such are involved in controlling synaptic plasticity (Groc, Choquet, et al. 2007). Marked reduction of PNN was detected *postmortem* in the brain of SCZ subjects (Pantazopoulos et al. 2010; Mauney et al. 2013). Shah et al described PNN alteration in MAM-treated rats, and demonstrated that *in vivo* digestion of PNN by chondroitinase ABC is sufficient to mimic

SCZ-like symptoms (Shah & Lodge 2013). Interestingly, decreased expression of Reelin represents a robust alteration in SCZ (Berretta 2012). Downregulation of the reelin gene has been detected in several brain regions of patients with SCZ and is often accompanied by alterations of GABA markers (Berretta 2012), consistent with the hypo-GABAergic transmission observed in SCZ. The reelin gene (RELN) is a strong risk gene for SCZ (Impagnatiello et al. 1998) and impairment in reelin signaling has been hypothetized to increase susceptibility to psychiatric disorders. In addition, Reelin haploinsufficient juvenile mice exhibited anomalous LTP concomitant to reduced dendritic spine density, which led to associative learning deficits in older animals (Iafrati et al. 2014). Therefore, it is likely that PNN physiologically restrict CNS plasticity, notably by controlling receptor surface trafficking, and disruption of ECM elements might lead to inadaptive plasticity as it is the case in SCZ.

NMDAR co-agonists

Astrocytes are active modulators of neuronal activity by releasing several molecules among which gliotransmitters like D-serine, a coagonist of the NMDAR (Van Horn et al. 2013). NMDAR co-agonists D-serine and glycine tightly control NMDAR surface dynamics (Papouin et al. 2012). Accordingly, abnormal D-serine levels can potentially impair NMDAR surface trafficking and subsequent distribution and function. Different genes implicated in regulation of D-serine level have been shown to be at risk for SCZ. Association studies have identified several mutations in human D-serine metabolic enzymes as risk factors for SCZ. These include SNP variants of SR involved in D-serine synthesis and the degrading enzyme DAAO) (Van Horn et al. 2013). Several studies have reported decreased D-serine levels in the plasma and CSF of SCZ patients (Hu et al. 2015), whereas DAAO activity has been found to be elevated in *postmortem* hippocampus and cortex from SCZ patients (Madeira et al. 2008). D-serine has been tested as a potential therapeutic agent for SCZ. In combination with antipsychotics, D-serine was reported to be more efficient than antipsychotics alone (Lin et al. 2012; Heresco-Levy et al. 2015). In rodents, administration of D-serine improves

impaired pre-pulse inhibition and cognitive defects induced by NMDAR antagonists (Van Horn et al. 2013) or genetic deletion of SR (Balu et al. 2013).

Kynurenic acid

Kynurenic acid (KA) is a naturally occurring antagonist of NMDAR and α7nAChR in the human brain (Hilmas et al. 2001). Kynurenic acid is a metabolite in the neuroprotective branch of the kynurenine pathway of tryptophan degradation (Stone 1993) whereas quinolinic acid, an NMDAR agonist is a neurotoxic metabolite (Stone 1993). Elevated KA has been described in the CSF (Erhardt et al. 2001; Nilsson et al. 2005; Linderholm et al. 2012) and brains of SCZ patients (Schwarcz et al. 2001; Sathyasaikumar et al. 2011). Kynurenine, the precursor of KA, is also elevated in the CSF of SCZ patients (Linderholm et al. 2012) whereas quinolinic acid is found at normal levels (Kegel et al. 2014). Thus, it is proposed that increased concentration of KA could cause NMDAR hypofunction, thereby leading to symptoms of SCZ. In that way, mutant mice with a deletion of the kynurenine aminotransferase II, a major biosynthetic enzyme of KA, showed reduced brain concentrations of KA and better cognitive performance compared to control animals (Potter et al. 2010).

b. Interactions with intracellular partners

PSD proteins

Among the numerous proteins present in the PSD, MAGUK scaffolding proteins have been demonstrated to bridge surface receptors with their intracellular effectors, and to regulate receptor distribution (van Zundert et al. 2004). Within the synaptic area, NMDAR are anchored *via* their GluN2 C-terminal tails to PDZ-containing scaffolding proteins such as PSD-95, PSD-93 and SAP102. PSD scaffold abnormalities might thus directly contribute to the NMDAR dysfunction proposed in SCZ. A recent genotypic study has revealed that several *de novo* copy number variants (CNVs) mutations affect genes encoding for PSD scaffolding proteins (Kirov et al. 2012). These mutations were found to

prominently affect the PSD proteome and were related to the NMDAR-dependent signaling (Kirov et al. 2012). *Postmortem* studies also revealed abnormal expression of PSD-95 in SCZ individuals, a crucial organizer of the PSD structure (see table). PSD-95 mRNA expression was found to be increased in the DLPFC of SCZ patients (Dracheva et al. 2001). In the thalamus, Clinton et al. reported differential results depending on the age of the patients. While in elderly SCZ patients PSD-95 and SAP102 are upregulated (Clinton et al. 2003; 2006), the same transcripts are downregulated in younger subjects (Clinton & Meador-Woodruff 2004). Reduced PSD-95 protein was also described in the hippocampus, the prefrontal cortex and the anterior cingulate cortex of SCZ individuals (Toro & Deakin 2005; Kristiansen et al. 2006; Catts et al. 2015; Funk et al. 2009). A significant reduction of PSD-95, Homer1b/c and synaptophysin protein levels whereas Homer1a is increased, has been detected in hippocampal *postmortem* tissues from SCZ patients (Matosin et al. 2016). PSD scaffold protein alterations were also observed in pharmacological models of SCZ. After acute ketamine treatment, both Homer1b and PSD-95 transcripts expression are reduced in the cortex (de Bartolomeis et al. 2013). By contrast, PSD-95 and SAP-97 mRNA levels are increased in the cortex of rats that received MK-801 (Lindén et al. 2001). Cortical PSD-95 mRNA is also increased after an acute treatment with PCP (Anastasio & Johnson 2008) whereas no modification of protein expression is reported in a subchronic paradigm (Anastasio & Johnson 2008; Owczarek et al. 2011b; Carty et al. 2012). These contradictory observations may reflect compensatory mechanisms occurring during development. Indeed, the only decrease of PSD proteins expression was reported in the adult, whereas studies conducted in young animals show higher or stable protein expression. Either way, PSD proteins may support an aberrant anchoring of NMDAR, potentially leading to NMDAR hypofunction in the synapse. Altogether, these findings strengthen the view that the PSD may represent a crucial site for SCZ pathophysiology, and PSD scaffolding proteins may underlie NMDAR-related deficits.

Table 5 . PSD proteins changes after psychotomimetic treatments

↘ Decrease ↗Increase = No change

Study	Dose	Duration	Animals	Brain region	mRNA	Protein
Acute injection						
MK-801						
(Lindén et al. 2001)	MK-801 5mg/kg i.p.	4h – 8h	Wistar rats 200-250g	Cortex	↗ PSD-95 4h after MK801 2.5 and 5mg/kg ↗ SAP-97 4h and 8h after MK801 5mg/kg in the entorhinal cortex ↘ SAP-97 4h after MK801 2.5 and 5mg/kg in the parietal cortex = SAP102	
PCP						
(Kajimoto et al. 2003)	10mg/kg s.c.	Killed 24h after injection	Sprague-Dawley rats 300-500g	NAc, ACC, HPC, putamen	↗SAPAP1 in NAc & HPC	
(Anastasio & Johnson 2008)	10mg/kg s.c.	0h, 4h, 8h,24h Killed 24h after last injection	Sprague-Dawley rats P7	Frontal cortex		↗ PSD-95 after 8h & 24h
Ketamine						
(de Bartolomeis et al. 2013)	Ketamine 25-50mg/kg i.p MK-801 0.8mg/kg i.p	Killed 90min after injection	Sprague-Dawley rats P60	Cortex (MC, IC, SS, ACC), NAc, putamen	↘ Homer1a KET25 in IC ↘ Homer1b KET25 in MC & KET50 in MC and putamen ↘ PSD-95 KET25, KET50 and MK-801 in putamen	
Subchronic/Chronic injections						
PCP						
(Carty et al. 2012)	5mg/kg i.p.	Injections twice daily for 7 days Killed 24h after the last injection	C57BL/6 mice (6–8 months)	Frontal cortex		= PSD-95 in WT ↗PSD-95 in STEP KO
(Owczarek et al. 2011)	10 mg/kg s.c.	P7 + P9 + P11	Rat >P80 (12weeks)	PFC HPC		= SAPAP, Shank1, Shank2, Shank3, Homer
(Anastasio & Johnson 2008)	10mg/kg s.c.	P7 + P9 + 11 Killed 24h after last injection	Sprague-Dawley rats P7	Frontal cortex		= PSD-95
(Pickering et al. 2013)	5mg/kg i.p.	Daily injections for 5days + withdrawal 2 days	Wistar rats 190–210 g	PFC		↗ synapsin-1

N.A: Not Available; PCP: phencyclidine; MK-801: dizocilpine; i.p.: intraperitoneal; s.c.: subcutaneous; P7: Post-Natal day 7; HPC: Hippocampus; PFC: Pre Frontal Cortex;; ACC: Anterior Cingulate Cortex; IC: Insular Cortex; MC: Motor Cortex; NAc: Nucleus Accumbens; SS: Somato-Senseory Cortex; WT: Wild-Type; KO: Knock-Out

DISC-1

DISC1 is a multifunctional anchoring molecule that is implicated in the regulation of many physiological mechanisms such as neuronal progenitor cell proliferation, neuronal migration, dendritic arborization and outgrowth (Ishizuka et al. 2006). DISC1 is also a gene locus originally identified in a Scottish family, in which a balanced translocation between chromosomes 1 and 11 has been associated with SCZ, bipolar disorder, and major depression (Karam et al. 2010). This translocation directly disrupts the DISC1 protein and leads to a C-terminal truncated mutation of DISC1 (Millar et al. 2000). Linkage and association studies support a role for DISC1 locus in SCZ (Karam et al. 2010) although recent GWAS and meta-analysis studies failed to observe significant association between DISC1 and SCZ (Brandon & Sawa 2011). Nevertheless, SCZ-like phenotypes have been reported in DISC1 transgenic mice (Li et al. 2007; Hikida et al. 2007; Pletnikov et al. 2008). Wei et al. (2013) recently described specific NMDAR alterations after DISC1 knockdown. DISC1 downregulation leads to the enhancement of NMDAR-mediated current, accompanied by a selective increase of GluN2A subunit synaptic expression, indicating that DISC1 deficiency triggers GluN2A subunit upregulation which could lead to aberrant NMDAR-dependent synaptic plasticity and cognitive processes (Wei 2013). These results contrast with previous findings that described a 40% loss of synaptic DISC1 in a murine model of NMDAR hypofunction (Ramsey et al. 2011). DISC1 is also downregulated in mice chronically exposed to MK-801, and accompanied by a dose-dependent reduction of spine density (Ramsey et al. 2011). Additionally, DISC1 has been shown to regulate D-serine level by controlling SR degradation (Ma et al. 2013). In a conditional mouse model expressing mutant DISC1 selectively in astrocytes, Ma et al. (2013) found that mutant DISC1 is no more able to bind to SR, enhancing its degradation via the ubiquitin pathway. D-serine production is then reduced, leading to molecular and behavioral deficits (Ma et al. 2013). Among the 127 proteins and 158 interactions it is interacting with, DISC1 and dysbindin, another major susceptibility gene for SCZ, have been suggested to share a number of common binding partners (Camargo et al. 2007). Interestingly, mice lacking dysbindin-1 protein, which is reduced in SCZ, display NMDAR

hypofunction, disrupted inhibitory transmission, hyperexcitability in the PFC, as well as deficits in working memory and learning (Karlsgodt et al. 2011). Seshadri et al. (2015) provided further evidence that DISC1 modulates NRG1-ErbB4 signaling in interneurons, by competing with ErbB4 for binding to PSD-95. DISC1 thus represents a direct modulator of the excitation-inhibition balance, known to be impaired in SCZ. Overall, through its sprawling interactions, DISC1 undoubtedly influences NMDAR trafficking and function, and in this respect might play a critical role in the etiology of SCZ.

c. Interactions between NMDAR and membrane proteins

NRG1/ErbB4

Together with DISC1 and dysbindin, NRG1 and its receptor ErbB4 are among the major risk genes for SCZ (Corfas et al. 2004; Harrison & Law 2006; Mei & Xiong 2008). *Postmortem* studies revealed an increased expression of NRG1 isoform type I (Hashimoto et al. 2004) and upregulated expression of ErbB4 in the DLPFC of SCZ patients (Law et al. 2007; Joshi et al. 2014). A marked increase in NRG1-induced ErbB4 signaling was reported in the prefrontal cortex of SCZ patients, whereas the total level of NRG1 and ErbB4 are not altered (Hahn et al. 2006b). ErbB4/PSD-95 and ErbB4/GluN1 interactions are also more pronounced in SCZ, suggesting an enhanced modulation of NMDAR function by NRG1 (Hahn et al. 2006b). Activation of ErbB4 receptor by NGR1 is indeed known to inhibit NMDAR-mediated currents through a mechanism involving NMDAR internalization (Gu et al. 2005). In addition, GluN2A subunit tyrosine phosphorylation levels induced by NMDAR stimulation are decreased in the prefrontal cortex of SCZ subjects compared to healthy brain tissues, supporting the idea that inhibition of NMDAR signaling by ErbB4 could contribute to NMDAR hypofunction in SCZ (Hahn et al. 2006b). Accordingly, cto*Nrg1* mice expressing Nrg1 transgene, which mimics high levels of NRG1 observed in forebrain regions of SCZ patients, exhibit characteristic behavioral and cellular deficits found in SCZ (Yin et al. 2013). Remarkably, restoring normal levels of NRG1 reversed the

behavioral deficits (Yin et al. 2013), further demonstrating the detrimental role of the NRG1- ErbB4 pathway in SCZ. ErbB inhibitors ameliorate the behavioral deficits in a rodent model of SCZ (Mizuno et al. 2013). Hypo-phosphorylation of the GluN2B subunit was also found in the hippocampus of heterozygous *Nrg1*$^{+/-}$ mice, as well as in ErbB4$^{+/-}$ mutant mice, and was associated with altered hippocampal synaptic plasticity (Bjarnadottir et al. 2007; Long et al. 2015). Such functional deficits were normalized by clozapine treatment (Bjarnadottir et al. 2007), which also reversed the behavioral deficits previously observed in *Nrg1*$^{+/-}$ and ErbB4$^{+/-}$ mutant mice (Stefansson et al. 2002). Besides, ablation of ErbB4 in PV-positive interneurons prevents NRG1 from inhibiting pyramidal neuron firing and causes SCZ-relevant phenotypes in mutant mice (Wen et al. 2010), supporting the idea that a disruption of NRG1-ErbB4 signaling may disturb the E/I balance in the brain. Of particular interest, the NRG1-ErbB4 pathway interacts with many other proteins, which constitutes as many potential ways of dysregulation. NRG1 regulates the expression of the α7nAChR (Liu 2001). ErbB4 also interacts with the PDZ-containing protein PSD-95 (Huang et al. 2000) and has therefore the potency to alter interactions between NMDAR and MAGUK proteins. As previously mentioned, ErbB4/PSD-95 interaction is modulated by DISC1 (Seshadri et al. 2015), adding a layer of complexity in NMDAR regulation. NRG1 molecule was also found to interact with ECM components (Corfas et al. 2004), another controlling pathway for NMDAR surface trafficking.

Dopamine receptors

Dopamine was the first neurotransmitter system to be strongly implicated in SCZ. Classical antipsychotics mostly act on D2R, and provide symptomatic improvement, particularly for positive symptoms (Beaulieu & Gainetdinov 2011). In a recent large GWAS study where 108 loci associated with SCZ were identified, the DRD2 gene coding for the D2R, was one of the most relevant associations with the etiology of SCZ (SCZ Working Group of the Psychiatric Genomics Consortium 2014). D2R density is slightly elevated in the striatum of patients with SCZ compared to healthy subjects (Oda et al. 2015), although the majority of studies included patients under antipsychotic

treatments known to impact dopamine receptor expression (Seeman & Seeman 2014). PET brain imaging studies showed that dopamine synthesis is increased in drug-naive SCZ patients compared with healthy matched subjects (Carlsson et al. 2001). According to the current glutamatergic hypothesis of SCZ, dopamine dysfunction would be secondary to a primary NMDAR disruption. Numerous studies have demonstrated extensive crosstalk between dopamine receptors and NMDAR *via* direct physical association between them (Beaulieu & Gainetdinov 2011). D1R binds to NMDAR C-terminus on separate sites of GluN1 and GluN2A subunits (Lee et al. 2002), and this physical interaction reciprocally regulates receptor properties and trafficking (Dunah & Standaert 2001; Scott et al. 2002; Fiorentini 2003; Ladépêche, Julien P Dupuis, et al. 2013). D1R activation, which reduces D1R-GluN1 interaction at the perisynapse, allows NMDAR to laterally diffuse into the PSD where they favor LTP (Ladépêche, Julien P Dupuis, et al. 2013). D1R stimulation also promotes GluN1-CaMKII coupling and enhances CaMKII activity, which upregulates NMDAR-mediated LTP (Nai et al. 2010). Regarding the D2R, it interacts with NMDAR through intracellular coupling with the GluN2B subunit (Liu et al. 2006). Contrary to D1R which favors glutamatergic transmission, activation of D2R inhibits NMDAR currents (Liu et al. 2006). Acute treatment with cocaine enhances D2R-GluN2B coupling in the striatum, which in turn disrupts GluN2B-CaMKII binding, resulting in decreased CaMKII activity, reduced GluN2B phosphorylation and thereby reduced NMDAR currents (Liu et al. 2006). By binding different dopamine receptor subtypes, dopamine can thus increase or decrease NMDAR function simultaneously. The “revised dopamine hypothesis” proposes that the dopamine transmission is elevated in the mesolimbic areas and decreased in the prefrontal cortex of SCZ patients (Howes & Kapur 2009). Therefore, one could hypothesize that decreased activation of D1R concomitant with D2R overactivation can fuel and worsen the initial glutamatergic dysfunction by favoring abnormal NMDAR trafficking and function.

mGlu receptors

Another protein interacting with the NMDAR which could appear as a potential pathogenic partner in SCZ is the metabotropic glutamate receptor (mGluR). mGluR are a family of G-protein-coupled receptors composed of eight heterogeneous subtypes, which have been divided into three groups based on sequence homology, intracellular signal transduction mechanisms, and pharmacological profiles (Pin & Duvoisin 1995). Group I mGluR, composed of mGluR1 and mGluR5, is preferentially coupled *via* Gq protein to activate phospholipase C (PLC). Group II and III mGluR are coupled negatively *via* Gi protein to adenylyl cyclase second messenger system. At the post-synaptic density, mGluR5 interacts with NMDAR via Homer, Shank and PSD-95 (Perroy et al. 2008), and functionally regulates NMDAR function as mGluR5 activation enhances NMDAR phosphorylation and NMDAR-mediated currents (Takagi et al. 2012). Genetic studies suggest that mGluR may be involved in the pathogenesis of SCZ. GWAS studies have shown that mGluR3 gene (GRM3), among other genes involved in glutamatergic neurotransmission and synaptic plasticity, is associated with SCZ (Stefansson et al. 2009; Ripke et al. 2014). *Postmortem* studies reported differential results depending on the mGluR subtype. Normal mGluR3 mRNA levels have been found (Richardson-burns et al. 1999; Harrison et al. 2008) although Gupta et al. (2005) reported increased expression of mGluR1 and mGluR2/3 in the PFC of SCZ patients. By contrast, total mGluR1 protein levels were found to be lower in the CA1 region of SCZ subjects relative to controls (Matosin et al. 2016). Pharmacological studies at the pre-clinical and clinical levels show that mGluR may be potential targets for new antipsychotic drugs (Muguruza et al. 2016). Group II mGluR agonist LY2140023 successfully reduced positive and negative symptoms in SCZ patients (Patil et al. 2007). However, a recent study reported the inefficacy of AZD8529, a positive allosteric modulator of mGluR2, to improve symptoms in SCZ patients (Litman et al. 2016), suggesting that positive modulation of mGluR may not be sufficient to provide an antipsychotic effect.

These several examples illustrates the intricate nature of NMDAR controlling pathways, and shows that NMDAR surface trafficking and function might be affected at multiple levels, and through many ways.

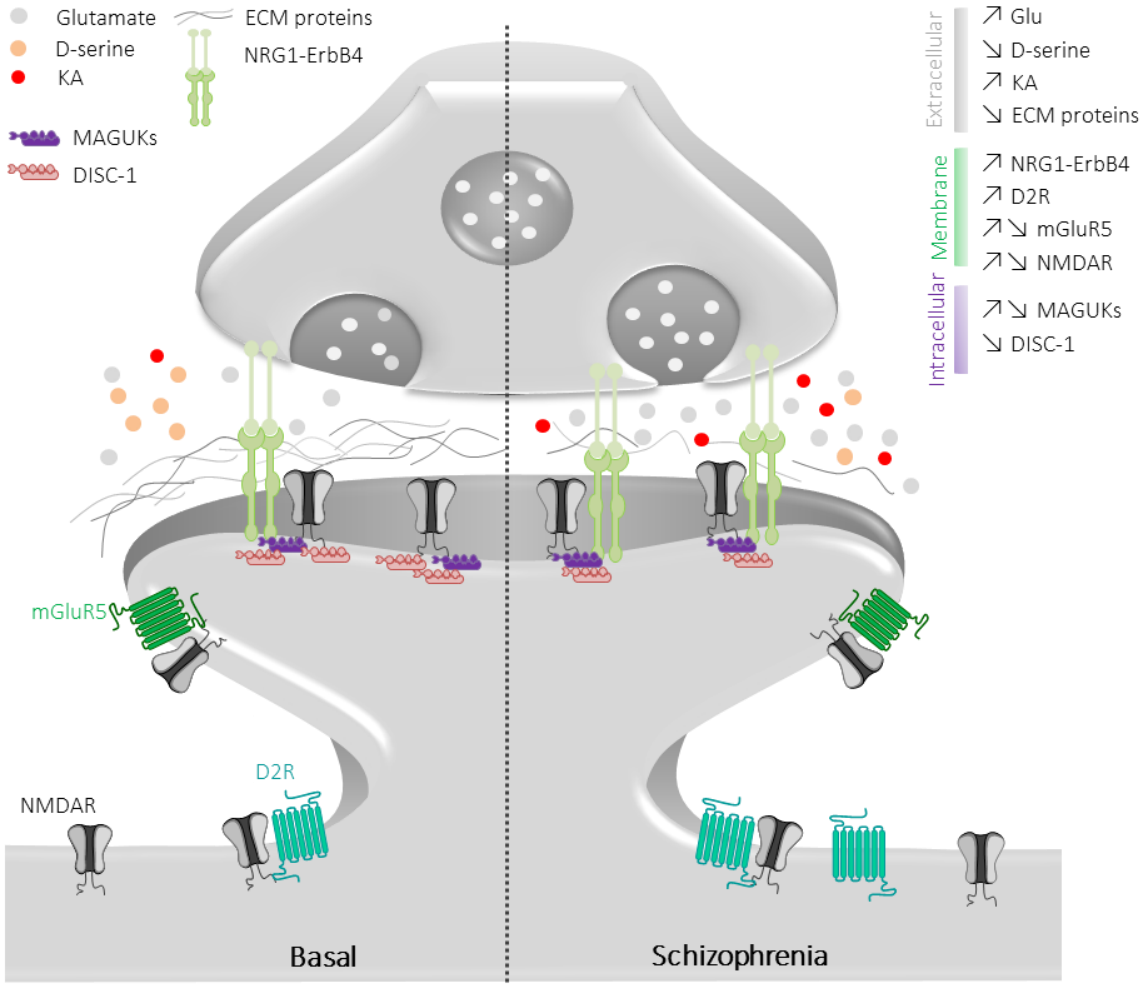

Figure 10. Many regulators of NMDAR surface trafficking are altered in schizophrenia
NMDAR are part of a large multiprotein complex at the glutamatergic synapse. Their assembly with diverses proteins through extracellular, membrane and intracellular interactions provide multiple regulation levels of NMDAR surface trafficking. Many of these interactors are dysregulated in schizophrenia, which could contribute to an alteration of NMDAR surface dynamics, and eventually to NMDAR dysfunction as described in the disease. Major modifications are summarized in the box.

III – Autoimmunity and psychotic disorders

« Cogito, ergo sum » *(I think, therefore I am),* René Descartes.

The Self defines the conditions of identity that make one subject distinct from all others. John Locke, an English philosopher and physician claimed: "*what makes me today the very same person as I was yesterday, is, basically, the fact that I can now remembe*r *what I did or experienced yesterday*". Then, for Locke, memory determines who we are. But memory is not only a brain capacity. The immune memory which resides in our body represents a biological print of any past physical experience. How ironical is the situation when the "immune-self" backfires on itself and attacks the "mental self"…

A. Overview of immune disorders associated with psychotic symptoms

1. The immune system

The immune system consists of a complex organization of cells and mediators that has largely evolved to protect human beings from infection (Khandaker et al. 2015). The immune system resides in the lymphatic system, which is composed of primary lymphatic organs (thymus, bone marrow), secondary lymphatic organs (lymph nodes and spleen), lymphocytes and antigen-presenting cells. These cells circulate in the blood throughout the body to fight infections. A mature immune system normally does not respond to its own tissue. This acceptance of self-cells is known as immunological tolerance. Accordingly, self-molecules can be distinguished from foreign substances by the immune system. Conversely, non-self-molecules or antigens (for antibody generators) are recognized as foreign molecules and elicit an immune response, which can be categorized into:

Glossary for basic knowledge in immunology

Infection: Invasion and multiplication of microorganisms such as bacteria, viruses, and parasites that are not normally present in the body.
Inflammation: Localized, nonspecific response to infection. Inflammation aids the mobilization of defensive cells at infected sites.
Antigen: Molecule that provokes a specific immune response.
Antibody: Protein in a class called immunoglobulin (Ig), divided into subclasses based on the structures and functions of the antibodies.
Lymphocyte B cell: Responds to antigens by producing antibodies to provide humoral immunity. When a B cell encounters the antigen, it divides and differentiates into plasma cells and memory cells.
Lymphocyte T cell: Attacks the cells that carry the specific antigens, producing cell-mediated immunity.
Macrophage: White blood cell that digests cellular debris, foreign substances, microbes, cancer cells through phagocytosis.
Dendritic cell: Antigen-presenting cell, acting as a messenger between the innate and the adaptive immune systems.

- *An innate response*, acting as a rapid and non-specific first line of defense, which is mediated by macrophages, dendritic and natural killer (NK) cells that recognize and clear invading organisms.

- *An adaptive response,* that is slower, but which recognize and remember specific pathogens. If an antigen gets through the innate immune system, macrophages, dendritic cells, and other antigen-presenting cells present short peptide sequences (epitopes) derived from this foreign antigens to specific lymphocytes T cells. T cells become activated and are able to direct the immune response against the cells expressing the epitope sequence. In parallel, B cells secrete antibodies against the antigen as part of the humoral response. The adaptive response is dependent upon the innate response. Inflammatory cytokines, secreted by macrophages and other cells, as well as activation of complement proteins help this process (Garay & McAllister 2010). Although the main role of the immune system is to protect against infection, this can have adverse effects if the expression of microbial antigens within cells leads to T cell- or macrophage-mediated damage (Vincent et al. 2006). This is the case when the immune response initiates an inflammatory infiltration in the antigen-expressing tissue, and if the antibodies activate the complement proteins that lead to cell lysis. Immune diseases of the nervous system can be associated with infections (such as HIV-AIDS-associated encephalitis) or with an inflammatory response against the brain caused by unknown mechanisms, such as multiple sclerosis. There is another way the immune system can fail: it can itself be the agent of disease. Autoimmune diseases are produced by failure of the immune system to recognize and tolerate self-antigens. Myasthenia gravis, for example, is an autoimmune disease in which individuals produce antibodies directed against acetylcholine receptors on their own skeletal muscle cells, causing paralysis.

Glossary for basic knowledge in immunology

Microglia: Type of glial cell which is a tissue-resident macrophage in the CNS.
Cytokine: Autocrine regulatory molecule involved in both pro- and anti-inflammatory responses.
Major histocompatibility complex (MHC): Proteins expressed at the surface of any body cell, essential for the acquired immune system to recognize foreign molecules.
Human leukocyte antigens (HLA): MHC proteins specific to humans. Serve as self-marker enabling self-versus-nonself recognition.

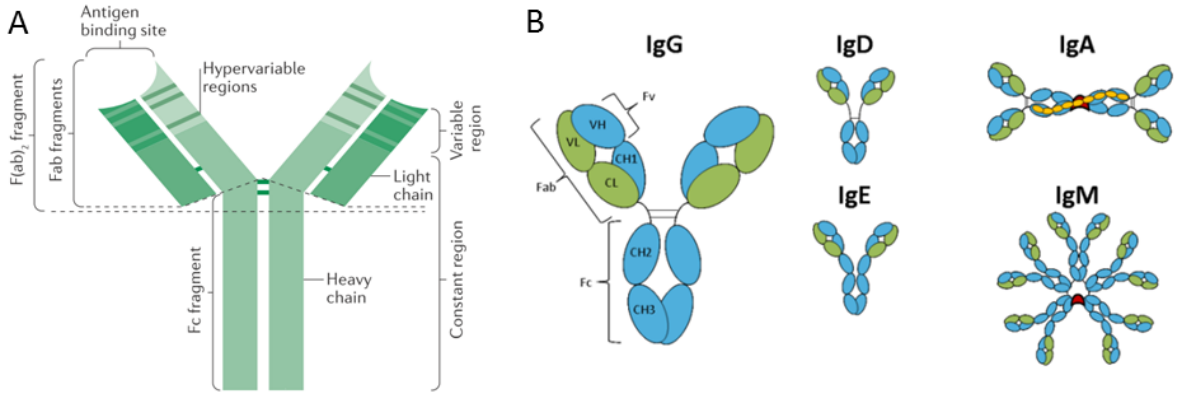

Figure 11. Immunoglobulin structure and its different isotypes
All immunoglobulins (Ig) or antibodies are large, Y-shaped proteins composed of 2 heavy identical polypeptide chains and 2 identical light polypeptide chains, connected by disulfide bonds. The variable or Fab (Fragment, antigen-binding) domains of the light and heavy chains,, form the antibody variable region that determines antigen binding specificity. The Fc (crystallisable fragment) domains form the constant region of the antibody that determines its ability to bind to complement and Fc receptors on phagocytic cells. There are 5 different types of heavy chains that define the 5 different types of Fc domains, allowing antibodies to be grouped into different isotypes:
IgA (α), the major form of antibody in external secretions
IgG (γ), the major form of antibody in the blood plasma
IgD (δ), which serves as a receptor for antigens on the B cell surface. Its other functions are unknown
IgE (ε), which promotes the release of histamine and other agents that aid in attacking a pathogen. It sometimes triggers a full-blown response when a harmless antigen enters the body producing allergic symptoms.
IgM (μ), the first type of antibody to be secreted during the primary immune response.
The basic functional unit of each antibody is a monomer containing only one Ig unit, like IgA, IgG and IgD. Secreted antibodies can also be dimeric with two Ig units as IgA or pentameric with 5 Ig units like IgM. (From Crisp et al., 2016)

2. The immune hypothesis of schizophrenia

a. The infectious theory of schizophrenia

Psychotic symptoms, mood disturbance and cognitive dysfunction are often observed during and shortly after an infectious illness. The hypothesis that infectious agents may cause psychotic disorders was first formulated in the 19th century. In 1845, the French neurologist Esquirol described the "epidemic" appearance of psychiatric disorders. In the 1870s, Kraepelin, among others, theorized that bacteria might be etiologically linked to *dementia praecox* or other psychiatric diseases (Yolken & Torrey 2008). The fact that psychoses occasionally accompanied bacterial diseases such as typhoid fever, tuberculosis and diphtheria supported this infectious theory. Following the 1918 influenza epidemic, attention then shifted to viruses. Menninger (1926) described a series of 200 cases of SCZ-

like psychosis in victims of influenza. This early neuroimmune hypothesis of psychosis was reanimated by the seminal work of Torrey et al. in the 1970s suggesting that latent viruses might be involved in the development of SCZ (Torrey & Peterson 1972). Since then, various infectious agents are being considered to play a role in the etiology of SCZ and related disorders (Benros et al. 2014). SCZ is associated with increased prevalence of various infections including neurotropic viruses from the *Herpes viridae* family (Yolken & Torrey 1995) and the intracellular parasite, *Toxoplasma gondii* (Yolken & Torrey 2008). Infection during fetal and childhood development is also associated with the risk of psychotic illness in adult life (Khandaker & Dantzer 2016). A systematic review of population-based studies indicated that prenatal exposure to a range of pathogens is associated with the risk of SCZ-related psychosis in adult offspring (Khandaker et al. 2012). These include *Herpes simplex* virus type-2 (HSV-2), influenza cytomegalovirus, and the intracellular parasite *Toxoplasma gondii* (Khandaker et al. 2012). These observations have resulted in different forms of infectious hypotheses of SCZ. One hypothesis states that a retrovirus causes direct structural or functional damage to the brain, eventually leading to psychosis. Endogenous forms of human retroviruses are expressed in brain tissues of individuals with SCZ and other neuropsychiatric diseases (Yolken 2000). Several studies have documented the expression of HERV-W in the brain, CSF and serum of patients with SCZ (Karlsson et al. 2001, 2004). A study evidenced the presence of two major antigens, HERV-W Env (envelope) and Gag

Mini glossary of immuno-psychiatry

Psychosis: Psychosis is a symptom, not an illness. It includes a range of symptoms but typically involves hallucinations and/or delusions.

Dementia praecox: for "premature dementia". First used in 1891 by Pick, then popularized by Kraepelin it in his first detailed textbook descriptions of a condition that eventually became a different disease concept and relabeled as SCZ.

Neurotropic viruses: Viruses with a specific tropism for the nervous system, *i.e.* capable of infecting nerve cells. For example, include polio, influenza, rabies, herpes, cytomegalo-viruses, and retroviruses like HIV.

Human Endogenous retroviruses: Endogenous silent viral elements in the genome that closely resemble and can be derived from retroviruses. They represent about 8% of the human genome.

Antigenic cross-reactivity: Ability of an immune cell to attack an antigen different from the one it originally targets. If a different type of disease-causing substance has similar chemical properties to a previously encountered invasive agent, then cross-reactivity can occur allowing the immune cell to attack the new invader.

(capsid/matrix), concomitantly present in the serum of nearly 50 % of a group of 49 individuals diagnosed with SCZ (Perron et al. 2008). Very recently, a first case of an HIV-infected patient with psychosis associated with neuronal surface antibodies was reported (Arboleya et al. 2016). Infectious insults, a well-described risk factor for psychiatric disorders, are in fact strong inducers of inflammatory responses and potentially of autoimmunity (Yolken & Torrey 2008).

b. Immune dysregulation and psychosis

Anyone who has experienced a viral or bacterial infection knows what it means to feel sick. Sickness is a normal response to infection, which is triggered by soluble mediators produced at the site of infection. These peripheral mediators known as pro-inflammatory cytokines can also act on the brain to cause the "sickness behavior" (Dantzer 2001). The brain has long been considered as an "immune-privileged" organ but this immune status is far from absolute, and current research suggests that there is extensive communication between the nervous and the immune systems in both health and disease (Garay & McAllister 2010). The brain contains immune cells, such as macrophages, dendritic cells, and microglia. It is now accepted that cytokines are also synthesized in the CNS and can act as mediators of normal physiological functions in the CNS although their constitutive expression is low (Zhao & Schwartz 1998). Several studies have shown that cytokines can alter excitatory and inhibitory synaptic transmission, are able to inhibit hippocampal LTP and have been associated with cognitive decline and dementia (Garay & McAllister 2010). The results of clinical studies showed that patients exposed to cytokine therapies for cancer or viral infection developed depressive symptoms and other psychiatric adverse effects (Capuron & Dantzer 2003). Furthermore, animal models of maternal infection lead to behavioral abnormalities, some of which are relevant with SCZ and related psychotic illnesses (Meyer 2014). Studying communication between the brain and the immune system is actually a hot topic in psychiatry and neuroscience research, and has led to the concept of

"immunopsychiatry" (Pariante 2015; Leboyer et al. 2016). The idea that altered immune mechanisms might play a role in the development of SCZ and related psychotic illnesses has regained popularity in recent years. Several studies have now demonstrated the presence of altered molecular profiles in serum or plasma from SCZ patients compared with controls (Schwarz et al. 2012; Schwarz et al. 2014). Changes in cytokines levels and cytokine receptors have been found in blood and CSF of unmedicated schizophrenic patients (Upthegrove et al. 2014; Müller et al. 2015). However, an important limitation of these studies is that they cannot determine whether cytokine alteration is a cause or consequence of illness. Signs of immune dysregulation in SCZ have also been observed using *in vivo* brain imaging. Although debated, neuroimaging PET studies provide evidence for neuroinflammation, especially in the gray matter and the hippocampus of patients with recent-onset SCZ and acute exacerbations of SCZ (Tomasik et al. 2014; Khandaker et al. 2015). Finally, recent investigations aimed at targeting the immune- and inflammation-related pathways as a potential alternative treatment approach in SCZ (Fond et al. 2014; Schwarz et al. 2014; Khandaker et al. 2015). Thus, chronic malfunctioning of the immune system seems to possibly contribute to the syndromic presentation of SCZ and to the development of several psychiatric and other medical comorbidities, including autoimmune disorders (Leboyer et al. 2016).

c. Immune genes mutations in schizophrenia

The links between SCZ and a wide range of infections suggest a common underlying pathway, probably involving the inflammatory immune response. Support for an immune-mediated cause in SCZ comes from recent genome-wide association studies (GWAS) that report significant associations between SCZ and markers close to the major histocompatibility complex (MHC) region on chromosome 6 (Ripke et al. 2011, 2013). The MHC region spans more than 200 genes, many of which encode key regulators of immune system function, such as the human leukocyte antigen (HLA) genes, TNF superfamily genes and complement cascade genes (Corvin & Morris 2014). Three GWAS

simultaneously published in 2009, provided evidence implicating the MHC in SCZ (Stefansson et al. 2009; International SCZ Consortium et al. 2009; Shi et al. 2009). Although an overlap has been observed between different psychiatric conditions, in particular between SCZ and bipolar disorder (Lee et al. 2013), polymorphisms within the MHC region are specific for SCZ (Ripke et al. 2013). Recent genomic studies strengthen this association between risk alleles in immune genes and psychotic disorders (Corvin & Morris 2014; Ripke et al. 2014), complementing the link between infections, autoimmunity, and SCZ (Bergink et al. 2014). The MHC association also raises interesting questions about gene-environment interaction, even if like Stefansson et al. who reported no significant link between MHC and season of birth (Stefansson et al. 2009), most of SCZ studies have not generally reported such interaction.

3. Autoimmunity in schizophrenia

Several epidemiologic studies demonstrate a co-occurrence of autoimmune diseases, chronic inflammation and psychiatric disorders (Benros et al. 2014). A Danish population-based study on 7704 patients with SCZ showed that the relative risk of SCZ for an individual with a history of autoimmune disease was elevated by about 45% (Eaton et al. 2006), and about 30% in a subsequent larger population-based study on 39076 patients with SCZ (Benros et al. 2011). A combination of infection and autoimmune disease showed a synergistic effect as the risk of SCZ was increased even further (Benros et al. 2011). Positive associations between SCZ and a wide range of autoimmune/inflammatory diseases, like celiac disease, psoriasis, type I diabetes, hepatitis, multiple sclerosis, Guillain-Barré syndrome have been reported (Benros et al. 2014). However, the majority of subjects do not have overt clinical symptoms of an autoimmune disorder even if they are serologically positive, but they are at higher risk to develop autoimmune disease. In the same way, recent screening studies of patients with SCZ, without autoimmune diseases or infections, have detected autoantibodies or antibodies against infectious agents in the CSF of 3.2-6% of patients

(Bechter et al. 2010). Since in many cases the elevated prevalence of inflammatory diseases was demonstrated before the onset of psychosis, one hypothesized that the onset of psychosis was induced by an inflammatory process elicited by the autoimmune reaction (Davidson & Diamond 2001). In some examples (*e.g.* Guillain-Barré, Multiple Sclerosis or diabetes), infection could cause the initial activation of the lymphocytes, and autoantigens could sustain the activation that persists even after the eradication of the infectious agent. Yet, there is no compelling evidence that such antigenic cross-reactivities are of pathogenic importance in most of the autoimmune diseases in humans. Moreover, most of the autoimmune diseases associated with SCZ are characterized by the presence of antibodies directed against intracellular targets (cf table below). These antibodies might contribute to the persistence of a chronic inflammatory state, known to favor the emergence of psychiatric disorders.

Beyond the fact that autoantibodies can contribute to a pathological environment and a chronic inflammatory state in SCZ, some evidence also suggest that antibodies can be directly pathogenic, as it was proved for some autoimmune diseases.

B. Antibodies in autoimmune synaptopathies

1. <u>Autoantibodies in the CNS and psychosis</u>

Neurological and neuropsychiatric diseases associated with autoantibodies can be divided into two groups according to the location of the antigenic targets: 1) antibodies to intracellular neuronal antigens, which usually have an underlying malignancy and are more likely markers of an inflammatory process, 2) antibodies to neuronal surface antigens, which are more responsive to immunotherapy as the antibodies are directly pathogenic (Vincent et al. 2006).

a. Anti-brain antibodies

A possible role of brain-reactive autoantibodies in SCZ has been discussed since the early 20th century. In 1937, Lehmann-Facius described evidence for the presence of circulating anti-brain antibodies in sera from schizophrenic patients (Lehmann-Facius 1937). In the late 1950s, Heath isolated a protein (taraxein) from the serum of schizophrenic patients that produced EEG and behavioral alterations, similar to findings in SCZ, when injected to monkeys or healthy human subjects (Baumeister 2011). Although the existence of taraxein is controversial and has never been confirmed by other groups, Heath later described the presence of anti-brain antibodies in SCZ (Heath & Krupp 1967), strengthening the autoimmune basis for SCZ popularized by Burch in the early 1960s (Burch 1964). Several groups indeed found increased concentrations of anti-brain antibodies in the sera of patients with SCZ compared to healthy controls (Fessel 1962; Heath & Krupp 1967; DeLisi et al. 1985). However, other studies have found no significant differences in the levels of antibodies directed against brain, brain septal regions, and hippocampus or brain lipids, between SCZ and control populations (Knight et al. 1990; Teplizki et al. 1992; Schott et al. 1998; reviewed in Jones et al. 2005). Autoantibodies binding to brain specific regions, including the amygdala, frontal cortex, cingulate gyrus, and septal area and, to a lesser extent, the hippocampus, parahippocampal gyrus, entorhinal cortex, putamen, mammillary bodies, and caudate nucleus have been detected in the sera and CSF of patients with SCZ (Henneberg et al. 1994; reviewed in Rothermundt et al. 2001). Antibodies against cellular proteins such as gangliosides, dietary antigens (*e.g*. gliadin and casein), neurotransmitters (*e.g*., 5HT, ACh) and their receptors have also been examined in SCZ but many of these brain-antibodies turned out not to be clinically relevant, with high prevalence in healthy subjects or variability in detection (Goldsmith & Rogers 2008). These limitations may be due in part to the method of antibody measurement, using nonspecific small peptide enzyme-linked immunosorbent assays (ELISA), or to the intracellular location of most antigens (Deakin et al. 2013; Benros et al. 2014).

Table 6. Autoimmune diseases associated with psychosis

Disease	Targets	Target nature/function	Target localization
Celiac disease	α-Gliadin α-Transglutaminase	Class of proteins present in wheat Enzyme converting Glutamine into Glutamic acid	EC IC
Rheumatoid arthritis	α-Ro (SSa) α-Sa α-RA33 α-p68 α-Keratin α-Calpastatin	Nuclear antigen Nuclear antigen Nuclear ribonucleoprotein Nuclear antigen Fibrous structural protein protecting epithelial cells Endogenous calpain inhibitor	IC
Thyroid-gland disease	α-TPO	Enzyme involved in thyroid hormone synthesis	IC
Type I diabetes	α-GAD (65 & 67)	Enzyme catalyzing glutamate decarboxylation to GABA and CO_2	IC
Systemic Lupus Erythematosus	α-DNA α-NMDAR	Nucleic acid, carrying the genetic information Glutamatergic receptor	IC EC
Multiple Sclerosis	α-MOG α-Proteolipid protein α-Myelin	Glycoprotein involved in CNS nerves myelination Major myelin protein Fatty white substance surrounding nerve cell axon	EC EC EC
Autoimmune Hepatitis	α-Actin α-ANA α-ANCA α-SMA α-ASGP-R	Major component of eukaryotic cell cytoskeleton Binds to contents of the cell nucleus Targets the cytoplasm of neutrophils and monocytes Targets smooth muscle Lectins binding to glycoproteins (in order to remove glycoproteins from the circulation)	IC IC IC IC EC
Guillain-Barré syndrome	α-Ganglioside	Glycosphingolipid found in the CNS	EC
Psoriasis	α-Calpastatin	Endogenous calpain inhibitor	IC

TPO: Thyroperoxidase; GAD: Glutamate Decarboxylase; DNA: Deoxyribonucleic acid; NMDAR: N-Methyl-D-Aspartate Receptor; MOG: Myelin Oligodendrocyte Glycoprotein; ANA: Anti-Nuclear Antibodies; ANCA: Anti-Neutrophil Cytoplasmic Antibodies; SMA: Smooth Muscle Antibody; ASPG-R: Asialoglycoprotein receptor

b. Intracellular antigens

Nuclear antigens

A large number of studies have investigated the presence of autoantibodies targeting nuclear antigens such as DNA and heat-shock proteins because these antibodies are often increased in patients with an autoimmune disease (Goldsmith & Rogers 2008). A significantly higher frequency of circulating antinuclear antibodies in people with SCZ compared to controls has been found in a number of studies, whereas others have found no significant increase of such antibodies in unmedicated people with SCZ compared to healthy controls (Amanda L Jones et al. 2005). Several possible reasons could account for these discrepancies. The diagnostic criteria used to classify patients could influence results, patients with different presentations (that could be indicative of

different disease processes or causes) being compared against one another. The medication status of patients could also influence results (unmedicated *vs* anti-psychotic treated patients). The techniques used have also significantly changed over the last decades in which the tests have been done, with some studies using much more sensitive techniques than others.

Cytoplasmic antigens

In addition to antinuclear antibodies, increased concentrations of anti-cardiolipin antibodies have been detected in patients with SCZ and their healthy relatives (Goldsmith & Rogers 2008). A recent study by Dahm et al. (2014) describes circulating antibodies against several intracellular antigens in a large cohort of 1378 patients with SCZ (some of whom also positive for NMDAR-Ab): amphiphysin 2%, ARH-GAP26 1%, GAD65 0.7%, Ma1 0.2%, Ma2 0.6%, Yo 0.4% . Frequencies of antibodies were similar between healthy and schizophrenic subjects and titer ranges were comparable. In line with this, Endres and colleagues reported reactivity of antibodies against intracellular onconeural antigens (Yo, Hu, CV2) in 3.5% of CSF samples from schizophrenic patients (Endres et al. 2015). Concomitant NMDAR and VGKC antibodies were detected in the serum and CSF of these patients (3.2% of patients). It was previously assumed that onconeural antibodies are mostly associated with paraneoplastic syndromes and might indicate a tumor-induced immune response (Pittock et al. 2004; Graus et al. 2010). Surprisingly, none of the patients included in this study had a cancer (not specified in Dahm et al. 2014). Moreover, the similar seropositivity in healthy controls raises the question about the pathophysiological meaning of these antibodies. One possible explanation could be that the presence of such intracellular antibodies may indicate the presence of a cancer that is not yet detectable with standard diagnostic means.

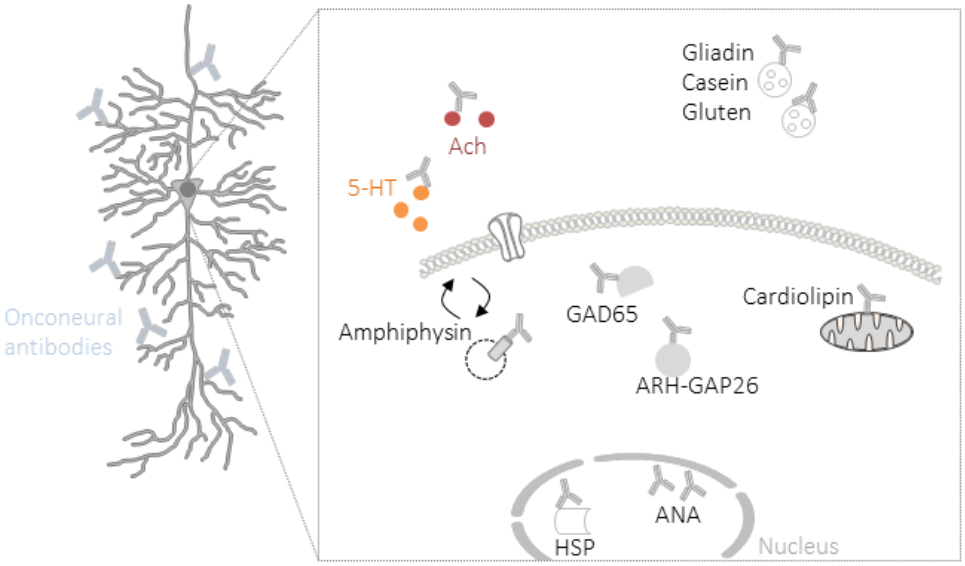

Figure 12. Anti-brain and intracellular antibodies detected in schizophrenia
Brain-reactive antibodies have been found in early studies from the 20th century. Numerous onconeural antibodies have also been detected in patients with schizophrenia, as well as antibodies against cellular proteins. However, whether these antibodies possess a mechanism to enter the cell or an unrecognized cross-reactivity to an extracellular/membrane antigen is not know. Thus, the relevance of these targets is not certain. 5-HT: serotonine, Ach: acethylcholine; GAD65: Glutamate Decarboxylase 65; ARH-GAP26: Rho GTPase activating protein 26; HSP: Heat Shock Protein; ANA: Anti-Nuclear Antibodies; (see Goldsmith & Rogers 2008)

c. Surface synaptic antigens

Relevant autoantibodies in psychosis and SCZ are likely related to cell surface synaptic proteins in which the likelihood of pathogenicity is greater (Deakin et al. 2013). Indeed, patients with intracellular antibodies respond poorly to tumor removal and immunotherapy, including plasmapheresis and corticosteroids (Dalmau et al. 2008). Antibodies against several neurotransmitter receptors have been identified in SCZ. Several groups have reported significantly higher levels of antibodies to M1 and M2 muscarinic cholinergic receptors, nicotinic acetylcholine receptors, dopamine D2 receptors, mu-opioid and serotonin (5-HT1A) receptors in sera from people with SCZ when compared to healthy controls (Jones et al. 2005; Goldsmith & Rogers 2008; Deakin et al. 2013; Benros et al. 2014; also see table). One group found increased serum titers of autoantibodies against nerve growth factor (NGF) in patients with positive symptoms, but not in patients with negative

symptoms (Goldsmith & Rogers 2008). Additional antigens including AMPAR, $GABA_B$ receptor and DPPX, an auxiliary subunit of the potassium channel Kv4.2, have been described in neuropsychiatric disorders associated with psychosis expression (Deakin et al. 2013; also see paragraph III.B.3). Relevance of an antibody is obvious especially when the phenotype of the patients correlates well with the specific antibody. For example, muscle weakness is caused by antibodies against acetylcholine receptors in the neuromuscular junction, whereas the phenotype of patients with intracellular onconeural antibodies is usually broad and unrelated to the antigens function. These antibodies can also be classified according to their relevance for the pathology (Diamond et al. 2009a): antibodies that have a causal relationship with the development of symptoms, antibodies that are generated as a secondary symptom during the disease, and antibodies that are not associated with the disease. Indeed, not all cell surface antibodies may be clinically relevant.

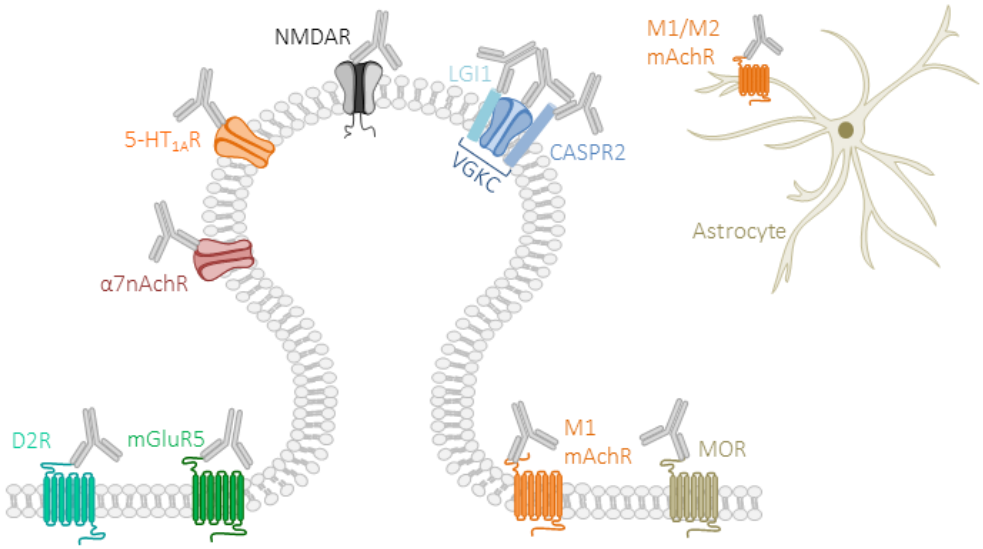

Figure 13. Membrane antibodies detected in psychosis-associated disorders
Antibodies against several neurotransmitter receptors have been detected in the serum of patients with schizophrenia and psychosis-related disorders. Whether all these antibodies are relevant for psychosis remains an open question. NMDAR: N-Methyl-D-Aspartate receptor; 5-HT1A: Serotonine 1A receptor; α7nAchR: α7 nicotinic Acetylcholine receptor; D2R: Dopamine D2 receptor; mGluR5: metabotropic receptor 5; M1/M2 AchR: M1/M2 muscarinic Acetylcholine receptor; MOR: mu-opioid receptor; LGI1: Leucine-rich, Glioma Inactivated 1. CASPR2: Contactin Associated Protein 2;.VGKC: Voltage Gated Potassium Channel; (see Goldsmith & Rogers 2008

2. What defines an autoimmune disease?

Autoimmunity is a misdirected immune response that occurs when the immune system goes awry and attacks the body itself. Any disease that results from such an aberrant immune response is termed an autoimmune disease. There are several levels of evidence required to establish the pathogenicity of an autoantibody in an autoimmune disease (Rose & Bona 1993; Zuliani et al. 2012; Coutinho et al. 2014). Autoimmune diseases can be defined according to principles that are similar to Koch's postulates (1893) in microbiology, originally designed to establish a causative relationship between a microbe and a disease (Rose & Bona 1993). These 4 criteria were further adapted by Witebsky (1957) to describe the types of experimental evidence used to assess whether a particular disease is of autoimmune origin; and if so, if it is caused by pathogenic autoantibodies. The first described synaptopathy of autoimmune origin was myasthenia gravis (MG). MG is a paradigm autoantibody-mediated disease in which autoantibodies targeting acetylcholine receptors (AChR) disrupt synaptic transmission at the neuromuscular junction (NMJ), leading to muscle weakness and fatigue. How myasthenia gravis became recognized as an autoimmune disease? Experimental evidences were accumulated over time to fill Witebsky's criteria (Vincent 2002):

Criteria #1- The autoantibody must be present with the clinical manifestation and detectable in the blood and/or affected tissue: Antibodies to the ACh receptor are found in 85% of patients with generalized muscle weakness. The demonstration of the presence of AChR antibodies in patients was obtained by immunoprecipitation assay using radiolabeled α-bungarotoxin, a competitive antagonist of AChR.

Criteria #2- Autoantibodies must target a receptor or other protein expressed on the membrane surface: Both IgG and complement are present at the NMJ of MG patients, and co-localize with the remaining Ach receptors.

Criteria #3- Antibody transfer must replicate the disease in an animal experimental model or in humans: Indirect evidence of antibody pathogenicity may be obtained from "active immunization", *i.e.* animal studies in which immunization of the animal with an autoantigen induces expression of the disease symptoms. Immunized rabbits with purified AChR became weak, a feature similar to MG.

This muscular weakness could be reversed by acetylcholinesterase inhibitors, and was shown to be due to antibodies cross-reacting with muscle AChR, leading to a decrease of functional AChR. Direct evidence of autoantibody pathogenicity was later demonstrated by passive antibody transfer in mice injected with IgG from MG patients, who developed a typical MG phenotype (weakness and a reduced number of AChRs at the NMJ).

Criteria #4- Elimination or suppression of the autoimmune response prevents disease progression or improves the clinical manifestations: Removal of the IgG antibodies from MG patients by plasma exchange produced a significant clinical improvement, inversely correlated with the level of AChR antibodies.

A number of diseases are designated “autoimmune” even though they do not meet all the criteria described above. Indeed, these criteria are not restrictive and other clinical observations can help to suspect a disorder of autoimmune origin. As discussed before, the occurrence of other autoimmune diseases in the same individual or aberrant expression of MHC antigens on the affected organ constitute risk factors. Lymphocyte infiltration in the target organ can also be a sign of immune dysregulation. These circumstantial evidences, as named by Rose et al. (1993), cannot define an autoimmune disease *per se* but are strong indicators that can strengthen Witebsky’s criteria in some cases where they are not all fulfilled.

3. Antibodies against membrane targets in psychosis-associated disorders

a. Voltage-gated potassium channel (VGKC) complexes

A wide variety of clinical syndromes has been associated with antibodies to VGKC (Irani & Vincent 2016). VGKC antibodies (VGKC-Ab) were first detected in neuromyotonia by radioimmunoassay (Shillito et al. 1995), which still represents the gold standard assay to detect such antibodies (Vincent et al. 1995). Most VGKC-Ab do not bind to VGKC themselves, but rather target proteins tightly complexed to VGKC (Sarosh R. Irani et al. 2010). The VGKC complex contains, among others, the leucine-rich, glioma-inhibited 1 (LGI1) protein and contactin-associated protein 2 (CASPR2). Patient antibodies were shown to bind to extracellular domains of one or occasionally more of these antigenic targets (Irani & Vincent 2016). Detection of one or the other predicts a differential clinical phenotype but noteworthy, they all show neuropsychiatric symptoms (Pollak et al. 2016; Prüss & Lennox 2016). Antibodies to CASPR2 are more commonly associated with peripheral nerve hyperexcitability including neuromyotonia and Morvan syndrome (Deakin et al. 2013; Irani & Vincent 2016). LGI1 antibodies are found almost exclusively in patients with limbic encephalitis, a non-paraneoplastic disease characterized by amnesia, disorientation, psychiatric features, and seizures (Irani & Vincent 2016). One study reported that 26% of patients with neuromyotonia present psychiatric symptoms (Hart et al. 2002). Somers and colleagues (2011) reported that 44% of patients seropositive for VGKC-Ab presented one or more neuropsychiatric symptoms, and patients with medium or high antibody values were significantly more likely to present with neuropsychiatric manifestations than patients with low antibody values (Somers et al. 2011). However, the prevalence of VGKC-Ab in purely psychiatric populations has not been fully explored. Still, some patients with predominantly psychiatric presentations associated with VGKC-Ab responded positively to immunotherapy treatment (Prüss & Lennox 2016). On the other hand, a recent study reported a lack of response to immunotherapy in patients with low levels of VGKC-Ab, and advanced that VGKC-Ab in the absence of LGI1 or CASPR2 antibodies are likely to be irrelevant (van Sonderen et al. 2016). A number of VGKC-positive but CASPR2 or LGI1-negative cases respond to immunotherapy (Kruse et al.

2015), suggesting a potential contribution of yet unidentified antigens. Indeed, about half (ranging from 16% to 77% depending on the studies) of the VGKC-positive patients lack antibodies to LGI1 and CASPR2 (van Sonderen et al. 2016). Thus, future work is needed to identify antigenic targets that may be particularly relevant for those with neuropsychiatric presentations. Of great interest, mutations in the gene CNTNAP2 encoding CASPR2 are associated with SCZ, epilepsy and autism (Prüss & Lennox 2016), reflecting the fact that genetic and autoimmune conditions often target the same protein.

b. Dopamine receptors

In the early 1980s, Knight proposed that acute positive symptoms of SCZ are caused by autoantibodies which would either interact with and stimulate dopamine receptors, or block presynaptic autoreceptors, and thus suggested to look for the presence of antibodies in schizophrenic patients CSF (Knight 1982). Thirty years later, Dale et al. detected D2R antibodies (D2R-Ab) surface in the serum of 12 of 17 children with basal ganglia encephalitis, associated with psychiatric disturbance like agitation, psychosis and hallucinations (Dale et al. 2012). Pathmanandavel et al. (2015) confirmed the detection of serum antibodies to D2R in 3 of 43 children with first episode of psychosis. One third of the IgG antibody-positive patients also had IgM D2R-Ab, whereas no IgA D2R-Ab were detected. Very few studies have analyzed the potential role of dopamine receptor antibodies in adult patients. Von Kirchbach (1987) failed to detect any autoantibodies against dopamine receptors in the serum of 15 schizophrenic patients in acute state. Similarly, Tanaka et al. (2003) observed no serum D2R-Ab in SCZ patients (N = 44) using a radioligand assay. It is not clear whether the analyzed blood samples came from acutely ill or clinically stable SCZ patients. Additionally, in a cohort of 62 patients with an acute psychosis, 1 serum reacted with dopamine receptor-enriched membranes, but the authors ignored this result (Teplizki et al. 1992). These negative results might be taken with caution as the methods used might not be sensitive enough to detect low levels of antibodies. Still, a recent study using classical CBA reports the absence of D1R- and D2R-Ab in blood samples from acute schizophrenic patients (Müller et al. 2014).

Whether D2R-Ab could be responsible for psychiatric symptoms expression has not yet been demonstrated, but D2R-Ab appear to stimulate rather than antagonize dopamine receptor signaling, consistent with the evidence of hyperdopaminergic activity in psychosis (Carlsson 1978). Brimberg et al (2012) observed an increase of dopamine levels in the medial prefrontal cortex of a rodent model of GAS (Group A Streptococus) infection, involved in pediatric autoimmune disorders. In line with this, human sera containing IgG D2R-Ab induced inhibitory signaling of the D2R, like in presence of dopamine (Cox et al. 2013).

c. Cholinergic receptors

Elevated serum levels of antibodies to the α7nAchR have been reported in schizophrenic patients (Mukherjee et al. 1994; Chandley et al. 2009). Lieberman et al. (1984) found a longer duration off neuroleptics to be associated with higher anti-nAchR antibodies levels, consistent with the idea that neuroleptic-resistant SCZ could have an underlying autoimmune origin. Unfortunately none of those patients benefited of immunotherapy, leaving this hypothesis as pure speculation. Muscarinic acetylcholine receptors also represent antigenic targets in SCZ (Borda et al. 2004; Jones et al. 2014). Circulating autoantibodies against muscarinic receptors most likely display an agonist-like activity as they would bind irreversibly to the receptor, modifying the intracellular events associated with specific mAChR activation (Borda et al. 2004; Ganzinelli et al. 2006). The persistent activation due to antibodies binding could eventually induce desensitization, internalization and/or intracellular degradation of the mAChR, leading to a progressive decrease of neuronal M1 mAChR expression and activity, as observed in SCZ (Dean 2012).

d. Other receptors

Glutamatergic mGluR5 receptors were identified as a target antigen of antibodies either in the serum or the CSF of patients with Ophelia syndrome, a neuropsychiatric disorder associated with Hodgkin lymphoma (Lancaster et al. 2011; Mat et al. 2013). mGluR5 antibodies have also been detected in the serum and CSF of a patient with limbic encephalitis associated with neuropsychiatric disturbances, who was responsive to immunotherapy (Pruss et al. 2014). Notably, mGluR5 dysfunction seems to be part of the pathogenesis of glutamate dysfunction in psychiatric disorders (Pollak et al. 2016). Serotonin 5-HT_{1A} receptor autoantibodies have been found in 6.8 % of patients with SCZ (Tanaka et al. 2003), using a recombinant peptide resembling an extracellular epitope of the receptor in a radioligand assay. Antibodies against ionotropic $GABA_A$ or metabotropic $GABA_B$ receptors have also been described in the serum and CSF of patients with psychiatric manifestations (Lancaster et al. 2010; Tsuchiya et al. 2014; Dahm et al. 2014). A recent study retrospectively identified $GABA_AR$ antibodies in the sera of 40 out of 2,046 patients with varied clinical features, whose sera were negative for other antibodies (Pettingill et al. 2015). Psychiatric features were present in 5 out of 15 subjects, and one patient with new-onset treatment-resistant catatonia who presented with anxiety, obsessionality and psychosis symptoms made substantial improvement after plasma exchange (Pettingill et al. 2015). However, GABA antibodies are typically present in patients with seizures that are refractory to antiepileptic treatment (Jain & Balice-Gordon 2016), and their relevance in the expression of neuropsychiatric symptoms is unknown. In addition, $GABA_BR$ are associated with an underlying tumor in approximately half of patients (Höftberger & Titulaer 2013). Similarly, psychotic symptoms are expressed by some patients with AMPAR antibodies, typically found in epileptic forms of limbic encephalitis (Lai et al. 2009; Graus et al. 2010; Höftberger & Titulaer 2013). Overall, these case studies raise the possibility of a predominantly psychiatric presentation with limited neurological associations, potentially amenable to immunomodulation (Graus et al. 2010). However, the significance of AMPAR antibodies to psychiatric symptoms is unknown, and these antibodies remain rare and associated with cancer (Höftberger & Titulaer 2013).

Table 7. Synaptic antigens found in neuropsychiatric disorders

Antigen detected	Associated disorder	Ig subtype	Major symptoms	Psychiatric symptoms at presentation	Detection method	Reference
AMPAR (extracellular part) Epitope unknown	Limbic encephalitis	IgG (serum)	Prominent seizures, memory deficits & psychiatric disturbance	Confusion, depression, anxiety, hallucinations, delusions, psychosis, personality change	IHC, WB, CBA (fixed) IP, mass spectrometry, CBA (fixed)	Hofberger 2015 Graus 2010 Lai 2009
$GABA_BR$ ($GABA_{B1}$&$_{B2}$ subunits)	Limbic encephalitis	Serum			IHC, ICC and WB CBA (fixed), Euroimmun	Lancaster 2010, Hoftberger 2013 Dahm 2014 Tsuchiya 2014
	NPSLE	IgG Serum/CSF	Neuropsychiatric syndromes (CNS&PNS) Prominent seizures, psychosis	Confusion, psychosis other psychiatric syndromes	ELISA	
$GABA_AR$ (α1/β3/γ2 subunits)	Limbic encephalitis	IgG (1/3), IgM Serum/CSF	Prominent seizures, memory deficits & psychiatric disturbance		IP, tandem mass spectrometry, CBA (live)	Pettingill 2015
mGluR5	Limbic encephalitis + Hodgkin lymphoma (Ophelia syndrome)	IgG Serum/CSF			IHC, ICC, IP, mass spectro, CBA (fixed)	Lancaster 2011, Mat 2013, Pruss 2014
D2R (extracellular part) Epitope unknown	Basal ganglia encephalitis	IgG (serum)	Dominant movement (100%) & psychiatric disorder (75%)	Paranoia, psychosis, hallucinations, agitation, anxiety, ataxia	CBA (live HEK)	Dale 2012
	First episode Psychosis	IgG, IgM		Delusions, hallucinations, disorganized behavior	Flow-cytometry (live HEK)	Pathmanandavel 2014
	Schizophrenic disorders Schizophrenia	IgG	Acute episodes	N.A	Competitive binding ELISA Radioligand assay	Von Kirchbach 1987 Teplizki 1992 Tanaka 2003
LGI1	Peripheral nerve excitability					
CASPR2, LGI1	Morvan syndrome					
VGKC	Limbic encephalitis	IgG (serum)	Prominent seizures, memory deficits & psychiatric disturbance	Confusion, depression, anxiety, hallucinations, delusions, psychosis, personality change	Radioligand assay	Somers 2011
nAChR α7 subunit	Myasthenia gravis Schizophrenia	IgG Serum (CSF not tested)		N.A	Radioligand assay ELISA	Mukherjee 1994, Chandley 2009
Muscarinic M1/M2 AChR (astrocytic - 2nd extracellular loop of the human M1/M2 mAChR) M1 Neuronal	Schizophrenia	IgG (serum)			ICC, flow cytometry, radioligand assay IHC, ELISA, WB ELISA, radioligand assay	Borda 2002, 2004 Jones 2014 Ganzinelli 2006

4. NMDAR-Ab in neuropsychiatric disorders

a. Systemic Lupus Erythematosus (SLE)

Between 14% and 75% of patients with SLE are estimated to experience neuropsychiatric symptoms, including mood and behavioral disturbances, and psychotic symptoms (Amanda L Jones et al. 2005). Neuropsychiatric SLE (NPSLE) is a generic term for cerebral manifestations that may arise in patients with SLE. The manifestations of NPSLE vary in presentation, and are often difficult to distinguish from other neuropsychiatric conditions with different etiologies. The neuropsychiatric symptoms are suspected to be induced by brain-reactive antibodies, as hypothesized almost four decades ago (Bluestein 1978). A recent meta-analysis unveiled strong links between NPSLE and the presence of anti-neuronal antibodies, as patients with NPSLE are more likely to have elevated serum (9.50 times) and CSF (36.84 times) levels of anti-neuronal antibodies compared with SLE patients (Ho et al. 2016). Lymphocytotoxic antibodies, anti-phospholipid antibodies, anti-ribosomal P-protein antibodies and many other antibodies have been linked to global and focal abnormalities of the brain, but their pathogenic role has remained controversial (Yaniv et al. 2015). A subset of DNA-specific antibodies has been detected in the serum of 30-60% of patients with SLE (Diamond et al. 2009). Pioneering studies by Diamond's laboratory demonstrated that a murine monoclonal anti-DNA antibody (R4A) recognizes a consensus sequence (DWEYS) present in the extracellular, ligand-binding domain of both murine and human GluN2 subunits of the NMDAR (Degiorgio et al. 2001). Intrathecal injection of this antibody into mouse brains led to neuronal death by apoptosis. Likewise, the injection of human sera or CSF from SLE patients into mouse brains resulted in neuronal death (Degiorgio et al. 2001). Originally, anti-DNA antibodies were described as cross-reacting with NMDAR (Aranow, Diamond 2011). It is now proposed that there are ''pure'' anti-GluN2 antibodies in addition to cross-reacting anti-DNA/NMDAR antibodies (Lauvsnes & Omdal 2012). It has recently been demonstrated that NMDAR-reactive antibodies function as modulators that preferentially bind to the open NMDAR pore, thus antibody binding can be presumed to increase the open-state duration and enhance the calcium influx (Faust et al. 2010). The effect of anti-GluN2 antibodies correlates with titers: at low

concentrations, GluN2 antibodies act as agonists and enhance NMDAR currents, while at higher concentrations they lead to excitotoxic cell death (Faust et al. 2010). This neuronal overexcitation may then lead to neurocognitive disturbances. Blood levels of NMDAR-Ab in patients with SLE were associated with cognitive dysfunction and depressive mood (Omdal et al. 2005; Lapteva et al. 2006), although this is a matter of debate (Lauvsnes & Omdal 2012). Mice with circulating anti-NR2 antibodies did not show neuronal death before they were injected with the inflammatory agent lipopolysaccharide (LPS), which abrogated the BBB and led predominant cell death in the hippocampus, and associated impaired memory (Lauvsnes & Omdal 2012). The same effect was observed after administration of sera from SLE patients and LPS (Kowal et al. 2006).

b. Neurodegenerative disorders

A recent study reported the presence of NMDAR-Ab in the serum of 16 % of dementia patients, including patients with Alzheimer disease (AD), and 7.1 % of age-matched healthy volunteers (Busse et al. 2014). IgG NMDAR-Ab were present in only 1 AD patient while IgM and IgA levels were similar between dementia and healthy aged subjects, suggesting that seropositivity for these Ig subtypes was associated with aging rather than with psychosis presentation. Likewise, Hammer et al. (2013) detected IgG NMDAR-Ab in one patient (0.4%) suffering from Parkinson disease (Hammer et al. 2013). By contrast, IgM and IgA were detected equally in patients and age-matched controls (Parkinson vs control: IgA 6.5% vs 5.7%; IgM 9.5 vs 5.7%). The serum samples from Hammer's publication were re-tested for the purpose of another study (Dahm et al. 2014). In agreement with their previous work, high levels of circulating IgM and IgA were found in Parkinson patients as in healthy aged controls (Parkinson vs control: IgA 7.75% vs 4.46%; IgM 6.2 vs 4.35%). While using the same detection method as in Hammer's study (Hammer et al. 2013), IgG NMDAR-Ab were detected in 4.65% of Parkinsonian subjects and 1.17% of healthy controls. These differences could be explained by the fact that fluorescence detection, and by extension sample seropositivity, relies on a

visual criteria dependent on the experimenter. Moreover, the group of Parkinson patients might include individuals with and without dementia, which can influence the statistics due to the low amount of IgG NMDAR-Ab positive samples in each subgroup.

c. Demyelinating disorders

Patients afflicted with the autoimmune disease Neuromyelitis Optica (NMO) have antibodies directed against astrocytes in their serum. Lennon and colleagues discovered that these astrocyte antibodies bind the antigen aquaporin-4 (AQP4), a water channel protein expressed on astrocytic foot processes surrounding blood vessels which controls the water flow in and out of the brain (Lennon et al. 2005). Evidence points towards a possible association of NMDAR-Ab encephalitis with demyelinating disease. NMDAR-Ab encephalitis was described in young female patients with NMO (Alam et al. 2015; Luo et al. 2016) and confirms previous case reports (Kruer et al. 2010). One of the patients showed psychiatric symptoms including confusion, incoherent speech, hallucinations, delusions and prominent catatonia at presentation (Alam et al. 2015). The second patient was a Chinese female who developed NMDAR-Ab encephalitis during the course of NMO spectrum disorders (NMOSD). She presented fulminant neuropsychiatric manifestations and behavioral dysfunction, as the NMDAR-Ab titers increased in both the serum and CSF (Luo et al. 2016). Consistently, a previous review reported that 3.3% NMDAR-Ab encephalitis patients had prominent MRI features of demyelination, including patients in whom NMDAR-Ab encephalitis was preceded or followed by independent episodes of NMOSD (Titulaer et al. 2014). NMO can closely resemble other diseases such as multiple sclerosis (MS). Several cases of MS overlapping with NMDAR-Ab encephalitis have been reported these last years (Uzawa et al. 2012; Fleischmann et al. 2014), strengthening evidences of wide range overlapping disorders with NMDAR-Ab encephalitis.

d. Psychiatric disorders

Autism spectrum disorders (ASD) are a severe group of neurodevelopmental disorders. The etiology of ASD remains unclear with some implications of genetic, neurological, environmental factors, together with signs of an altered immune response (Kobeissy & Moshourab 2015). NMDAR-Ab encephalitis was diagnosed in a 9-year old patient with autistic regression (Creten et al. 2011). By the time his symptoms worsened, NMDAR-Ab titers raised in the serum and CSF. Electroconvulsive treatment combined to immunotherapy significantly improved his clinical condition. A couple of years later, Scott and colleagues (Scott et al. 2013) described a 33-month-old patient with autistic regression associated with NMDAR-Ab encephalitis. Similarly, a case report recently described two toddlers who presented with developmental regression, mimicking an autistic regression, who were found to have NMDAR-Ab in serum and CSF (Hacohen et al. 2016). Immunotherapy was beneficial in both patients. On the contrary, a recent work failed to detect NMDAR-Ab in children with autism, as none of the 42 children with autism or 21 healthy controls had positive NMDAR-Ab (Bayram et al. 2016). The great heterogeneity in presentations of autistic spectrum disorder suggests that its etiology is complex and can involve an underlying autoimmune process (Scott et al. 2013). Furthermore, emerging genetic data together with clinical trials suggests a potential role of the NMDAR in autism (Scott et al. 2013). Several studies have found increased levels of autoantibodies in bipolar disorder (BD) patients compared to controls (Eaton et al. 2010; Sidhom et al. 2012). An autoimmune process involving the NMDAR has been recently outlined as a possible biological correlate of the pathophysiology of BD in a cohort of 60 manic patients, who presented elevated serum levels of autoantibodies against the GluN2 subunit of the NMDAR (Dickerson et al. 2012). None of the patients exhibited NMDAR-Ab encephalitis at the time of the 6-months follow-up. Usually, patients with NMDAR-Ab encephalitis have psychiatric manifestations at onset, and later develop the typical symptoms spectrum of the disease (Kuo et al. 2012). The presence of IgG NMDAR-Ab has been reported in the serum of one BD subject (titer 1:100), with no further clinical information (Hammer et al. 2013). From their part, Choe and colleagues (2013) described the first

case of IgM NMDAR-Ab coincident with symptoms of bipolar disorder. Patient's serum reduced NMDAR expression on neuronal cells and decreased cell survival, similar to IgG pathogenic effects classically described in NMDAR-Ab encephalitis (while no IgG were found in this patient). Very recently, the first case of HIV-infected patient with bipolar I disorder (manic orientation) and associated psychosis has been reported (Arboleya et al. 2016). In this study, 2 out 61 patients (3.3%) with first episode psychosis were seropositive for NMDAR-Ab, one of them eventually met the criteria for NMDAR-Ab encephalitis. Narcolepsy type 1 (NT1) is a disabling orphan sleep disorder characterized by excessive daytime sleepiness and cataplexy, and is frequently associated with hypnagogic (transition from wakefulness to sleep) hallucinations and sleep paralysis. Recent findings suggest an overlapping autoimmune pathogenesis between NT1 and SCZ-like psychosis, associated with both HLA and autoantibodies. In line with this, Tsutsui et al. (2012) found NMDAR-Ab in 3 out of 5 (60%) patients with a comorbid diagnosis of psychosis and narcolepsy, and considered a shared autoimmunity between these disorders. On the opposite, no antibodies against the GluN1/2B heteromers of the NMDAR were found in a large cohort of 542 narcoleptic patients including 10 patients (1,8%) diagnosed with a comorbid SCZ-like psychosis (Dauvilliers et al. 2016). These negative results corroborate a recent study in 10 patients with NT1 and psychosis (Canellas et al. 2014). Overall, the nature and extent of the possible relationship between NMDAR-Ab encephalitis, NMDAR-Ab and psychiatric disorders is yet to be ascertained. One of the most controversial areas of psychiatric research today concerns the further question of whether neuronal autoantibodies have a causal role in psychiatric disorders such as SCZ. There are presently a number of case studies, albeit uncontrolled, demonstrating immunotherapy-responsive psychiatric presentations associated with neuronal autoantibodies (Pollak et al. 2015). Though, due to the fact that a main antigen is often associated with 1 or several other antigens, symptoms expression and antibodies detection are not direct correlative elements. As described above, NMDAR-Ab have been detected in a wide range of neuropsychiatric disorders. However, there is no clear evidence to link their presence to the etiology of the disease.

5. NMDAR-Ab encephalitis

a. Characteristics

Antibodies against the GluN1 subunit of NMDAR are associated with a characteristic syndrome called NMDAR-Ab encephalitis that develops in several stages of illness and recovery (Dalmau et al. 2007, 2008, 2011). First, most of the patients experience a non-specific viral-like prodrome consisting of headache, fever, nausea, vomiting, diarrhea, or upper respiratory-tract symptoms (Dalmau et al. 2011; Wandinger et al. 2011). Within a few days, patients develop psychiatric symptoms. At presentation, a psychiatric disorder is usually considered and patients are often admitted to psychiatric centers. Short-term memory loss is common but underestimated because psychiatric symptoms and speech problems often interfere with the assessment of memory. This initial phase of the illness is usually followed by decreased responsiveness that can alternate between periods of agitation and catatonia. At this stage, abnormal movements, seizures and autonomic instability are usual manifestations (Dalmau et al., 2008). During such stages, dissociative responses to stimuli are noted. Some patients were unresponsive to painful stimuli but resisted passive opening of the eyes (Iizuka et al. 2008). This dissociative state is similar to that caused by NMDAR antagonists, such as phencyclidine or ketamine, which are called dissociative anesthetics (Lahti et al. 1995, 2001). If not treated, the syndrome naturally evolves towards coma, but can also occur earlier when the level of consciousness is relatively preserved. Strikingly, the time course of clinical features is uniform between patients and draws a very typical and recognizable syndrome (Irani et al. 2010). About 80% of patients with NMDAR-Ab recover or have mild sequelae (Titulaer et al. 2013). Most patients receive corticosteroids, intravenous immunoglobulins or plasma exchange as first-line of immunotherapy. Of patients who did not improve with first-line treatment, the majority improved with second-line immunotherapy, most often rituximab or cyclophosphamide (Ishiura 2008; Dalmau et al. 2011; Titulaer et al. 2013). Despite substantial clinical remission of NMDAR-Ab encephalitis, most patients still show persistent cognitive impairment several years later (Finke et al. 2011). The persistence of these long-term deficits suggests that full recovery may be limited in some patients

and underlines the need for immediate and aggressive therapy, knowing that early treatment had a significantly better cognitive outcome (Finke et al. 2011).

b. Epidemiology and diagnosis

Since the first cases described in 2007, the number of documented cases has increased manifold, suggesting that NMDAR-Ab encephalitis is not rare. Initially, the disorder was thought to exclusively be a paraneoplastic disorder, occurring in young females with an underlying ovarian teratoma (Dalmau et al. 2007). However, over the last decade, it has become clear that NMDAR-Ab encephalitis can occur with or without a tumor, and can arise in children and young adults, both male and female (Dale et al. 2009; Florance et al. 2010 ; Irani & Vincent 2011). Confirmation of the clinical diagnosis of NMDAR-Ab encephalitis requires the detection of NMDAR-Ab in the serum and/or the CSF of the patient. There is an ongoing controversy as to whether serum or CSF is best tested. Dalmau's group recommends testing both when possible (Dalmau et al. 2011) whereas Vincent and colleagues report that serum levels of NMDAR-Ab were similar or higher to those of CSF (Irani & Vincent 2011). Neurological features usually do not help for diagnosis as brain MRI is normal in 50% of patients and EEG are abnormal in most patients, usually showing non-specific, slow, and disorganized activity sometimes with electrographic seizures (Dalmau et al. 2011). The clinical symptoms of NMDAR-Ab encephalitis correlate well with the antibody titers (Dalmau et al. 2007, 2008, 2011) and high titers are usually associated with poor outcome (Gresa-Arribas et al. 2013). However, Murdie and colleagues reported a case of negative serum reactivity in NMDAR-Ab encephalitis (Murdie et al. 2015), in which blood samples were negative at first presentation, as the EEG and brain MRI. While symptoms worsened, repeated blood tests eventually revealed the presence of NMDAR-Ab both in the patient's CSF and serum (Murdie et al. 2015). This intriguing case illustrates seronegativity in the acute stage of the illness, and confirms that seropositivity is indeed related to the timing of the blood test, albeit does not fully correlate with the acute state of the disease (Murdie et al. 2015; Beck et al. 2015 but see Steiner et al. 2013).

c. Pathogenic effects of NMDAR-Ab

Molecular impact of NMDAR-Ab

Compelling clinical and laboratory evidence attest that NMDAR-Ab are pathogenic. Antibody-mediated pathogenesis is indisputable as pathogenic effects are no longer observed when NMDAR-Ab are removed (Zhang et al. 2012) and are titer-dependent (Dalmau et al., 2008; Hughes et al. 2010). Furthermore, human autopsy of NMDAR-Ab encephalitis patients showed deposits of IgG but a lack of complement immunoreactivity (Dalmau et al. 2007; Martinez-Hernandez et al. 2011). The main and robust alteration observed postmortem in patients with NMDAR-Ab encephalitis (Hughes et al. 2010) and replicated both in vivo and in vitro is a massive loss of NMDAR. In 2008, a first study showed that cultured hippocampal neurons exposed chronically to patients' CSF showed a strong but reversible decrease of surface NMDAR clusters density (Dalmau et al., 2008). NMDAR-Ab likely mediate their pathogenic effect by binding to, and cross-linking NMDAR, resulting in their internalization (Hughes et al. 2010; Mikasova et al. 2012; Moscato et al. 2014). Patients' antibodies do not alter other membrane receptors, synaptic proteins, neuron morphology or cell survival (Hughes et al. 2010). IgG pathogenicity do not appear to be specific to excitatory neurons as NMDAR on inhibitory neurons are similarly internalized (Moscato et al. 2014). Interestingly, NMDAR internalization reached a "steady state" after 12h of treatment with patients' antibodies; a phenomenon which is independent of receptor activity as application of APV, an antagonist of the NMDAR, failed to potentiate NMDAR endocytosis (Moscato et al. 2014). Once internalized, antibody-bound NMDAR traffic through both recycling endosomes and lysosomes, following a physiological degradation path (Moscato et al. 2014). If NMDAR-Ab do not perturb intracellular receptor trafficking, they rapidly alter NMDAR surface trafficking (Mikasova et al. 2012). A rapid dispersal of synaptic GluN2A subunit is observed in presence of NMDAR-Ab while GluN2B subunit dynamics were highly reduced in the extrasynaptic area, favoring their endocytosis and degradation. The lateral escape of synaptic NMDAR would most likely result from the disruption of NMDAR-EphB2R interactions, essential for its synaptic anchorage. Of great interest, activation of EphB2R by its ligand EphrinB2, known to increase the clustering of synaptic NMDAR (Dalva et al. 2000),

prevented NMDAR-Abs' effects on their target (Mikasova et al. 2012). Consistently, such molecular alterations lead to a decrease in synaptic NMDAR currents (Hughes et al. 2010), the abolishment of synaptic plasticity (Mikasova et al. 2012; Zhang et al. 2012; Dupuis et al. 2014; Würdemann et al. 2016), and the acute suppression of global neuronal network activity (Jantzen et al. 2013). These effects are most likely caused by receptor internalization rather than through a direct action on NMDAR as no antagonist-like effect of NMDAR-Ab was reported (Mikasova et al. 2012; Moscato et al. 2014). Noteworthy, in vitro network activity was impaired already after 15 min with patient CSF, suggesting additional dysfunction prior to antibody capping and receptor internalization (Jantzen et al. 2013). Results from in vivo studies confirmed that NMDAR density was significantly reduced in the hippocampus of rats infused with patients' antibodies, a finding comparable to that observed in autopsied patients (Hughes et al. 2010; Mikasova et al. 2012). Another study observed an increase of the extracellular glutamate concentration following NMDAR-Ab infusion in CA1 (Manto et al. 2010), suggesting a hyperactivity of glutamatergic pathways as shown in pharmacological or genetic models of SCZ. More recently, an elegant work from Dalmau's group demonstrated that a chronic intraventricular infusion of patients' CSF induced progressive memory and behavioral deficits in mice (Planagumà et al. 2015), which were maximal around 14 days when the highest concentration of brain-bound IgG NMDAR-Ab and lowest density of NMDAR occurred. In a follow-up study (Planagumà et al. 2016), in vivo administration of EphrinB2, an endogenous ligand of the EphB2R, consistently prevented the behavioral impairments due to NMDAR-Ab, as already demonstrated in vitro (Mikasova et al. 2012). Still, these studies could not discriminate the effect of NMDAR-Ab and the contribution of other anti-neuronal autoantibodies potentially present in patients samples (CSF, serum or purified Ig). Using single cell cloning, Kreye and colleagues (2016) generated a panel of recombinant monoclonal GluN1 antibodies from CSF memory B cells and antibody secreting cells of NMDAR-Ab encephalitis patients, and showed that these monoclonal antibodies are sufficient to cause NMDAR downregulation and subsequent impairment of NMDAR-mediated currents, providing the ultimate proof of NMDAR-Ab pathogenicity (Kreye et al. 2016).

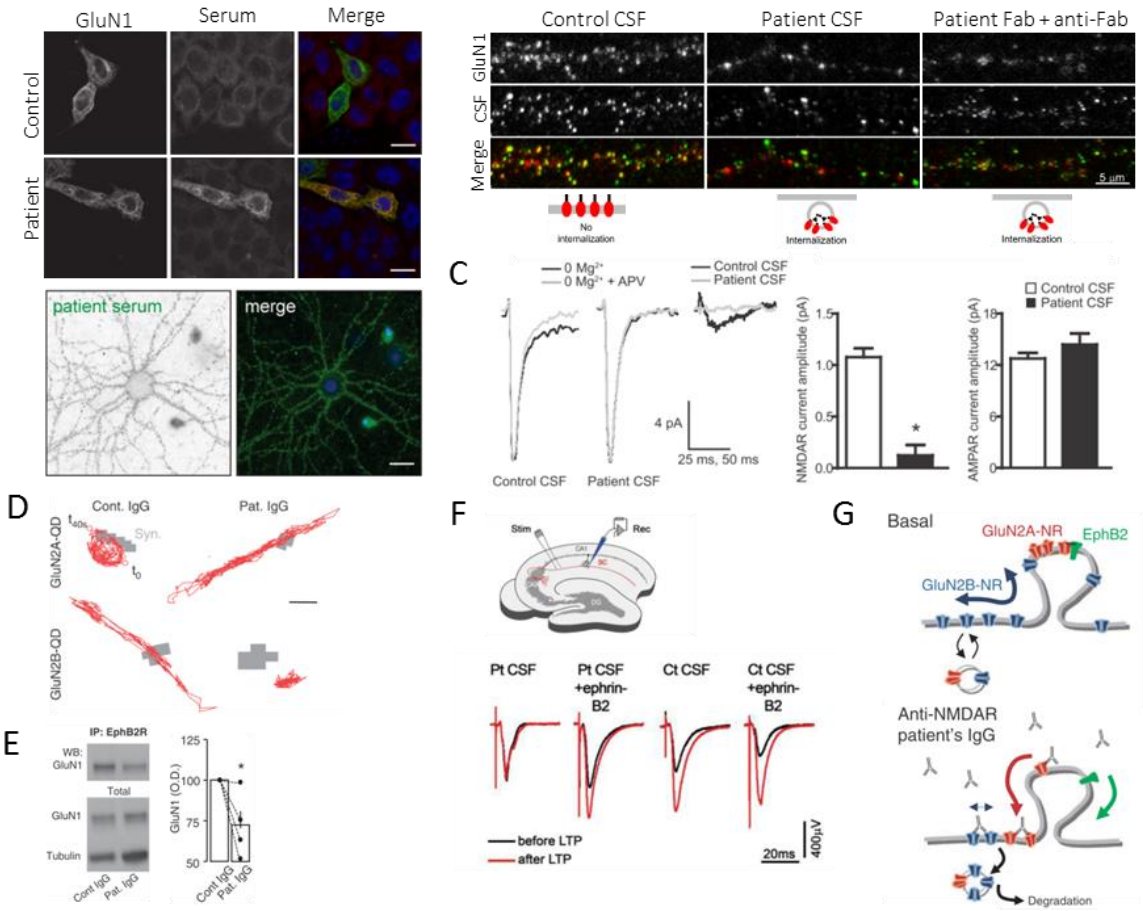

Figure 14. Pathogenic effects of NMDAR-Ab in NMDAR encephalitis

(A) Classical detection of NMDAR-Ab in patients serum using cell-based assay (CBA) on fixed HEK293 cells and hippocampal neurons. Scale bar HEK= 500µm, scale bar neuron= 20µm. (B) NMDAR-Ab cross-link and internalize NMDAR. Treatment with patients CSF positive for NMDAR-Ab decreases surface NMDAR density. Scale bar= 5µm. Similar outcome is observed after treatment with divalent patient Fab fragments (Fab fragments plus anti-Fab secondary antibodies). (C) Patient NMDAR-Ab selectively decrease synaptic NMDA currents without affecting the AMPA component. (D) Patient NMDAR-Ab differentially affect the surface diffusion of NMDAR subunits. In presence of NMDAR-Ab, synaptic GluN2A diffuse much more and escape from the synapse while extrasynaptic GluN2B which are usually very diffusive , are cross-linked in the extrasynaptic compartment. Scale bar= 500nm. (E) Patient NMDAR-Ab disrupt the synaptic interaction between NMDAR and EphrinB2R. (F) Ephrin-B2T treatment partially prevents LTP impairment caused by patients' NMDAR-Ab in the hippocampus; Pt CSF: patient CSF; Ct CSF: control CSF. (G) Model of NMDAR-Ab pathogenic action proposed by Mikasova et al. (2012). (From van Coeverden-Hameete et al., 2016; Hughes et al., 2010; Mikasova et al., 2012; Planaguma et al., 2016)

Epitopes

While the cellular alterations caused by NMDAR-Ab in the eponymous encephalitis have been well described, there is still no clear answer regarding the binding site(s) of such autoantibodies. In the princeps study, Dalmau and colleagues report that antibodies found in patients CSF recognize GluN1/GluN2 heteromers but do not react with GluN subunits expressed individually (in HEK cells), suggesting that NMDAR-Ab only bind to functional receptors. Further work from Dalmau's lab showed that serum or CSF reactivity remained unchanged no matter the GluN2 subunit co-expressed with GluN1 (Dalmau et al., 2008). Interestingly, samples reactivity was fully abrogated when the mutant NR1d4 lacking amino-acid residues 25-380 was expressed, confirming that the extracellular part of the GluN1 subunit is necessary for NMDAR-Ab binding (Dalmau et al., 2008). To better explore the antigenic region of the NMDAR, Gleichman and colleagues created a series of GluN1/2 subunit mutants (Gleichman et al. 2012). None of the GluN2B subunit variants or mutations in the transmembrane domains impacted antibody staining, confirming the importance of the GluN1 subunit in antibody recognition. By contrast, the residues N368/G369, located in the bottom lobe of the ATD, near the hinge of the two lobes, may be a necessary component of the epitope (Gleichman et al. 2012). Indeed, the GluN1-N368Q mutation as well tunicamycin treatment, an inhibitor of N-acetylglucosamine transferases, abolished antibody staining indicating that glycosylation may play a role in the creation of the immunoreactive region. Furthermore, a series of mutations of the residue G369 involved in N368 residue deamidation, also strongly decreased antibody staining. A similar outcome was obtained when residues 144-156 located in close proximity to N368/G369 were deleted, while top lobe deletions resulted in mixed staining (Gleichman et al. 2012). Further work confirmed that patients' antibody reactivity depends on the integrity of the N368/G369 epitope region, as the mutant GluN1-G396I abolished the staining of 27 of 36 sera or CSF samples (and substantially decreased the reactivity of the other 9) (Gresa-Arribas et al. 2013). Of note, polymorphism of GRIN1, coding for the GluN1 subunit of NMDAR, do not affect NMDAR-Ab antigen (Day et al. 2015). More recently, a very innovative work generated a panel of recombinant

monoclonal GluN1 antibodies from CSF B cells and antibody secreting cells of NMDAR-Ab encephalitis patients (Kreye et al. 2016). In line with previous data, binding to the N368Q mutant expressed in HEK cells was eliminated for all GluN1 clones from different patients. Of interest, persistent serum reactivity after deletion of the GluN1-NTD was observed in about 30% of cases (Castillo-Gómez et al. 2016), pointing out the possibility that NMDAR-Ab would target several epitopes, yet unidentified. Though, epitope mapping is a fastidious process. Because each single mutation can trigger major conformational changes, it is very difficult to conclude whether a lack of staining is indeed due to the deletion of the epitope or conformational changes that could hide the binding site. It is thus important to cautiously interpret the data and to keep expanding our knowledge regarding NMDAR-Ab nature and function.

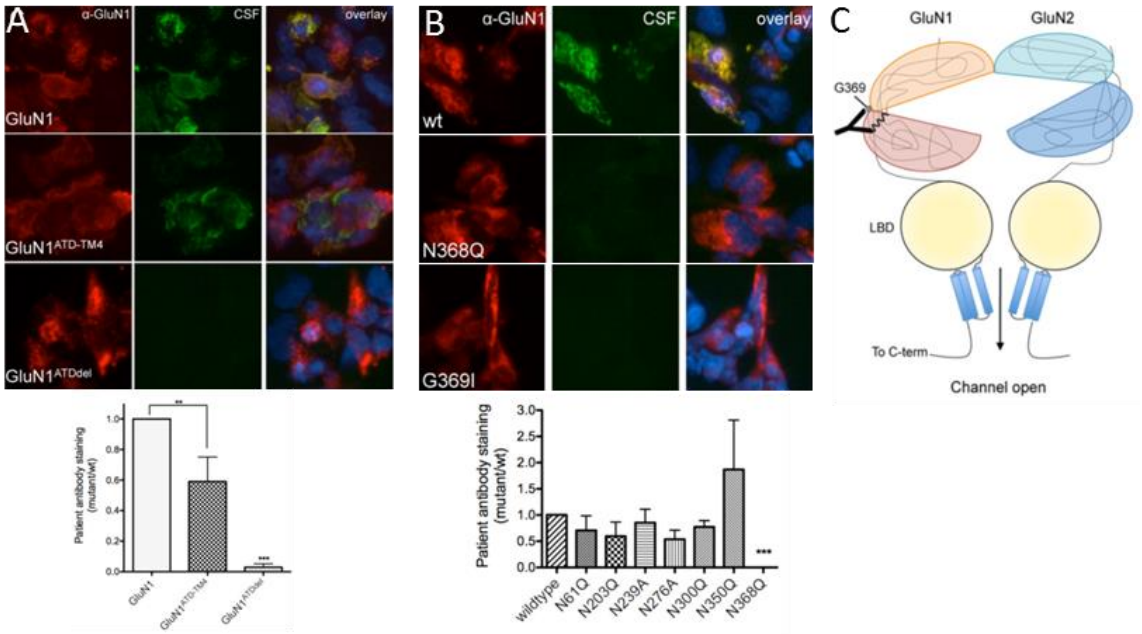

Figure 15. Epitope identification of NMDAR-Ab in NMDAR encephalitis
(A) Antibodies present in patients CSF recognize the ATD of GluN1 subunit. The lack of transmembrane domains does decrease antibody staining but does not abolish it. (B) GluN1-N368 contributes to the epitope formation. Both glycosylation and deamidation mutations of the N368 region affect antibody staining. Among the N-linked glycosylation mutants designed (mutation within the GluN1 ATD), only the mutation of N368 to Q blocks antibody staining. (C) Model of NMDAR-Ab binding to the N368/G369 region on the GluN1 ATD proposed by Gleichman et al. (2012). Open-cleft conformation of the ATD would allow antibody binding, which would then stabilize the open-conformation and prolong receptor open time. (From Gleichman et al., 2012)

d. NMDAR-Ab encephalitis or misdiagnosed schizophrenia?

The occurrence of severe behavioral changes reminiscent of a SCZ-like illness has fueled speculation that this disorder might define a subset of patients misdiagnosed as having a primary psychiatric disease (Chapman & Vause 2011; Kayser & Dalmau 2011). Can patients with NMDAR-Ab encephalitis have pure psychiatric episodes without neurological involvement? Indeed, milder or incomplete forms of NMDAR-Ab encephalitis in which patients develop predominant or apparently isolated psychiatric symptoms can occur (De Nayer et al. 2009; Irani et al. 2010; Dalmau et al. 2011). A recent study revealed that 4% of patients with NMDAR-Ab encephalitis develop isolated psychiatric episodes without eventual neurological symptoms (Kayser et al. 2013). Among them, 83% had good outcomes owing to immunotherapy, supporting the autoimmune origin of the psychiatric symptoms. On the other hand, do some patients diagnosed as having primary psychiatric disorders harbor NMDAR-Ab and respond to immunotherapy? Recent studies indeed revealed the presence of NMDAR-Ab in the serum of schizophrenic patients (Pollak et al. 2013; Ezeoke et al. 2013; Pearlman & Najjar 2014). Of particular interest, Hammer and colleagues showed a similar antibody-dependent endocytosis process (Hammer et al. 2013), supporting an aberrant NMDAR trafficking due to the presence of these NMDAR-Ab. However, we still do not know if the autoantibodies produced in encephalitis share the same properties as the one found in schizophrenic patients. Thus, it is crucial to better explore this question in order to understand their possible implication in the etiology of psychotic disorders.

6. NMDAR-Ab in schizophrenia

a. NMDAR-Ab prevalence in schizophrenia: negative *versus* positive detection studies

Most previous publications have described the full picture of the NMDAR-Ab encephalitis, but not all patients go through all of the stages described above. Accordingly, mild cases of this disease with prominent psychiatric disturbances might occur (Kayser et al. 2013), analogous to the "mild encephalitis hypothesis of SCZ" proposed by Bechter (Bechter 2013). This description led to the hypothesis that NMDAR autoimmunity could be relevant to idiopathic psychosis (Sinmaz et al. 2015). Idiopathic psychosis have been estimated to affect 0.1-0.5% of the population (Masdeu et al. 2016). While the hypothesis of a subgroup of psychosis or SCZ patients associated with anti-brain antibodies is not novel *per se,* recent studies have rekindle discussion about the immune hypothesis of psychiatric disorders. The possibility of finding NMDAR-Ab among patients with psychosis, who might respond to immunosuppressive treatment, has recently incited several groups to look for NMDAR-Ab in the sera of patients with SCZ. So far, studies have produced contradictory outcomes, with detection prevalence ranging from 0 to less than 20% (see table). Several meta-analyses summarize the discordant data accumulated over the last years (Pollak et al. 2013; Ezeoke et al. 2013; Pearlman & Najjar 2014). They all report an increased prevalence of NMDAR-Ab in schizophrenic patients compared to healthy controls, one of these studies even claimed that patients with SCZ were about 3 times more likely to have elevated NMDAR titers compared with healthy subjects (Pearlman & Najjar 2014). However, a large cohort study found a similar prevalence for serum NMDAR-Ab between SCZ (n= 1081) and control (n= 1272) groups (Hammer et al. 2013). Similarly, a large screening study looking for several neuronal antibodies (targeting both surface and intracellular antigens) detected serum NMDAR-Ab in 0.6% of schizophrenic patients and 1.2% of healthy controls (Dahm et al. 2014). In line with this, Steiner et al. re-examined samples for a previous work (Steiner et al. 2013) using a different detection method and, this time, found comparable levels of NMDAR-Ab in patients with acute psychosis and healthy controls (Steiner et al. 2014). Thus, the authors previously described the presence of NMDAR-Ab of IgG subclass in 2 out 121 patients with acute psychosis who were

eventually re-diagnosed as NMDAR-Ab encephalitis cases, while only one healthy subject (out of 230 individuals) was seropositive for IgM NMDAR-Ab (Steiner et al. 2013). Of note, around 10% of patients with SCZ were found to have IgA and/or IgM subtype antibodies reacting with GluN1/GluN2 subunits (Steiner et al. 2013). Several other studies did not find any IgG antibodies against NMDAR neither in SCZ subjects, nor in healthy controls when such a control group was present (Rhoads et al. 2011; Masdeu et al. 2012; Haussleiter et al. 2012; Titulaer et al. 2013; van Mierlo et al. 2015; Masopust et al. 2015). Haussleiter and colleagues examined 50 chronic schizophrenic patients and found no evidence of NMDAR-Ab (Haussleiter et al. 2012). These results are consistent with another smaller study that failed to demonstrate IgG NMDAR-Ab in patients with SCZ (Rhoads et al. 2011). Only one study (Masdeu et al. 2012) applied Dalmau's criteria established for NMDAR-Ab encephalitis diagnosis (Dalmau et al., 2008). Examination of serum obtained at presentation of 80 patients with new-onset psychosis who met 1 year later criteria for SCZ-spectrum illness revealed no presence of IgG NMDAR-Ab in either patients or controls (Masdeu et al. 2012). Of note, both groups had 4 cases with sera reactivity to other still unidentified neuronal surface antigens. The absence of antibodies in first psychotic episodes and SCZ was further confirmed (van Mierlo et al. 2015; Masopust et al. 2015). By contrast, two studies did detect such autoantibodies in the serum of first episode of psychosis (FEP) cases (Zandi et al. 2011; Pathmanandavel, Starling, Merheb, Ramanathan, Sinmaz, Russell C. Dale, et al. 2015). The first study detected 3 out of 46 seropositive patients (6.5%), one of whom showed a significant clinical improvement after immunotherapy (Zandi et al. 2011). The second study report 2.3% (1 out of 43) seropositive FEP cases while no antibodies were measured in the serum of healthy controls using a combination of live flow cytometry and immunocytochemistry (Pathmanandavel, Starling, Merheb, Ramanathan, Sinmaz, Russell C. Dale, et al. 2015). Other groups have reported presence of serum NMDAR-Ab in a subset of patients with psychosis. Tsutsui and colleagues examined 51 chronic schizophrenic patients and found 7.8% seropositive samples (4/51) after detection assays performed in 2 distinct laboratories (Tsutsui et al. 2012). Similar seroprevalence (7%) was found in a study conducted on 43 patients with chronic refractory psychosis

(Beck et al. 2015). Likewise, Heresco-Levy et al. (2015) observed the presence of both IgG and IgM NMDAR-Ab in a patient with chronic SCZ, whose clinical condition significantly improved following D-Serine treatment (Heresco-Levy et al. 2015). Only a few studies explored the presence of autoantibodies in the CSF of schizophrenic patients (Kayser et al. 2013; Zandi et al. 2014; Kruse et al. 2015). In a large study including 213 psychiatric inpatients with various psychiatric presentations, 13 IgG NMDAR-Ab seropositive patients and 173 healthy subjects were tested for NMDAR-Ab in sera and CSF. None of the psychiatric patients had NMDAR-Ab in their serum or CSF whereas 62% of the IgG NMDAR-Ab seropositive patients had NMDAR-Ab in their CSF (Kruse et al. 2015). In Zandi's study, 1 patient (out of 18) with acute psychosis showed NMDAR-Ab both in serum and CSF (Zandi et al. 2014). Interestingly, Kayser et al. showed that NMDAR-Ab are present in 94% of patients diagnosed with NMDAR-Ab encephalitis exhibiting pure psychiatric, and even reached 100% in patients at initial presentation (Kayser et al. 2013). Besides, serum IgA and IgM NMDAR-Ab were similarly elevated in healthy and SCZ groups in all cohorts examined (Hammer et al. 2013; Steiner et al. 2014; Dahm et al. 2014), suggesting that they may have no relevance for diagnosis. Overall, some studies have detected NMDAR-Ab in schizophrenic patients, whereas others reported negative findings. These discrepancies can be due to the huge heterogeneity between studies in terms of the assay used to detect the presence of the autoantibodies (Pollak et al. 2013). Many other factors can influence autoantibody production including age, medication effects, genetics among others, and could also contribute to discordant findings (Ezeoke et al. 2013b). Therefore, it is imperative that future studies control for these factors in order to be able to discuss comparable data.

Table 8. NMDAR-Ab detection in schizophrenia

	Demographic data	Detection	Ig type	Protocol	NMDAR-Ab prevalence
Negative detection					
(Rhoads et al. 2011)	SCZ n=7 - under treatment Healthy n=3	CBA (HEK fixed) Euroimmun	IgG	Serum 1:20	SCZ 0% Healthy 0%
(Haussleiter et al. 2012)	SCZ (chronic) n=50 47/50 under treatment No control group	GluN1/GluN1, GluN1/2B CBA (HEK fixed) Euroimmun	IgG	Serum 1:10	SCZ 0% 0% AMPAR, $GABA_{B1}R$, LGI1 & CASPR2
(Masdeu et al. 2012)	SCZ n=80 (recruited when FEP, but blood collection 1year after when they met DSM-IV-TR criteria for SCZ spectrum disorders) Healthy n=40	GluN1/2B CBA (HEK fixed) Rat HPC slices (fixed) Rat HPC cultures (fixed) → Dalmau's criteria	IgG	cf Dalmau 2008 Lancet	SCZ 0% Healthy 0% SCZ 4/80 & Healthy 4/40 seropositive to other unidentified neuronal surface antigens
(Titulaer et al. 2013)	SCZ n=80 Healthy n=200	GluN1 NA	IgG	Serum	SCZ 0% Healthy
(Steiner et al. 2013)	n=459 acute patients, unmedicated for >6weeks SCZ n=121 Healthy n=230	GluN1/GluN1, GluN1/2B CBA (HEK fixed & permeabilized)	IgG, IgA, IgM	Serum 1:10 Undiluted CSF 30'min incubation RT	SCZ all Ig types 9.9% (12/121) IgG+ 1.65% (2/121) Healthy 0.4% IgM (1/230) 2 SCZ with CSF positive for NMDAR-Ab reclassified as encephalitis
(Steiner et al. 2014)	SCZ n=184 (117 overlapping samples (OL) i.e. samples from Steiner 2013 re-examined) Healthy n=357 (126 OL)	GluN1 CBA - BIOCHIP assay (Euroimmun)	IgG, IgA, IgM	NA	SCZ 9.8% All Ig subtypes (10/184) 0.9% (1/184) 1 OL Healthy 7% All Ig subtypes (25/357) 5 OL 0.6% IgG (2/357), 3.1% IgA (11/357), 3.1% IgM (11/357)
(van Mierlo et al. 2015)	SCZ = 104 No control group	GluN1 CBA (HeLa fixed) HPC cultures (live)	NA	Plasma 1:50 1h 37°C	SCZ 0% in both tests But positive for AMPAR (used as a positive control)
d(de Witte et al. 2015)	Cohort 1 Van Winkel 2006 Cohort 2 Schwarz 2012 Cohort 3 Korver 2012				Publication not relevant. Cohort 2 included while the authors do not even look for NMDAR-Ab …
(Masopust et al. 2015)	FEP n=50 Healthy n=50	CBA Euroimmun	IgG	NA	SCZ 0% Healthy 0%
(Kruse et al. 2015)	Group 1: Psychiatric inpatients n=213 Group 2: NMDAR-IgG seropositive patients n=13 Healthy n=173	CBA (fixed), IHC Radio IP Enzyme-linked immunosorbent assay WB	IgG	Serum 1:10 CSF (10/213 from group 1, 13/13 from group 2)	Serum Group 1 (SCZ)0% Group 2 62% (8/13) Healthy 0% CSF Group 1 0% Group 2 100%
Positive detection					
(Zandi et al. 2011)	FEP n=46 No control group	GluN1/2B CBA (HEK live) Fluorescent IP	IgG	Serum 1:20 1h Undiluted CSF Serum O.N. 4°C	SCZ 6.5% (3/46) 1 patient showed a significant clinical improvement after plasmapheresis and prednisolone → first description of a SCZ case responding to immunotherapy
(Tsutsui et al. 2012)	SCZ n=51 No control group	Detection by Dalmau & Tanaka's labs	IgG	Serum & CSF	SCZ 7.8% (4/51)
(Hammer et al. 2014)	SCZ n=1081 Healthy n=1272	GluN1 or GluN1/2B CBA (HEK fixed) - Euroimmun	IgG, IgA, IgM	Serum	SCZ 8.6% all Ig subtypes 0.7% IgG (7/1081), 5.2% IgA (56/1081), 4.3% IgM (46/1081) Healthy 10.8% all Ig subtypes 0.4% IgG (5/1272), 5.9% IgA (75/1252), 6.3% IgM (80/1272)

(Kayser et al. 2013)	NMDAR encephalitis n=571 Isolated psychiatric symptoms n=23 (4%) Initial episode n=5 Relapse n=18	GluN1/2B CBA (HEK fixed) Rat HPC slices (fixed) Rat HPC cultures (fixed) → Dalmau's criteria	IgG	Serum/CSF	Isolated psychiatric symptoms 17/18 CSF positive (1NA) 61% (11/18) seropositive, 33% (6/18) seronegative - 1/18 NA Initial episode 100% CSF positive 2 seropositive (3 NA)
(Pollak et al. 2013)	SCZ n=1441 FEP cases n=272 Healthy n=1598	Meta-analysis	IgG	Serum	SCZ 8% (115/1441) all Ig subtypes 1.5% IgG (21/1441), 3.5% IgM (50/1441), 4.3% IgA (62/1441) FEP 5.1% (14/272) all Ig subtypes 1.8% IgG (5/272), 2.2% IgM (6/272), 1.5% IgA (4/272) Healthy 9.0% all Ig subtypes 0.3% IgG (5/1598), 5.3% IgM (84/1598), 4.9% IgA (78/1598) Prevalence for IgG SCZ > controls
(Ezeoke et al. 2013)	SCZ n=355 Healthy n=273	Meta-analysis 6 studies included for NMDAR-Ab	IgG	Serum	SCZ 5.4% (19/355) Healthy 0.4% (1/273) Positive IgG NMDAR Ab ↗ in FEP
(Dahm et al. 2014)	SCZ n=1378 Healthy n=1703	Metanalysis – CBA (HEK, BIOCHIP assay) Euroimmun	IgG, IgA, IgM	Serum 1:10 30min	SCZ 9.4% (129/1378) all Ig subtypes 0.6% IgG (8/1378), 5.3% IgA (73/1378), 5.0% IgM (69/1378) Healthy 8.5% (145/1703) all Ig subtypes 1.2% IgG (20/1703), 4.5% IgA (76/1703), 4.3% IgM (74/1703) Seroprevalence comparable between controls and SCZ
Pearlman & Najjar 2014		Meta-analysis 9 studies included	IgG, IgA, IgM	Serum	SCZ patients are about 3 times more likely to have elevated NMDAR Ab titers compared with healthy controls
Zandi 2014	SCZ (acute psychosis) n=18	CBA (live HEK)	IgG	Serum CSF	18/18 IgG 9/18 treated with immunotherapy – 8/9 responders 1 CSF positive
(Beck et al. 2015)	N=43 patients with chronic refractory psychosis SCZ n=36 Schizoaffective n=7 No control group	GluN1/2B CBA (HEK live)	IgG	Serum	7% (3/43) with low titers (1:50, 1:50, 1:100)
(Heresco-Levy et al. 2015)	SCZ (chronic) n=17 No control group	GluN1/2B CBA (HEK & CHO live) WB	IgG, IgA, IgM	Serum 1:200 O.N 4°C	SCZ 5.9% (1/17) IgG & IgM Clinical improvement after D-Serine treatment
(Pathmanandavel et al. 2015)	FEP n=45 Healthy n=17 Pediatric cases <17 years	Flow cytometry (live CBA HEK) Mice HPC cultures (live) IP	IgG, IgA, IgM	Serum 1:50 30min RT Serum 1:50 1h	FEP 11.6% IgG (5/43) 2.3% (1/43) IgG D2R + NMDAR 2.3% IgM (1/43) 2.3% IgA (1/43) Healthy 0%
(Endres et al. 2015)	Group 1: schizophreniform n=132 among whom SCZ n=104 Group 2: Schizoaffective n=43 Group 3: Psychotic in other disorders n=5 No control group	CBA (HEK fixed) Euroimmun	IgG	Serum	Serum 0.8% (1/125 samples) Data for all groups pooled, no detail inside each group

(Castillo-Gómez et al. 2016)	SCZ n=2 NMDAR encephalitis n=2 Healthy n=6 Hypertension n=13 Diabetes/hypertension n=3	GluN1 CBA (HEK fixed) Euroimmun	IgG, IgA, IgM	Serum	SCZ 50% (1/2) - 100% IgG (titer 1:320) Encephalitis 2/2 – 100% IgG Healthy 33% (2/6) - 100% IgM Hypertension 7/13 - 57% IgM, 29% IgA, 14% IgG Diabetes/ Hypertension 2/3 – 100% IgA, 100% IgM

IgG: Immunoglobulin G; IgM: Immunoglobulin M; IgA: Immunoglobulin A; SCZ: schizophrenia; FEP: First Episode; CSF: CerebroSpinal Fluid; CBA: Cell-Based Assay; IP: ImmunoPrecipitation; HPC: Hippocampus; HEK: Human Embryonic Kidney cells; CHO: Chinese Hamster Ovary cells; O.N: overnight; RT: Room Temperature; NA: not available

b. How to detect NMDAR-Ab?

Which technique to use?

Traditional methods to detect antibodies, such as enzyme-linked immunosorbent assay (ELISA) and western blot have been used for antibodies detection in sera. Although classically used to identify intracellular antibodies, these methods detect linearized or denatured protein antigens, potentially leading to false-positive or false-negative results. Most antibodies against neuronal surface proteins likely recognize target epitopes if they are in their native conformation. CBA employing live mammalian cells have the advantage of exposing the patients' antibodies to the extracellular domains of native receptors. This assay was used in several positive studies above-mentioned (Zandi et al. 2011; Zandi et al. 2014; Heresco-Levy et al. 2015; Beck et al. 2015). A similar approach using flow cytometry cell-based assay was developed by Brilot's lab (Amatoury et al. 2013) and successfully detected several antibodies targeting surface antigens (Sinmaz et al. 2015), like NMDAR-Ab in the serum of schizophrenic patients (Pathmanandavel, Starling, Merheb, Ramanathan, Sinmaz, Russell C. Dale, et al. 2015). Dalmau who first developed methods for NMDAR-Ab detection in the context of NMDAR-Ab encephalitis has argued that the gold standard for antibody detection should involve 3 antigen-binding assays (Dalmau et al., 2008): 1) a CBA using transfected HEK cells expressing the antigen of interest, 2) cultured dissociated neurons, and 3) brain sections optimized for antigen presentation. Actually, they excluded the step 2 for routine testing, considering that cell surface reactivity was similar to that of any synaptic antibody (Gresa-Arribas et al. 2013). Of note, Dalmau's

lab routinely uses fixed and permeabilized CBA. This three-step methodology was used by only 2 studies that gave quite contradictory outcomes (Masdeu et al. 2012; Kayser et al. 2013). Currently, most diagnostic laboratories use commercial kits that provide fixed brain tissue and fixed antigen-expressing cells (Biochip assay from EUROIMMUN). Several groups used this assay to detect NMDAR-Ab in schizophrenic patients and obtained opposite outcomes as presented in the previous paragraph (Negative detection: Rhoads et al. 2011; Haussleiter et al. 2012; Masopust et al. 2015; positive detection: Hammer et al. 2013; Steiner et al. 2014; Dahm et al. 2014; Endres et al. 2015; Castillo-Gómez et al. 2016). Despite the 100% sensitivity and specificity in detecting patients diagnosed with Dalmau's criteria claimed by Wandinger et al. (2011), one major issue (as for the non-live CBA) of these commercial kits is that they do not use intact neuronal cell surfaces. The detection of autoantibodies in this system may then differentially target cell surface epitopes in favour of intracellular, thus, non relevant epitopes.

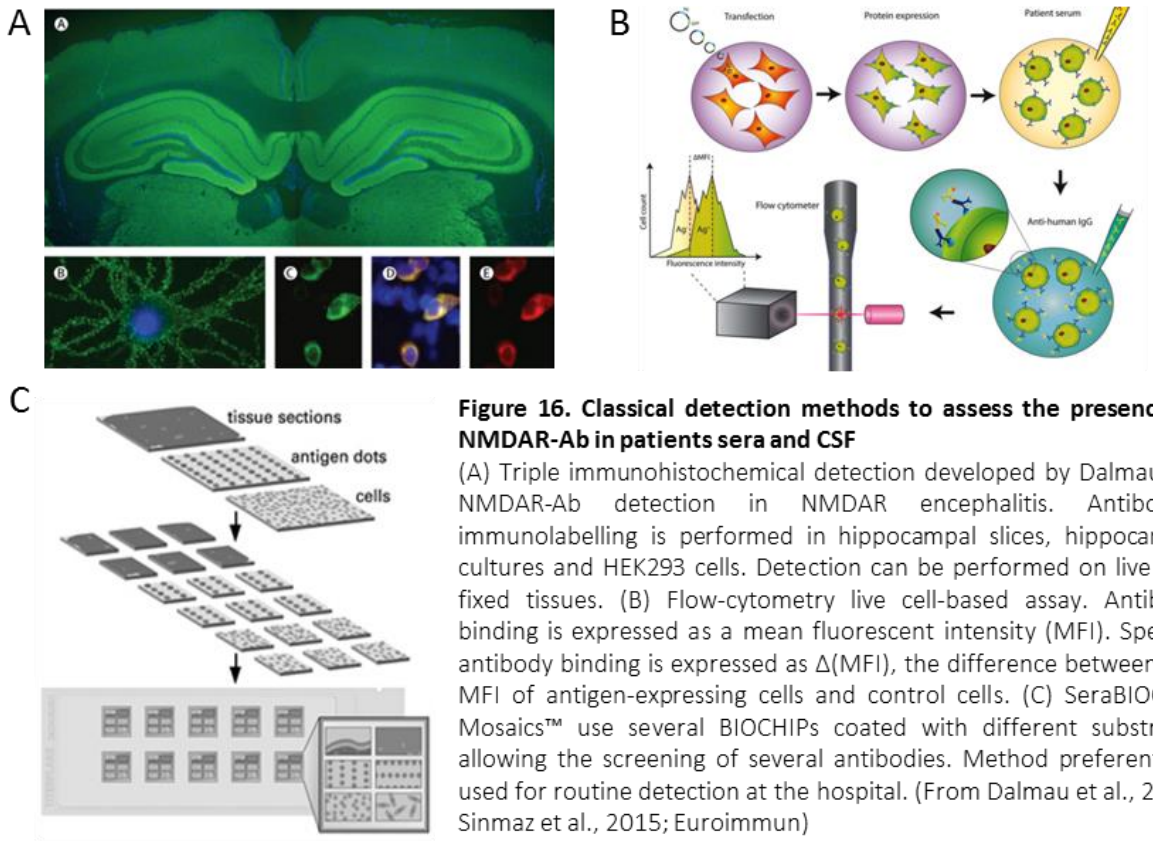

Figure 16. Classical detection methods to assess the presence of NMDAR-Ab in patients sera and CSF

(A) Triple immunohistochemical detection developed by Dalmau for NMDAR-Ab detection in NMDAR encephalitis. Antibodies immunolabelling is performed in hippocampal slices, hippocampal cultures and HEK293 cells. Detection can be performed on live and fixed tissues. (B) Flow-cytometry live cell-based assay. Antibody binding is expressed as a mean fluorescent intensity (MFI). Specific antibody binding is expressed as Δ(MFI), the difference between the MFI of antigen-expressing cells and control cells. (C) SeraBIOCHIP Mosaics™ use several BIOCHIPs coated with different substrates allowing the screening of several antibodies. Method preferentially used for routine detection at the hospital. (From Dalmau et al., 2011; Sinmaz et al., 2015; Euroimmun)

Serum or CSF testing?

Another controversy concerns whether the presence of antibodies in CSF must be demonstrated to prove pathogenicity (Dalmau et al., 2008) or whether serum testing is in fact more sensitive than CSF testing (Irani et al. 2010). In the case of NMDAR-Ab encephalitis, antibodies are present in both CSF and serum samples. Gresa-Arribas and colleagues have compared the sensitivity of classical detection techniques using either CSF or serum (Gresa-Arribas et al. 2013). 100% of CSF positive samples were detectable with both rat brain immunohistochemistry and CBA using fixed cells while only 85.6% serum samples were positive with both techniques. When using serum on live CBA, CBA with live cells was less specifically reactive than CBA with fixed cells (63/108 positive with live cells vs 77/108 with fixed cells). But one could ask what *"less specifically reactive than CBA with fixed cells"* means? Couldn't it be that CBA with fixed cells gives more false-positives than live CBA? As already mentioned, cell fixation can modify receptors conformation and reveal epitopes that would not be accessible on live and intact neuronal membranes. Dalmau's group recommends to use when possible, paired detection on serum and CSF (Lancaster & Dalmau 2013; Graus et al. 2016). However, what accounts for NMDAR-Ab encephalitis does not for SCZ. Indeed, CSF positive samples in SCZ are extremely rare. Unlike in neurology services, lumbar puncture is not a routine test in psychiatry services. Authorization for such a medical procedure is not easily obtained in many countries, explaining the low number of studies performing paired serum-CSF examinations.

c. NMDAR-Ab pathogenicity

Early experiments in the 1960s isolated antibodies targeting forebrain regions of the brain from the sera of people with SCZ. When administered intravenously into monkeys or healthy human volunteers, it caused EEG changes and the induction of psychotic symptoms comparable to those observed in SCZ (Heath & Krupp 1967). These studies support the hypothesis that autoantibodies from some patients with SCZ have the potential to transfer disease from on individual to another. The mechanisms by which these antibodies produce a psychiatric phenotype is not clear but NMDAR

dysfunction has been a plausible explanation as the underlying mechanism for SCZ over the last decades (Lennox et al. 2012). NMDAR blockade *in vivo* produces a range of SCZ-like symptoms in healthy individuals and exacerbates psychotic symptoms in SCZ (Lahti 1995, 2001). One could hypothesize that NMDAR-Ab reported in schizophrenic patients can be part of the etiology of the disease by inducing NMDAR hypofunction. In that way, Hammer et al. (2013) observed *in vitro* NMDAR endocytosis in presence of NMDAR-Ab from schizophrenic patients. Intravenous injection of NMDAR-Ab in ApoE$^{-/-}$mice, a model of deficient BBB, showed a reduced spontaneous activity in the open field and hyperlocomotion following MK-801 compared to wild-type animals (Hammer et al. 2013). The authors concluded that loss of BBB integrity may generally constitute a major risk factor for detrimental effects of peripheral NMDAR-Ab against CNS epitopes (Hammer et al. 2013). Recent work from the same group confirmed, using human-induced pluripotent stem cell-derived neurons, that circulating NMDAR-Ab from schizophrenic patients provoke NMDAR internalization and a reduction of glutamate-evoked currents (Castillo-Gómez et al. 2016). Such pathogenic effects were observed regardless of the clinical condition (Healthy controls, NMDAR-Ab encephalitis, hypertension, diabetes patients) and of the immunoglobulin class (IgG, IgA and IgM all showed the same effects), insisting on the fact that BBB permeability is crucial for autoantibodies pathogenicity (Castillo-Gómez et al. 2016). But once the data are categorized by disease, it clearly appears that IgG NMDAR-Ab are only present in NMDAR-Ab encephalitis and SCZ cases (and 1 patient with hypertension), while IgM and IgA are found in all hypertension and/or diabetes patients and healthy controls. Hence, the clinical significance of IgA and IgM antibodies is still uncertain (Busse et al. 2014; Titulaer & Dalmau 2014; Lancaster et al. 2015), and further work is needed to explore their pathogenicity and if so, to elucidate the molecular mechanisms engaged.

d. Relevance for the disease

Treatment-resistant SCZ is clinically defined as a non-response to at least two adequate trials of antipsychotic medication, and is estimated to affect about 30% of all patients with SCZ (Wimberley et al. 2016). A crucial question is whether treatment-resistant SCZ represents a distinct neurobiological entity that might respond to different treatments by comparison with treatment-responsive SCZ. Several studies reported a significant clinical improvement of schizophrenic patients with NMDAR-Ab after immunotherapy (Zandi et al. 2011; Zandi et al. 2014; Gungor et al. 2016; Senda et al. 2016). In 2011, Zandi et al described a 19 years old male patient with first psychotic episode who relapsed despite antipsychotic treatment (Zandi et al. 2011). His clinical condition rapidly improved after plasmapheresis and prednisolone administration. A couple of years later, the same group describe 18 cases of acute psychosis with NMDAR-Ab, including patients with history of treatment-resistant illness (Zandi et al. 2014). Nine out of these 18 patients received immunotherapy, among which 8 of 9 responded clinically even though 6 of 9 had a subsequent relapse of illness. Six achieved symptomatic remission, and one of the patients with a long history of resistance to antipsychotics significantly improved after immunotherapy (Zandi et al. 2014). Similarly, Gungor et al. recently reported a case presenting with psychosis attacks and suspicion of autoimmune encephalitis. The patient gave no response to mood stabilizers and antipsychotic drugs but his psychiatric symptoms rapidly disappeared after intravenous Ig treatment (Gungor et al. 2016). Still in line with this, NMDAR-Ab and various thyroid antibodies were detected in the CSF of a 31-year-old woman with psychotic symptoms. Treatment with 3 atypical antipsychotics was ineffective, so she received several rounds of immunosuppressive treatment until CSF NMDAR-Ab levels dropped and she recovered (Senda et al. 2016). Altogether, these case studies strengthen the autoimmune hypothesis of SCZ and support the potential pathogenic role for NMDAR-Ab in the emergence of psychotic symptoms. Another strong indication that NMDAR-Ab might cause NMDAR hypofunction resides in the efficacy of NMDAR modulators treatments. Of great interest, Heresco-Levy and colleagues administered D-serine, a NMDAR co-agonist thought to enhance NMDAR function, to an NMDAR-Ab

positive patient with chronic treatment-refractory SCZ. Following treatment with D-serine, the patient's EEG normalized and her psychosis improved (Heresco-Levy et al. 2015).

e. Parallel between schizophrenia, autoantibodies and NMDAR hypofunction

To date, the evidence for a direct pathological role of NMDAR-Ab has only been clearly demonstrated in NMDAR-Ab encephalitis (Hughes et al. 2010; Planagumà et al. 2015; Planagumà et al. 2016). The overlap between SCZ and NMDAR-Ab encephalitis in regard to the NMDAR hypofunction and psychotic symptoms raises a question about possible involvement of autoantibodies in the pathogenesis of, at least some of, the SCZ-spectrum illnesses. Several studies have investigated the presence of NMDAR-Ab in patients with SCZ and have found intriguing results. Although the mechanisms by which NMDAR-Ab induce NMDAR dysfunction remain to be elucidated, an association between NMDAR-Ab and SCZ is biologically plausible. In healthy volunteers, blockade of NMDAR with ketamine produced psychotic symptoms (Krystal et al. 1994; Lahti et al. 2001). A genetic model of NMDAR hypofunction revealed several behavioral and pathophysiological features in mice that resemble human SCZ (Belforte et al. 2010). Genetic studies also support a link between SCZ and the NMDAR. *De novo* mutations affecting glutamatergic post-synaptic proteins that are part of the NMDAR complex have been reported in SCZ (Fromer, Andrew J Pocklington, et al. 2014). Finally, it is interesting to note that psychotic symptoms in NMDAR-Ab encephalitis, drug-induced NMDAR blockade and SCZ occur more commonly around adulthood. One could hypothesize that NMDAR-Ab are present at low titers during an earlier developmental period, resulting in a more gradual and chronic exposure to NMDAR hypofunction.

IV. Objectives of the thesis project

Surface trafficking represents a key mechanism to control NMDAR synaptic localization, thus modulating the efficacy of excitatory synaptic transmission. NMDAR surface trafficking itself is subjected to multiple regulatory processes occurring through numerous interactions between the NMDAR and modulatory proteins, many of which are dysregulated in psychotic disorders. The primary aim of my thesis was thus to study the molecular impact of different psychotomimetic molecules on NMDAR surface trafficking, and assess whether a dysfunction of NMDAR surface dynamics could constitute a "pathogenic step" leading to the emergence of psychotic disorders.

The first part of my thesis project aimed at determining the molecular pathogenicity of circulating NMDAR autoantibodies (NMDAR-Ab). In a first study, we compared the effect of NMDAR-Ab purified from the serum of schizophrenic patients and healthy subjects on NMDAR surface trafficking and distribution. Several questions drove this research:

- Can we efficiently and specifically detect the presence of circulating NMDAR-Ab?
- Do NMDAR-Ab from schizophrenic patients and healthy subjects alter NMDAR surface trafficking and distribution? If yes, do they equally affect NMDAR surface dynamics?
- Through which molecular mechanism(s) NMDAR-Ab impact on NMDAR surface trafficking?

This study was performed in tight collaboration with the group of Pr Leboyer (Hôpital Mondor, Créteil, France) who conducted patients inclusion, and Pr Honnorat (CNRS, Lyon, France) who managed the detection and purification of NMDAR-Ab.

In parallel, we questioned the detection methods currently used to identify NMDAR-Ab in patients sera and/or CSF. The presence of NMDAR-Ab in the serum of first-episode psychotic patients and healthy subjects was assessed using different detection methods. This work was performed in collaboration with different laboratories taking part of the OPTIMISE consortium, and aimed at answering these main questions:

- Do detection on live cells *versus* fixed cells provide similar outcomes?
- Do these tests reliably detect NMDAR-Ab?
- Do circulating NMDAR-Ab isolated from first-episode psychotic patients exhibit a pathogenic potential?

The second part of my PhD project was dedicated to investigating the molecular impact of ketamine and MK-801, two NMDAR antagonists exhibiting psychotomimetic properties. We confronted the effects of psychotomimetic (ketamine and MK-801) *versus* non-psychotomimetic (AP5) NMDAR antagonists in order to tackle these points:

- Do psychotomimetic NMDAR antagonists differently impact NMDAR surface trafficking in comparison with non-psychotomimetic molecules?
- If yes, why NMDAR inhibition would trigger different diffusion behaviors of the NMDAR? What are the molecular mechanisms recruited by NMDAR antagonists to modulate NMDAR surface trafficking?
- Do psychotomimetic NMDAR antagonists alter specific NMDAR interactions?

Results

Functional heterogeneity of human anti-NMDA receptor autoantibody: nanoscale disorganization of synaptic receptors triggered by autoantibodies from schizophrenic patients

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Short title: Molecular pathogenicity of anti-NMDAR autoantibody in schizophrenia

Keywords : single molecule imaging, schizophrenia, autoantibodies, glutamate receptor

Highlights

- NMDAR-Ab are identified in the serum of 3% of healthy and 20% of schizophrenic individuals
- NMDAR-Ab from different origins show a heterogeneity of action
- NMDAR-Ab from schizophrenic patients impact NMDAR trafficking and nanoscale organization
- NMDAR-Ab from schizophrenic patients alter NMDAR-dependent synaptic plasticity

In brief

Jezequel el al. provide evidence that circulating NMDA receptor autoantibodies (NMDAR-Ab) from healthy and schizophrenic individuals have distinct molecular and functional impacts on synaptic NMDAR, shedding new mechanistic lights and calling for caution in using autoantibodies as biomarkers for diagnosis.

The flourishing identification of circulating autoantibodies against neuronal receptors in neuropsychiatric disorders has fostered new conceptual and clinical frameworks. However, their putative presence in different diseases, as well as in healthy subjects, has raised questions about detection liability, pathogenic role, and use as biomarkers. Using a combination of single molecule-based imaging approaches, we ascertain the presence of circulating autoantibodies against glutamate NMDA receptor (NMDAR-Ab) in less than 20% of acute phase schizophrenic patients and few healthy subjects. The NMDAR-Ab IgG from patients and healthy subjects did however not compete for binding on native receptor. Strikingly, NMDAR-Ab from patients, and not from healthy subjects, specifically altered the surface dynamics and nanoscale organization of synaptic NMDAR and its anchoring partner EphrinB2 receptor. Functionally, patients' NMDAR-Ab prevented chemically-induced AMPA receptor synaptic potentiation while leaving intact NMDAR-mediated calcium influx. Thus, by taking advantage of the single molecule imaging, we unveil that the nanoscale surface organization of NMDAR and EphrinB2 receptor is profoundly altered by autoimmune molecule from schizophrenic patients, supporting a pathogenically relevant role of this unsuspected pathway *in vivo*.

INTRODUCTION

Over the past decade, autoantibodies against neuronal receptors have been increasingly identified in neuropsychiatric disorders and constitute today one of the hottest topics in psychiatry (Benros et al., 2014; Coutinho et al., 2014; Deakin et al., 2014; Hoftberger et al., 2015; Kayser and Dalmau, 2014; Pollak et al., 2015). This generated great hope for a better understanding of the molecular and cellular dysfunctions underlying psychiatric disorders, and it fostered debate on how to identify the patients that may benefit from immunotherapy. Several neurological diseases are indeed well-defined and treated after the identification of autoantibodies against neurotransmitter receptors and ion channels (Crisp et al., 2016; Leypoldt et al., 2015). In the psychiatric field, the link between psychotic disorders, particularly schizophrenia, and immune system dysregulations, including autoimmunity, is an old concept that regained strong support thanks to the better characterization of brain inflammation-induced psychotic symptoms and autoimmune encephalitis (Khandaker et al., 2015). The best characterized encephalitis is the anti-N-methyl-D-aspartate receptors (NMDAR) one in which autoantibodies directed against the GluN1 subunit of NMDAR (NMDAR-Ab) are detected and associated to psychotic symptoms and catatonia, followed by profound neurologic deterioration (Dalmau et al., 2011; Titulaer et al., 2013). Studies exploring the presence of NMDAR-Ab in the serum of schizophrenic (SCZ) patients have produced contradictory outcomes, with detection prevalence ranging from 0 to less than 20% (Beck et al., 2015; Dahm et al., 2014; de Witte et al., 2015; Hammer et al., 2013; Haussleiter et al., 2012; Masdeu et al., 2012; Pathmanandavel et al., 2015; Pearlman and Najjar, 2014; Rhoads et al., 2011; Steiner et al., 2014; Steiner et al., 2013; Tsutsui et al., 2012; Zandi et al., 2011). Several reasons for these discrepancies have been proposed and debated, among which different sensitivities and specificities between detection methods (Coutinho et al., 2014; Gresa-Arribas et al., 2014; Sinmaz et al., 2015). From a clinical point-of-view, immunotherapy treatment of patients with acute psychosis and NMDAR-Ab was associated with good outcomes, especially for psychosis resistant to anti-psychotics (Kayser et al., 2013; Zandi et al., 2014). Although large randomized investigations are surely needed to ascertain NMDAR-Ab pathological role in psychosis,

the functional interplay between NMDAR-Ab and psychotic symptoms represents an exciting and promising area of research.

The understanding of the mechanism(s) underlying the molecular and behavioral dysfunctions triggered by NMDAR-Ab is still in its infancy. The glutamatergic model of psychosis and schizophrenia is increasingly accepted as part of the etiopathology since the discovery that some NMDAR blockers induce schizophrenia-like psychosis, reproducing both positive and negative symptoms (Kantrowitz and Javitt, 2012). The anti-NMDAR encephalitis patients' cerebrospinal fluid only slightly prolongs NMDAR open time and purified NMDA-Ab do not alter NMDAR-mediated calcium influx (Gleichman et al., 2012; Mikasova et al., 2012), suggesting that NMDAR-Ab-related psychosis is not directly due to receptor blockade. NMDAR-Ab mostly target extracellular epitopes of the GluN1 subunit, which is an obligatory subunit that associate with GluN2 and/or GluN3 subunits to form NMDAR (Paoletti et al., 2013). These receptors are constantly trafficked to and from the glutamatergic synapse in order to ensure the stability of the pool in basal condition (Lau and Zukin, 2007). In addition to the exocytosis/endocytosis cycle, membrane NMDAR laterally diffuse between synaptic and extrasynaptic areas in regulated and subunit-dependent manners (Groc et al., 2004; Groc et al., 2006b; Tovar and Westbrook, 2002). NMDAR-Ab from encephalitis patients strongly alter the NMDAR surface dynamics, synaptic retention, as well as NMDAR-dependent synaptic plasticity and cognitive tasks (Dupuis et al., 2014; Hughes et al., 2010; Mikasova et al., 2012; Planaguma et al., 2015). These deficits are mostly due to the disruption of the direct interaction between NMDAR and EphrinB2 receptor, leading to the receptor lateral escape (Mikasova et al., 2012; Planaguma et al., 2016). In the extrasynaptic compartment, NMDAR are cross-linked by NMDAR-Ab, reducing their diffusion and favoring their internalization (Dalmau et al., 2008; Hughes et al., 2010; Mikasova et al., 2012; Moscato et al., 2010). Thus, one may hypothesize that NMDAR-Ab-related psychosis results from a specific alteration of NMDAR trafficking rather than an intrinsic change of the channel activity. Here, we investigated the presence and impact of NMDAR-Ab purified from a cohort of healthy subjects and SCZ patients, excluding cases of autoimmune encephalitis. An original array of classical and high-resolution imaging

approaches was implemented in rat hippocampal neurons to provide in-depth information, at the single molecule level, of the impact of NMDAR-Ab from healthy subjects and SCZ patients on the surface dynamics and nanoscale organization of NMDAR.

RESULTS

Demographic and clinical features of seropositive patients

Forty eight SCZ patients were recruited during their hospitalisation and included after approval by a French ethical committee and written informed consent for their participation (Table S1). A healthy control group (n=104) with no personal or family history of schizophrenia and/or bipolar disorder was matched with the SCZ patient sample for age, gender and years of education (Table S1). For all groups, history of stroke, multiple sclerosis, epilepsy, or encephalitis constitutes exclusion criteria. Most of the SCZ patients were under an antipsychotic treatment (74.5%) and 8 also received a mood stabilizer (Tables S1 and S3). The mean age of onset of schizophrenia was 24.5 years and the mean duration of illness was 11 years (Table S2). The mean total score for the Positive and Negative Syndrome Scale (PANSS) was 68.6. After performing a series of serological tests to detect the presence of circulating NMDAR-Ab (see section below, Figure 1A), positive and negative patients' populations were identified and compared to each other's (seronegative SCZ, SCZ-: n=39; seropositive SCZ, SCZ+: n=9; Figure 1A, Table S1). Both groups were similar for age, gender, BMI, age of onset, duration of illness, and number of episodes. Both SCZ- and SCZ+ patients were under atypical antipsychotic medication, 89% for SCZ- and 100% for SCZ+ patients. Noteworthy, 78% of SCZ+ patients and only 61.5% of SCZ- patients were in acute state (Table S2). SCZ+ patient symptoms were consistently more severe than SCZ- patient ones as shown by the significant difference in PANSS total score, PANSS general psychopathology and positive (Table S2). The clinical files of the 9 SCZ+ patients were systematically reviewed to search for signs of ancient autoimmune encephalitis (e.g. abnormal movements, bad tolerance to antipsychotics, epilepsy, dysautonomia, and brain MRI). None of these signs or clinical history of encephalitis was found. The infectious screening and detection of other antibodies in SCZ- and SCZ+ patients revealed no obvious alteration, although SCZ+ patients have other autoantibodies (Table S3). Furthermore, since autoantibody titers can fluctuate over time in patients (Dalmau et al., 2011; Titulaer et al., 2013), 5 of the 9 SCZ+ patients

were re-hospitalised to perform further medical explorations at a second time point (Table S3) while they were outpatient, treated, compliant but remained symptomatic for psychosis. All were re-confirmed for NMDAR-Ab seropositivity. In addition, no neurological symptom was observed, EEG were unspecific and brain MRI normal. None of them had history of memory disturbance, epilepsy, dysautonomia, abnormal movement or catatonia. Finally, all the CSF from these patients were negative for NMDAR-Ab (Table S3). Altogether, the clinical examination of the seropositive patients confirmed diagnosis of schizophrenia and failed to detect clinical history of encephalitis.

Detection of circulating NMDAR-Ab using conventional and single molecule imaging approaches

The detection of NMDAR-Ab in the sera of neuropsychiatric patients has been vividly debated over the past years (Sinmaz et al., 2015). We here investigated the presence of NMDAR-Ab in the serum of SCZ patients and healthy subjects described above by using a unique combination of conventional and single molecule high-resolution imaging approaches. First, we used classical cell-based assays on live HEK cells ectopically expressing GluN1-GFP and GluN2 NMDAR subunits (Figure 1A). These assays were independently duplicated in two laboratories (Lyon, France and Barcelona, Spain). Sera from 9 SCZ+ patients and 3 healthy individuals (healthy+) stained GFP-positive HEK cells (Figure 1A, Table S1). Autoantibody titers were then estimated as end point dilutions. They ranged between 1/20 and 1/320, with no significant difference between SCZ+ patients and healthy+ subjects (Figure 1B). When these values were compared to the ones obtained from gold-standard anti-NMDAR encephalitis patients, a significant 20-fold decrease was observed (mean end point dilution: 1/3200; Figure 1B), indicating that levels of circulating NMDAR-Ab in SCZ+ patients and healthy+ subjects are lower than in anti-NMDAR encephalitis patients. Next, we tested the sample seropositivity by using a variant of the cell-based assay, i.e. fixed HEK cells overexpressing GluN1-NMDAR, which is a sensitive and reliable method used to detect NMDAR-Ab in anti-NMDAR encephalitis patients (Dalmau et al., 2007; Gresa-Arribas et al., 2014). None of the samples were positive using this method (data not shown). Thus, detection methods that are highly reliable to detect NMDAR-Ab in anti-NMDAR encephalitis patients provide

contradictory outcomes when used to detect NMDAR-Ab from SCZ+ patients or healthy+ subjects, strengthening the urgent need for new detection methods.

To further explore the presence of these antibodies, we compared the labeling of hippocampal slices obtained with purified type G immunoglobulins (IgG) from healthy+ subjects, SCZ+ patients, and commercial anti-GluN1 subunit IgG ($\alpha GluN1_{N\text{-}term}$, see Methods). A comparable labeling was observed between the three conditions (Figure S1), supporting the presence of NMDAR-Ab in both SCZ+ patients and healthy+ subjects. In cultured hippocampal neurons, the live staining with healthy+ and SCZ+ NMDAR-Ab showed a clustered-type distribution (Figure 1C), similar to the distribution of endogenous membrane NMDAR (Groc et al., 2006a). To further confirm the presence of NMDAR-Ab, we performed a live double immunostaining of GluN1-SEP, a genetically modified GluN1 subunit containing a superecliptic GFP (SEP) at its extracellular N-terminus, and healthy+ or SCZ+ IgG (Figure 1D). Surface clusters of GluN1-SEP clearly colocalized with both healthy+ and SCZ+ IgG staining (Figure 1D). These stainings were then compared to the ones obtained with the same concentration of IgG (5 μg/ml) from anti-NMDAR encephalitis patients. The type of staining was similar whereas the fluorescence intensity was significantly higher with encephalitis patients when compared to healthy+ subjects and SCZ+ patients (Figure 1D). Together, these classical immunocytochemical assays support the presence of NMDAR-Ab in healthy+ subjects and SCZ+ patients.

In search for a new sensitive assay in living neurons, we took advantage of the single molecule imaging as the molecular behavior of an individual membrane receptor (targeted by an antibody) is given by robust biophysical characteristics and is virtually independent of the antibody concentration. The target of the autoantibodies was examined by coupling together human IgG to single nanoparticle (Quantum dots, QD) (Figure 1E). The autoantibody-target complex was imaged with a sub-wavelength precision at video rate in live hippocampal cultured networks (Figure 1E), giving access to the live "signature" of the healthy+ subjects' and SCZ+ patients' IgG target (Dahan et al., 2003; Groc et al., 2007). The mean square displacement (MSD) curves, reflecting the surface explored and type of motion of the complex, revealed that healthy+ subjects, SCZ+ patients, and GluN1-NMDAR signatures

were undistinguishable whereas potassium channel Kv1.3 MSD was clearly different (Figure 1F). Collectively, our combination of conventional and single nanoparticle imaging approaches provides robust evidence that NMDAR-Ab are present in the sera of a subset of SCZ+ patients and very few healthy+ subjects.

NMDAR-Ab from healthy+ subjects and SCZ+ patients do not compete for target binding

To investigate whether NMDAR-Ab from healthy+ subjects and SCZ+ patients similarly bind NMDAR, we performed immuno-competition assays in hippocampal cultured neurons and acute slices. For the *in vitro* assay, cultured neurons were first incubated with NMDAR-Ab from SCZ+ patients followed either by identical SCZ+ NMDAR-Ab, different SCZ+ NMDAR-Ab, healthy+ NMDAR-Ab, or encephalitis NMDAR-Ab (Figures 2A and 2B). Based on a dose-dependent competition assay and fluorescence background measurements (Figure S2), we selected the IgG concentration of 5 µg/ml for the immuno-competition assay. The fluorescence intensity of the secondary staining (i.e. IgG2) was measured within the area of the primary staining (i.e. IgG1). As expected, identical IgG1 and IgG2 highly compete, leading to a very low IgG2 staining and unimodal distribution of the IgG2/IgG1 ratio (Figure 2B). Strikingly, little, if any, competition was observed between different SCZ+ NMDAR-Ab, healthy+ NMDAR-Ab, and encephalitis NMDAR-Ab (Figure 2C). A significant shift in the cumulative distributions of IgG2/IgG1 ratio was observed between SCZ+ patients and healthy+ NMDAR-Ab, as well as between SCZ+ and encephalitis patients (Figure 2D), indicating that NMDAR-Ab of various origins differently bind NMDAR. Next, we confirmed part of these data in brain tissue by performing a competition assay in which hippocampal acute slices were first incubated with either SCZ+ or encephalitis NMDAR-Ab followed by biotinylated encephalitis NMDAR-Ab (Figure 2E). Encephalitis NMDAR-Ab competed with each other for the same binding site but they failed to compete with SCZ+ NMDAR-Ab (Figure 2E). Together, these data provide evidence that NMDAR-Ab from SCZ+ patients, healthy+ subjects, or encephalitis patients do not compete for binding on their target, suggesting different binding properties that can originate from polyclonal NMDAR-Ab and/or NMDAR-Ab with different affinities.

NMDAR-Ab differentially alter synaptic NMDAR surface dynamics

Based on the above difference in binding properties, we explored the potency of healthy+ and SCZ+ patients NMDAR-Ab to acutely alter NMDAR surface trafficking (Figure 3A). Indeed, antibodies directed against extracellular epitopes of the NMDAR can acutely alter the receptor surface distribution and dynamics (Dupuis et al., 2014; Mikasova et al., 2012; Potier et al., 2015). We specifically investigated the impact of autoantibodies on synaptic NMDAR by focusing on the GluN2A-NMDAR subtype located within postsynaptic density areas (PSD areas). Each patients' IgG (3 healthy+ and 4 SCZ+) was tested separately. Noteworthy, only 6% variability was found between patients (Figure S3). Strikingly, NMDAR-Ab from SCZ+ patients increased GluN2A-NMDAR surface dynamics when compared to healthy+ subjects (Figure 3B). This robust effect was mostly observed within the synaptic area as only a tendency was observed within the perisynaptic area (Figure 3C). Consistently, SCZ+ NMDAR-Ab shifted up GluN2A-NMDAR MSD curves, indicating lower confinement and higher explored area (Figure 3D). In addition, the exchange frequency, i.e. the number of receptor entries and exits between the synaptic area and its periphery, was significantly increased by SCZ+ NMDAR-Ab (Figure 3E). Thus, these data provide direct evidence, at the single molecule level, that IgG from different origins produce distinct molecular effects on the synaptic trafficking of surface NMDAR.

To assess the direct role of NMDAR-Ab from the purified IgG, we first test the impact of a diluted SCZ+ solution (50 ng/ml of IgG) on GluN2A-NMDAR dynamics within synaptic areas. Such a 100-fold dilution fully abolished the effect on surface dynamics (Figure 3F). Then, we specifically reduced the content of SCZ+ NMDAR-Ab by performing a pre-absorption experiment in which SCZ+ IgG solutions were repeatedly exposed to either untransfected or transfected (GluN1 subunits) live HEK cells (Figure 3G) (Graus et al., 2008; Zhang et al., 2012). The IgG solutions collected from untransfected HEK cells increased GluN2A-NMDAR surface dynamics within the synaptic area whereas no effect was observed in presence of the IgG solutions collected from GluN1-transfected HEK cells (Figure 3G). Our data indicate that it is the NMDAR-Ab from SCZ+ that acutely alter the surface dynamics of synaptic GluN2A-NMDAR. Finally, we tested whether SCZ+ NMDAR-Ab effect was specific for NMDAR by

investigating their impact on another receptor, i.e. GluA1-AMPA receptor (GluA1-AMPAR), and a potassium channel, i.e. Kv1.3 type, that are both present at the glutamatergic synapse and interact with similar scaffolds in the synapse (Lim et al., 2003). SCZ+ NMDAR-Ab did not alter the diffusion coefficients or the MSD curves of GluA1-AMPAR and Kv1.3 channel (Figure S4). Altogether, these data indicate that NMDAR-Ab from SCZ+ patients, and not healthy+ subjects, specifically and rapidly "destabilize" synaptic NMDAR.

Super-resolution and conventional imaging reveal that NMDAR-Ab from SCZ+ patients specifically alter the nanoscale organization and decrease content of synaptic NMDAR

An altered surface dynamics of a given receptor may, or may not, lead to change of its synaptic organization and content (Choquet and Triller, 2013). To investigate the fine organization of NMDAR within synapses we used super-resolution microscopy since it overcomes the diffraction limit and constitutes a powerful approach to reveal the molecular organization of postsynaptic molecules at nanoscale resolution (Dani et al., 2010). To test whether NMDAR-Ab alter the organization of NMDAR, in a uniform or non-uniform manner, we thus took advantage of the stochastic optical reconstruction microscopy (STORM) imaging to obtain the first super-resolved map of surface endogenous GluN2A-NMDAR in glutamatergic synapses (Figure 4A). Neurons were exposed either to healthy+ or SCZ+ NMDAR-Ab for 2h in order to leave a sufficient amount of time for putative nanoscale re-organization. Endogenous GluN2A-NMDAR were organized in 3-4 juxtaposed nano-objects (Figure 4B), resembling the organization of other glutamate receptors (MacGillavry et al., 2013; Nair et al., 2013). In neurons exposed to SCZ+ NMDAR-Ab, nano-objects areas were altered whereas their mean number and shape remained unaffected (Figures 4C). The SCZ+ NMDAR-Ab homogeneously shifted the nano-object size distribution, suggesting that all nano-objects were similarly affected (Figure 4C). In order to specifically characterize the synaptic nanoscale organization PSD-95, a core protein of the glutamate postsynaptic density, was also immunolabeled. The area of nano-objects was consistently reduced in PSD-95-containing synapses exposed to SCZ+ NMDAR-Ab without any effect on their number (Figures 4E and

4F). Together, these data reveal that SCZ+ NMDAR-Ab reduce the area of synaptic NMDAR nano-objects while leaving unaltered the nano-object organization (i.e. number and shape), supporting a model in which SCZ+ NMDAR-Ab decrease homogeneously the content of NMDAR within synaptic nano-objects.

Next, we investigated whether this nanoscale re-organization translated to a decrease of NMDAR content. Using immunocytochemical staining and confocal microscopy, we quantified the surface NMDAR content in synapses by immunostaining GluN1-SEP in live neurons after a 30 min (as the acute effect on surface dynamics), 2h, or 12h incubation period with the different NMDAR-Ab. At all incubation times, hippocampal neurons exposed to healthy+ NMDAR-Ab exhibited similar intensities of synaptic NMDAR cluster when compared to a control condition (incubation with $\alpha GluN1_{N\text{-}term}$) (Figure 5A). In contrast, SCZ+ NMDAR-Ab significantly reduced GluN1-NMDAR synaptic content following a 12h incubation period, whereas shorter incubation time had no effect (Figures 5A and 5B). The decrease after 12h incubation was restricted to the synaptic pool since GluN1-NMDAR extrasynaptic content remained stable (Figures 5C and S5), a striking difference with the effects of NMDAR-Ab from encephalitis patients (Mikasova et al., 2012). The postsynaptic density of glutamatergic synapses, estimated by the detection of Homer-1c, remained unaltered in all conditions (Figure 5D). Altogether, these data indicate that SCZ+ NMDAR-Ab specifically reduce GluN1-NMDAR synaptic nanoscale organization and content over time, leading to the loss of nearly half the receptors after several hours.

SCZ+ NMDAR-Ab disorganize EphrinB2 receptor within synapses

The destabilization and lateral displacement of synaptic receptors often originate from the disruption of interaction with anchoring partner, such as transmembrane receptors or scaffold proteins (Choquet and Triller, 2013). Because NMDAR-Ab bind extracellular epitope(s) of the receptor and can thus perturb interaction between NMDAR and an anchoring partner, we primarily focused our attention on the EphrinB2 receptor (EphB2R) as it strongly retains synaptic NMDAR through a direct interaction of their extracellular domains (Dalva et al., 2000). We first performed immunocytochemical detection of

surface EphB2R in neurons exposed to either healthy+ or SCZ+ NMDAR-Ab. The number of synaptic EphB2R clusters was decreased in presence of SCZ+ NMDAR-Ab when compared to healthy+ NMDAR-Ab (Figures 6A and 6B). The size of EphB2R clusters was also reduced by SCZ+ NMDAR-Ab (Figure 6C). We then performed single QD tracking to precisely assess EphB2R dynamics in synapses exposed to either NMDAR-Ab from healthy+ subjects or SCZ+ patients (Figure 6D). In presence of SCZ+ NMDAR-Ab, the distributions of EphB2R surface dynamics was significantly shifted (Figure 6E) and the diffusion coefficient was increased (median ± 25-75% IQR; Healthy+ = 0.13 $\mu m^2/s$, IQR = 0.043-0.26 $\mu m^2/s$, n = 1432 trajectories from 33 neurons; SCZ+ = 0.15 $\mu m^2/s$, IQR= 0.06-0.30 $\mu m^2/s$, n = 1104 from 39 neurons). These data indicate that SCZ+ NMDAR-Ab perturb EphB2R synaptic trafficking. Thus, these data indicate that NMDAR-Ab from SCZ+ patients specifically disturb EphB2R synaptic retention and content, suggesting that the loss of this anchoring partner of NMDAR is likely responsible for the destabilization of the receptor in presence of SCZ+ NMDAR-Ab.

SCZ+ NMDAR-Ab impair glutamate synapse plasticity without altering NMDAR activity *per se*

NMDAR-Ab from SCZ+ patients decrease over time the synaptic NMDAR content, likely altering crucial NMDAR-dependent processes such as synaptic long-term plasticity. To address this question, we first tested the direct impact of NMDAR-Ab on the receptor function of NMDAR by monitoring the spontaneous calcium transients mediated by the activation of NMDAR in live hippocampal networks. For this, the genetically encoded calcium indicator GCaMP3 was expressed in hippocampal neurons and calcium transients were recorded in spines in presence of an L-type voltage-dependent calcium channel blocker. The calcium transients were mediated by NMDAR since the NMDAR competitive antagonist APV (50 µM) fully abolished the events (Figure 7). A 5min incubation of neurons with NMDAR-Ab from healthy+ subjects or SCZ+ patients did not alter the frequency of NMDAR-mediated calcium events (Figure 7B), indicating that NMDAR-Ab are not acute modulators of NMDAR.

To assess the functional impact of NMDAR-Ab on the glutamate synapse, we monitored the synaptic content of surface GluA1-AMPAR in basal conditions and after an activity-induced synaptic AMPAR

potentiation, i.e. chemical long-term potentiation (cLTP) (Dupuis et al., 2014; Lu et al., 2001). Neurons were exposed to either healthy+ subjects or SCZ+ patients for 12h to alter the NMDAR synaptic content. The basal level of GluA1-AMPAR within synapses was significantly lowered by NMDAR-Ab from SCZ+ patients when compared to control (Figures 8A and 8B). After cLTP stimulation, the surface content of GluA1-AMPAR increased over time at synapses exposed to NMDAR-Ab from healthy+ subjects, as in the control condition (Figures 8C and 8D). Strikingly, at synapses exposed to NMDAR-Ab from SCZ+ patients cLTP-induced potentiation of GluA1-AMPAR was abolished (Figures 8C and 8D), even leading to a tendency toward a depression of the AMPAR synaptic content. Thus, NMDAR-Ab from SCZ+ patients alter the basal level of synaptic AMPAR and impair their recruitment during activity-dependent synaptic plasticity. Together, these data indicate that NMDAR-Ab from SCZ+ patients specifically impair the plastic range of glutamatergic synapses.

Discussion

In this case study, we identified circulating NMDAR-Ab in 3% of healthy subjects and 19% of SCZ patients that were in acute phase. Their titers were approximately 20-times lower when compared to the one in anti-NMDA encephalitis, and they were absent from patients CSF. Taking advantage of the single molecule imaging, we demonstrate that NMDAR-Ab from SCZ+ patients, but not from healthy subjects, destabilize synaptic NMDAR and its interacting partner EphB2R. Consequently, NMDAR-Ab from SCZ+ patients decrease over time the NMDAR synaptic content and impair NMDAR-dependent synaptic plasticity. Together with their different binding properties on NMDAR (no competition between NMDAR-Ab from healthy+ subjects and SCZ+ patients), our data support the view that NMDAR-Ab from SCZ+ patients target extracellular domain(s) of the receptor important for interactions with anchoring partner, such as EphrinB2R. Our findings also shed clinical lights as we demonstrate that circulating NMDAR-Ab can have distinct molecular impact, calling for greater caution in using these autoantibodies as generic biomarkers for autoimmune brain disorders.

Over the last years, several studies have reached different conclusions regarding the presence of NMDAR-Ab in psychiatric disorders (Coutinho et al., 2014; Kayser and Dalmau, 2014; Sinmaz et al., 2015). Although there is no straightforward explanation for these discrepancies, the different methods used to define the patient seropositivity have been at the centre of debates. In order to optimize the detection, a set of multiple tests is often performed in addition to CBA assays (Coutinho et al., 2014; Dalmau et al., 2007; Gresa-Arribas et al., 2014). In this line, we here confirmed using live CBA in transfected HEK 293 cells expressing GluN1/GluN2 heteromers, immunohistochemical detection in brain tissue and cultured neurons, the presence of circulating NMDAR-Ab in both healthy+ subjects and SCZ+ patients. In the same time, we also experienced that the choice of CBA assays (e.g. live *versus* fixed HEK cells) greatly influences the outcome of NMDAR-Ab seropositivity. For instance, live CBA appears more sensitive as it can detect all conditions (healthy+, SCZ+, and encephalitis), being thus less "disease-specific". On the other hand, fixed CBA appears less sensitive (higher signal-to-noise ratio) as only encephalitis patients are detected, being thus more "disease-specific". In search for a new

additional test, we implemented a single molecule tracking approach to define the live "signature" of the autoantibody membrane target. We could unambiguously demonstrate that the signature of the previously defined "NMDAR-Ab" was undistinguishable from the one of native GluN1-NMDAR. Implementing such an additional approach in the clinical diagnostic will thus be of great help although such a cutting-edge imaging approach is technically challenging and time-consuming. Future methods, based on similar principals but with high-screening capacity, should thus be developed in order to efficiently detect circulating autoantibodies.

Could the seropositive SCZ+ patients also suffer, or suffered, from anti-NMDAR autoimmune encephalitis? Indeed, acute psychosis in patients with NMDAR-Ab has been reported and could thus complicate the diagnostic for schizophrenia (Kayser et al., 2013; Zandi et al., 2014). Based on several characteristics, we feel confident that seropositive patients suffered from schizophrenia only. First, most seropositive patients were diagnosed 10 years before the study and treated accordingly (Table S1). One patient was enrolled after its first episode of psychosis, leaving open the possibility of an acute episode in this patient. The history of patients was examined for stroke, multiple sclerosis, epilepsy, and encephalitis, which constituted exclusion criteria if positive. We systematically reviewed the clinical files of the SCZ+ patients to search for signs of possible ancient autoimmune encephalitis (e.g. abnormal movements, bad tolerance of antipsychotics, epilepsy**,** dysautonomia, CSF or brain MRI). None of them presented any sign or clinical history of encephalitis. Five of the nine patients accepted to be examined at a second time point, when they were outpatient, treated, compliant but remained symptomatic. All these five patients had a serological confirmation for NMDAR-Ab seropositivity on a new serum sample, and still no sign of neurological perturbation. Furthermore, NMDAR-Ab were only detected in the serum and never in the CSF of the SCZ+ patients, which sharply contrasts with anti-NMDAR encephalitis (Gresa-Arribas et al., 2014; Viaccoz et al., 2014). Thus, our in-depth examination does not provide evidence of ancient or ongoing autoimmune encephalitis in these SCZ+ patients.

Do these circulating NMDAR-Ab have a pathogenic role in psychosis? Providing a complete answer to this question is obviously a major challenge for the next decade. The majority of seropositive SCZ+ patients were in acute phase when tested and their symptoms were more severe than seronegative ones (Table S2), supporting the hypothesis that circulating NMDAR-Ab contribute or worsen symptoms. Well-tolerated immunotherapy in seropositive NMDAR-Ab patients with acute psychosis provided positive clinical outcomes (Zandi et al., 2014), further supporting a potential pathogenic role. Here, we focused our attention on the molecular pathogenicity of the autoantibodies. The glutamatergic model of psychosis and schizophrenia is increasingly accepted as part of the etiopathology since the discovery that some NMDAR blockers induce schizophrenia-like psychosis, reproducing both positive and negative symptoms (Kantrowitz and Javitt, 2012). Alterations of the NMDAR synaptic content have consistently been reported in the brains of schizophrenic patients (Kristiansen et al., 2010; Li et al., 2015). Our findings that only NMDAR-Ab from SCZ+ patients have the potency to dynamically disorganize synaptic NMDAR and alter NMDAR-dependent network processes provide molecular evidence for the relevance of NMDAR-Ab in psychosis. Recently, it was shown that D-serine, a co-agonist of the NMDAR that strongly alter the receptor surface dynamics (REF), clinically improves the symptoms of a NMDAR-Ab SCZ+ patient (Heresco-Levy et al., 2015), fueling further the hypothesis that the NMDAR hypofunction in schizophrenia partly originate from an altered cellular trafficking (Lau and Zukin, 2007). Such an alteration can have genetic (e.g. dysfunction of proteins involved in the receptor transport, membrane delivery, and/or synaptic stabilization) and environmental (e.g. immune-inflammation, autoimmunity) origins. Here, we show that NMDAR-Ab from SCZ+ patients rapidly alter the organization and trafficking of NMDAR and anchoring EphB2R.

EPHB2?

These cellular deficits and their behavioral consequences could be prevented *in vitro* and *in vivo* by modulation of NMDAR trafficking through the ephrinB2 ligand (Mikasova et al., 2012; Planaguma et al., 2016), rising therapeutical interest in anti-NMDAR encephalitis and even autoimmune psychotic disorders. However, patients with anti-NMDAR encephalitis do not develop schizophrenia despite

remaining of low-level NMDAR-Ab in sera and even CSF for months to years in few patients (Gresa-Arribas et al., 2014; Hansen et al., 2013). This suggests that other, yet unknown, processes are additionally involved in patients suffering from schizophrenia, such as other autoantibodies (e.g. against dopamine receptors), genetically-based immune or synaptic instability, developmental stage(s) at which autoantibodies are present in the brain, or altered biological barriers.

The NMDAR-Ab were detected in the sera and not in the CSF of patients, leaving open the question of IgG brain penetration. It has long been documented that SCZ patients have history of infections, which may transiently favour breaches in the blood brain barrier (BBB) and thus the brain infiltration of circulating molecules (van Os et al., 2010). Three of the SCZ+ patients had elevated albumine CSF/ratio and one had oligoclonal bands suggesting an altered the BBB. The injection of NMDAR-Ab from neuropsychiatric patients in the circulation of mice with deficient BBB was associated with locomotor and behavioral impairments (Hammer et al., 2013; Kowal et al., 2006). In addition, it cannot be excluded that in schizophrenia NMDAR-Ab IgG slowly penetrate the brain parenchyma even with unaltered BBB since the presence of NMDAR at the surface of epithelial barrier cells can favour receptor-antibody mediated transcytosis (Andras et al., 2007; Niewoehner et al., 2014; Reijerkerk et al., 2010).

In conclusion, single nanoparticle imaging unmasked the specific alteration of the NMDAR synaptic complex by circulating NMDAR-Ab from SCZ+ patients in hippocampal neurons. Comparing the molecular pathogenicity of NMDAR-Ab identified in mania (Dickerson et al., 2012), systemic lupus erythematosus (DeGiorgio et al., 2001), slow cognitive impairment (Pruss et al., 2012) or Alzheimer's patients with psychosis (Busse et al., 2014; Doss et al., 2014), could also be of great fundamental and clinical interests (e.g. defining patient subgroups) for future work, as much deciphering the molecular pathogenicity of different Ig classes. Based on the heterogeneity of NMDAR-Ab molecular impact caution should be taken in using "generic" NMDAR-Ab as biomarkers in brain disorders, urging for further clinical and fundamental investigations in this blooming research field.

Experimental Procedures

Additional information are provided in the Supplemental Experimental Procedures

Participants

Schizophrenic patients (n= 48) meeting DSM-IV criteria (APA, 1994), consecutively hospitalised in an university-affiliated psychiatric departments in France (Mondor hospital, Créteil, University Paris-Est hospital, Paris), were included in this study after approval by a French ethical committee and written informed consent for their participation. Patients were interviewed with the French version of the "Diagnostic Interview for Genetic Studies" (DIGS) for the assessment of lifetime clinical characteristics of schizophrenia such as age at onset, duration of illness, number of hospitalisations, demographic characteristics and ongoing treatments. In addition, medical history was explored, in particular history of neurological or inflammatory disorders. Positive and negative symptoms were assessed with the Positive and Negative Syndrome Scale (PANSS). Cognitive function was assessed with the NART, a reliable tool estimating the premorbid ability level using a word reading test which provides an estimate of premorbid cognitive ability. Healthy subjects (n= 104) without any personal and family history of schizophrenia or bipolar disorder were enrolled through a clinical investigation center (Center for Biological Resources, Mondor hospital, France). All subjects were submitted to serological screening and infectious antibody analysis. Participants were included only if they were negative for HIV1/2, Hepatitis B and C, had no ongoing inflammatory, auto-immune or neurological disorders and no ongoing immunosuppressive or immune-modulating treatment.

Collection of blood samples and medical examination

Blood samples were collected from patient and control groups within one week of the clinical assessment. Sera were then purified in order to extract IgG isotype antibodies. Samples were dialyzed against phosphate-buffered saline and solutions were used at pH7.4 (Mikasova et al., 2012; Viaccoz et al., 2014). Seropositive patients for NMDAR-Ab were contacted for additional medical and

biological evaluations. The remaining volume of sera after infectious screening and NMDAR-Ab detection (see below) was however not sufficient in each patient to perform IgG purification (4/9 were then used for IgG-based assays).

NMDAR-Ab detection in heterologous cells and neuronal preparations

Serum samples were tested for the presence of NMDAR-Ab using a cell-based assay on human embryonic kidney cells (HEK293) expressing both GluN1 and GluN2B subunits of the NMDAR, using the method previously described (Mikasova et al., 2012; Viaccoz et al., 2014). To detect NMDAR-Ab in CSF, cells were fixed (4% PFA, 10 min) and then incubated with patients' CSF (1:50 in saturation buffer, 90min). Samples were considered as positive when a clear staining was confirmed by three different readers in three independent assays. Titers of positive sera were determined by end point dilutions.

For cellular imaging processes (for further details, see Supplemental Experimental Procedures), either sera or purified IgG from seropositive subjects were used. Cultures of hippocampal neurons were prepared from E18 Sprague-Dawley rats and were maintained at 37°C in 5% CO_2 for 15*div* at maximum as previously described (Mikasova et al., 2012). For exogenous protein expression, 7-10*div* hippocampal cultured neurons were transfected at least 48h before each experiment using either the Effectene (Qiagen) or phosphate calcium transfection (Jiang et al., 2006).

Immuno-absorption

Schematically, 24h after plating (150 000 cells/ml), HEK293 cells were transfected with GluN1-SEP and GluN2B (Lipofectamine LTX, Invitrogen). The following day, either transfected or non-transfected HEK cells were incubated for 1h with NMDAR-Ab (5 µg/ml). This step was repeated 6 times and the resulting absorbed fraction was kept at 4°C (max 24h) for further live experiments.

Immunochemistry

Mice (P22) hippocampal coronal sections of 50 µm were incubated overnight at 4°C with either a polyclonal antibody against the N-terminal part of the GluN1 subunit ($\alpha GluN1_{N\text{-}term}$ Alomone Labs, 20µg/ml) or human IgG (20 µg/ml). Images were obtained using a Nanozoomer and a confocal microscope (SP8, Leica). Human IgG labeling was obtained on fixed dissociated hippocampal neurons after incubation with purified IgG (5 µg/ml, overnight 4°C). Hippocampl cultured neurons expressing surface exogenous GluN1-SEP subunits were stained using a monoclonal antibody against GFP (1/500, 15 min). Surface endogenous EphB2R were labeled with an anti-EphB2R polyclonal antibody (1/200, 3h). Neurons were then incubated with an anti-Homer-1c antibody (1/500, 30min). All imaging sessions were done on a video spinning-disk system (Leica DMI6000B, 63X) and quantification analysis was performed using MetaMorph software (Molecular Devices) and ImageJ (NIH).

Immunocompetition

Sections (7µm) of hippocampal tissue were fixed and incubated with undiluted serum from patients or blood donors overnight at 4°C. Sections were then extensively washed with cold PBS and incubated for 1h with biotinylated IgG from a representative patient with NMDAR-Ab. After washing, the binding of biotinylated IgG was revealed with a standard avidin-biotin-peroxidase method (Vectastain ABC kit Elite, PK-6100, Vector). Slides were then mildly counterstained with hematoxylin, mounted, and results photographed with a digital camera (AxioCam MRc) adapted to a confocal microscope (Zeiss LSM710). Hippocampal cultured neurons were fixed and successively incubated with SCZ+ NMDAR-Ab (purified IgG 5 µg/ml, overnight, 4°C). Remaining antigen binding sites were blocked using anti-human Fab fragments (Jackson ImmunoResearch, 100 µg/ml, 1h). Cells were then incubated with competing IgG from either SCZ+, Healthy+, or Encep+ individuals (5 µg/ml, overnight, 4°C). All imaging sessions were done on a video spinning-disk system (Leica DMI6000B, 63X) and quantification analysis was performed using ImageJ (NIH).

Direct stochastic optical reconstruction microscopy (dSTORM)

Live hippocampal neurons were incubated with either human IgG for 2h at 37°C. Surface endogenous GluN2A-containing NMDAR were specifically stained using an anti-GluN2A antibody (0.1 mg/ml, 15 min). Cells were then successively incubated with an anti-PSD95 antibody (0.1 mg/ml, 45 min) and secondary anti-rabbit Alexa 647 (Invitrogen, 0.1 mg/ml, 30 min) and anti-mouse Alexa 532 (0.1 mg/ml, 30 min) antibodies. A second fixation was performed after incubation with the secondary antibodies. All imaging sessions were performed using a Leica SR GSD 3D microscope (Leica HC PL APO 160x 1.43 NA oil immersion TIRF objective) and an ANDOR EMCCD iXon camera. Localization of single molecules and reconstruction of the super resolved image was performed by applying a fitting algorithm determining the centroid-coordinates of a single molecule and fitting the point-spread-function (PSF) of a distinct diffraction limited event to a Gaussian function. The final achieved spatial resolution was 40nm.

Quantum dot (QD) tracking and surface diffusion calculation

QD labeling and microscopy were performed as previously described (Mikasova et al., 2012). See Supplemental Experimental Procedures for further details.

Calcium imaging

Dissociated neurons transfected with $GCaMP_3$ at 10div were transferred into a Tyrode solution at 12-13 div. 15min before imaging, cells were transferred to a Mg^{2+}-free Tyrode solution with 5 µM Nifedipine (Tocris), and 5 µM Bicuculline (Tocris). Time-lapse images were acquired at 20Hz. Three time-lapse movies (3000 frames) were successively recorded: 1) “Pre” (baseline period), 2) “Post” (5 min after bath application of buffer or purified IgG, 5 µg/ml) and 3) “APV” (5 min after bath application, 50 µM).

Chemically induced potentiation (cLTP)

Live hippocampal neurons transfected with GluA1-SEP were incubated overnight with human IgG (Healthy+ or SCZ+, 5 µg/ml, 37°C). After washing thoroughly, chemically induced long-term potentiation (cLTP) was elicited by a bath co-application of glycine (200 µM) and picrotoxin (5 µM) for 4 min (Dupuis et al., 2014). cLTP was always applied after a period of baseline acquisition and the medium was carefully replaced by fresh equilibrated and heated medium after induction. GluA1-SEP fluorescence signal was then recorded every 5 min during the 30 min following the stimulus. Synapses were defined using the synaptic protein Homer-1c DsRed. Synaptic GluA1-SEP clusters intensity and area values were normalized to the baseline values.

Author contributions

Conceptualization, J.J., E.M.J., J.H., M.L. and L.G.; Methodology, J.J., E.M.J., V.R., M.S., J.D., J.H., M.L., and L.G.; Clinical analysis, E.L., C.R., N.H., R.H., J.H., and M.L.; Analysis, J.J., E.M.J., H.G., V.R., M.S., M.L., B.K., E.M.; Investigation, J.J., E.M.J., H.G., V.R., M.S., M.L., B.K., N.H., E. L., C.R., E.M., R.H.Y., and D.B.; Resources – J.D., J.H., M.L. and L.G.; Writing - Original draft, J.J. and L.G.; Writing - Review and editing, J.J., E.M.J, J.D., J.H., M.L. and L.G.; Supervision, J.H., M.L. and L.G.; Funding, J.H., M.L. and L.G.

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Figure 1

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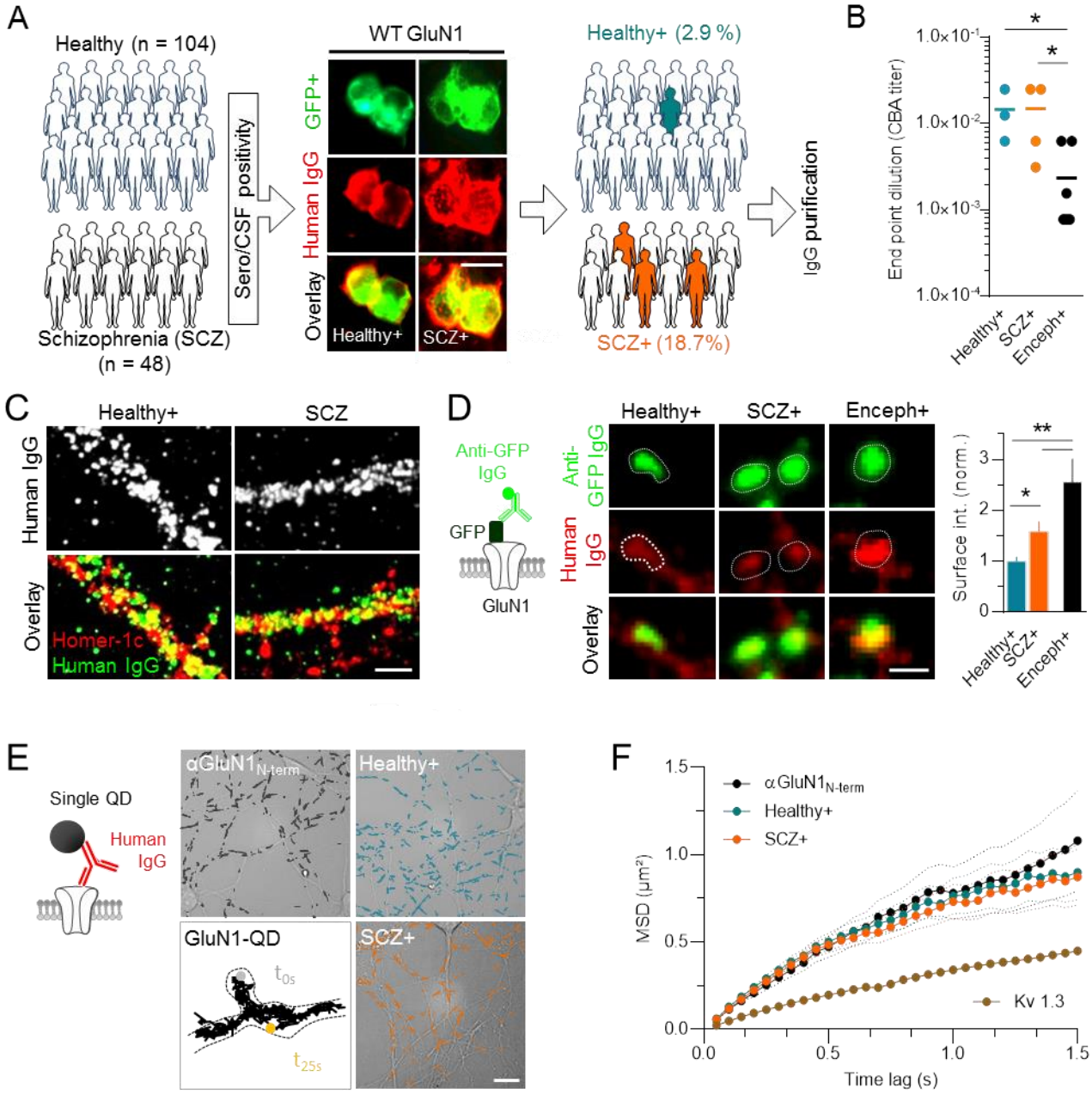

Figure 1. Multi-approach identification of NMDAR-Ab IgG in the serum of schizophrenic patients and healthy subjects

(A) Immunostaining of HEK293 cells expressing GluN1-GFP and GluN2B subunits with the sera of healthy subjects or schizophrenic patients (1/10, 3h incubation). Note the overlap between serum reactivity (red) and GFP-positive HEK cells (green) for seropositive samples. Scale bar, 10 µm.

(B) The titer of NMDAR-Ab was estimated by end-point dilutions. Each data point represents the value calculated for a subject or patient. Average data are represented as mean ± SEM. Healthy+= 0.014 ± 0.004 a.u., n= 3. SCZ+ = 0.017 ± 0.005 a.u., n= 4. Enceph+ = 0.0005 ± 0.0001 a.u., n= 7.

(C) Representative dendritic areas of cultured hippocampal neurons (12 *d.i.v.*) labelled with purified IgG (5 µg/ml, green) from Healthy+ subjects or SCZ+ patients. Homer-1c staining (red) localizes glutamate postsynaptic densities. Both staining reveal a cluster-type distribution with good colocalization with the synaptic area. Scale bar, 20 µm.

(D) Surface co-immunostaining for GluN1-SEP containing NMDAR and human IgG's target in live hippocampal neurons. Both Healthy+ and SCZ+ IgG detect a target that colocalizes with surface GluN1-SEP clusters (yellow). Scale bar, 1 µm. *Right panel*, Fluorescence intensity normalized to the cluster area. Data are represented as mean ± SEM. Healthy+ = 1 ± 0.09 a.u., n = 45 clusters. SCZ+ = 1.586 ± 0.191 a.u., n = 59 clusters. Enceph+ = 2.553 ± 0.4533 a.u., n = 38 clusters. *p < 0.05, **p < 0.01, one-way ANOVA followed by a Newman-Keuls multiple comparison test.

(E) Schematic representation of single QD complex approach used to label the surface target of patients' IgG (left panel). On the right panel, representative trajectories of control $\alpha GluN1_{N\text{-}term}$ (dark gray lines), Healthy+ (blue) and SCZ+ (orange) QD complexes. IgG-QD complexes were tracked during 500 frames with a 50 ms acquisition frequency on cultured hippocampal neurons (14-15 *div*). Scale bar, 20 µm.

(F) Mean Square Displacement (MSD) over time of GluN1-NMDAR (dark grey) and purified IgG's target. The curves are represented as mean ± SEM (note that the SEM are not visible). Surface targets of both Healthy+ and SCZ+ purified IgG exhibit diffusion behaviors similar to GluN1-NMDAR but different from Kv1.3, a potassium channel receptor also present in glutamatergic synapses. $\alpha GluN1_{N\text{-}term}$, n = 5 neurons. Healthy+, n = 6 neurons. SCZ+, n = 10 neurons.

Also see Tables S1-3, Figure S1.

Figure 2

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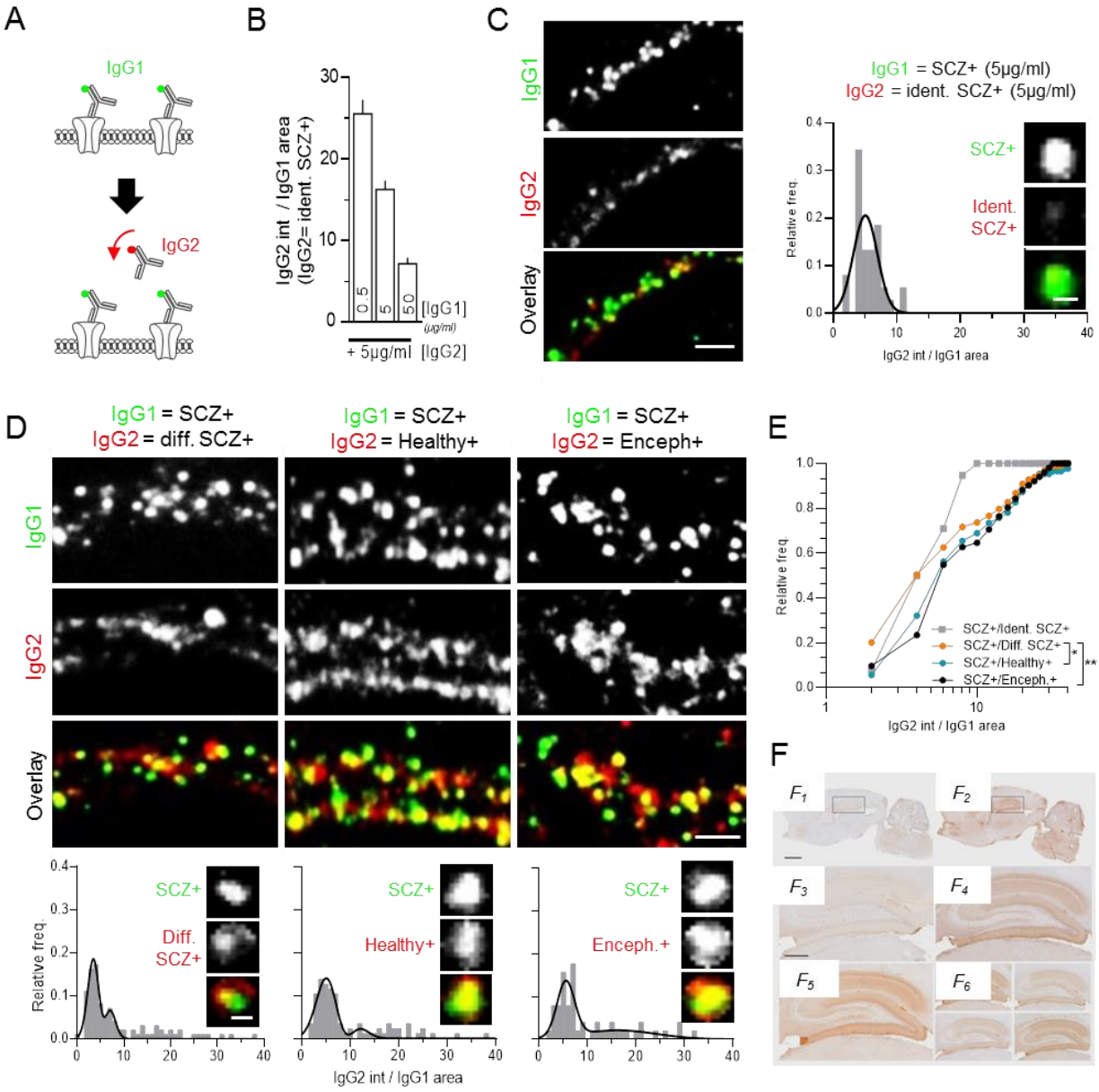

Figure 2. NMDAR-Ab from different origins target do not compete for target binding

(A) Experimental design of the *in vitro* immuno-competition test. Hippocampal cultures (12 *div*) were first incubated with SCZ+ NMDAR-Ab (IgG1) labeled in green. Cells were then incubated with a second Healthy+, SCZ+ or Enceph+ IgG (IgG2) labeled in red.

(B) Representative dendritic areas labeled with SCZ+ NMDAR-Ab (IgG1, 5 µg/ml, green) and treated with the same IgG (IgG2= ident. SCZ+, 5 µg/ml, red). Scale bar, 2 µm. *Right panel,* The fluorescence intensity of IgG2 within IgG1 area was measured for each competition challenge. The use of the same IgG (IgG2 = ident. SCZ+) results in a distribution fitted with a single Gaussian. *Insets,* staining from a single cluster. Scale bar, 500 nm.

(C) Representative dendritic areas labeled with a SCZ+ NMDAR-Ab (IgG1, 5 µg/ml, green) and treated with NMDAR-Ab from another SCZ+ patient (IgG2 = Diff. SCZ+), a healthy individual (IgG2 = Healthy+) or a patient with anti-NMDAR encephalitis (IgG2 = Enceph+). Scale bar, 2 µm. *Bottom panels*, Corresponding histograms of IgG2 fluorescence intensity within IgG1 area for the different competing conditions. *Insets,* staining from single clusters. Scale bar, 500 nm. Note that the application of a different SCZ+ IgG alter the distributions that were better fitted with a multi-peak curves.

(D) Cumulative distributions of IgG2 intensity within IgG1 cluster areas. SCZ+/Ident. SCZ+, n = 38 dendritic regions (10 neurons). SCZ+/Diff. SCZ+, n = 99 dendritic regions (32 neurons). SCZ+/Healthy+, n = 78 dendritic regions (26 neurons). SCZ+/Enceph+, n = 51 dendritic regions (15 neurons). *$p < 0.05$, **$p < 0.005$, Kolmogorov-Smirnov test.

(E) Pre-incubation of rat brain sections with serum from a patient with anti-NMDAR encephalitis blocks the reactivity of biotinylated IgG from another patient with anti-NMDAR encephalitis (E1 and E3). Pre-incubated with serum from a healthy control does not block the reactivity of biotinylated IgG from a patient with anti-NMDAR encephalitis (E2 and E4). Pre-incubation of sections of rat brain with serum of 5 patients with schizophrenia does not block the reactivity of biotinylated IgG from a patient with anti-NMDAR encephalitis (E5 and E6).

Also see Figure S2.

Figure 3

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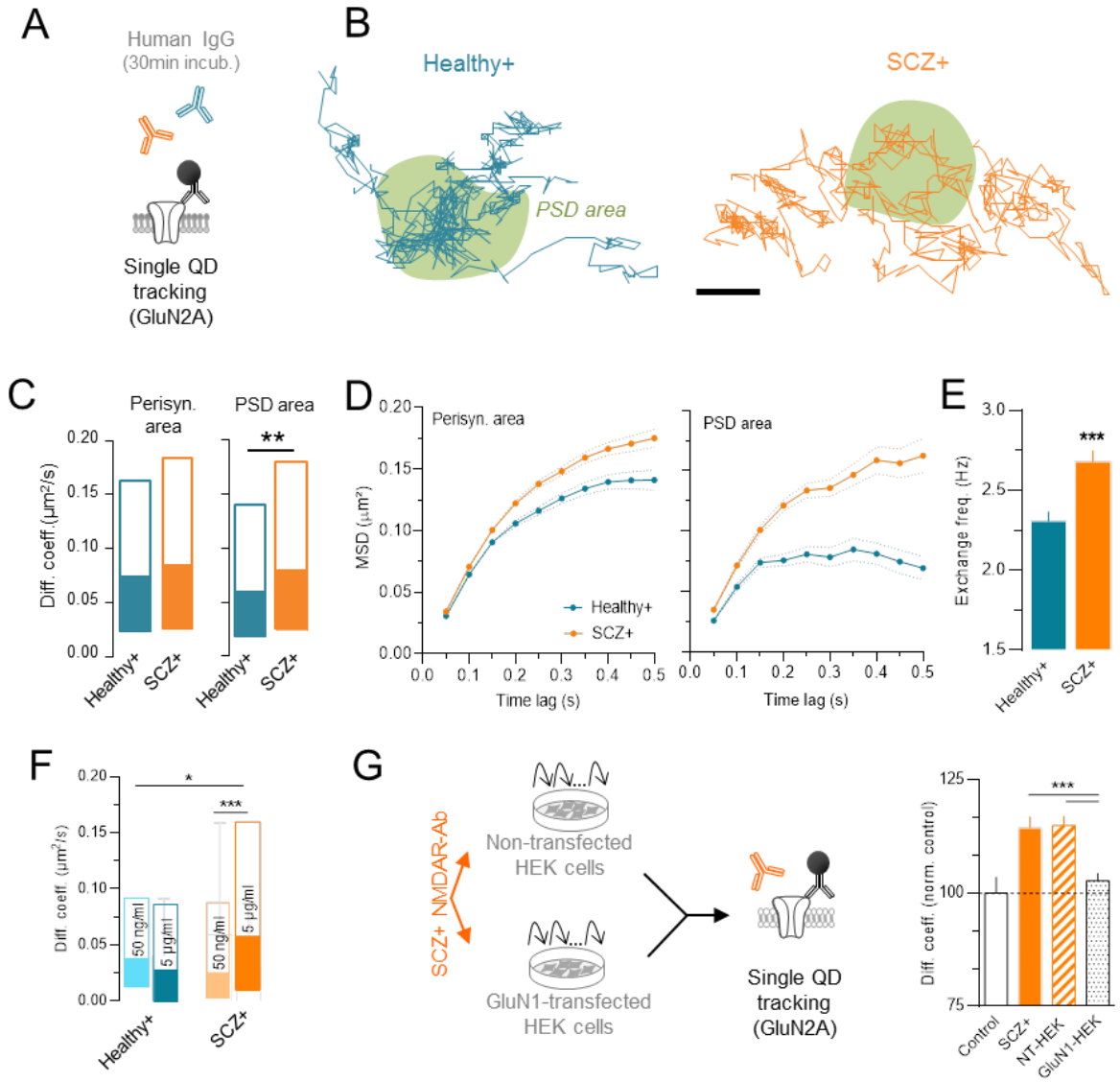

Figure 3. NMDAR-Ab from schizophrenic and healthy subjects display different effects on synaptic NMDAR dynamics

(A) Schematic representation of the experimental design.

(B) Representative trajectories of a single GluN2A-NMDAR-QD complex (500 frames, 50ms acquisition) within synaptic areas. Before tracking, hippocampal cultures (10-14 *d.i.v.*) were incubated for 30 min with different purified IgG samples (3 Healthy+ and 4 SCZ+). Scale bar, 500 nm.

(C) Comparison of GluN2A-NMDAR instantaneous diffusion coefficient (expressed in $\mu m^2/s$) in presence of healthy+ or SCZ+ NMDAR-Ab. Diffusions were compared within the perisynaptic and postsynaptic density (PSD) areas. Data are expressed as median diffusion coefficient ± 25-75% IQR. Perisyn. area healthy+ = 0.079 $\mu m^2/s$, IQR = 0.025-0.168 $\mu m^2/s$, n = 1344 trajectories (42 neurons). Perisyn. area SCZ+ = 0.086 $\mu m^2/s$, IQR = 0.026-0.184 $\mu m^2/s$, n = 2264 trajectories (64 neurons). P = 0.213, Mann-Whitney test. PSD area healthy+ = 0.055 $\mu m^2/s$, IQR = 0.013-0.135 $\mu m^2/s$, n = 313 trajectories (42 neurons). PSD area SCZ+ = 0.076 $\mu m^2/s$, IQR= 0.020-0.177 $\mu m^2/s$, n = 695 (59 neurons). **p = 0.003, Mann-Whitney test.

(D) Comparison of GluN2A-NMDAR MSD curves within the perisynaptic and PSD areas. Data are expressed as mean ± SEM (dotted lines). Perisyn. area, p = 0.076, Kolmogorov-Smirnov test. PSD area, p < 0.001, Kolmogorov-Smirnov test.

(E) Exchange frequency of diffusive GluN2A-NMDAR between the synaptic and extrasynaptic compartments. Data are expressed as mean ± SEM. Healthy+ = 2.31 ± 0.06 Hz, n = 241 trajectories. SCZ+ = 2.68 ± 0.07 Hz, n = 268 trajectories. ***p < 0.001, Mann-Whitney test.

(F) Comparison of GluN2A-NMDAR instantaneous diffusion coefficient after 30 min exposition to various concentrations of NMDAR-Ab. Data are expressed as median diffusion coefficient ± 25-75% IQR. Healthy+ 50 ng/ml = 0.040 $\mu m^2/s$, IQR= 0.0135-0.091 $\mu m^2/s$, n = 321 trajectories (21 neurons). Healthy+ 5 µg/ml = 0.030 $\mu m^2/s$, IQR= 0-0.085 $\mu m^2/s$, n = 69 trajectories (32 neurons). SCZ+ 50 ng/ml = 0.026 $\mu m^2/s$, IQR = 0.0025 - 0.087 $\mu m^2/s$, n = 187 (24 neurons). SCZ+ 5 µg/ml = 0.058 $\mu m^2/s$, IQR= 0.011-0.159 $\mu m^2/s$, n = 178 (32 neurons). *p < 0.05, ***p<0.001, Kruskal-Wallis followed by a Dunn's multiple comparison test.

(G) Experimental design of the NMDAR-Ab pre-absorption experiment. SCZ+ NMDAR-Ab (5 µg/ml) were incubated successively (6 times) on HEK cells transfected or not with GluN1 subunit (left panel). The collected fraction was then incubated 30 min on live hippocampal neurons (14 *d.i.v.*). Data are expressed as normalized mean of GluN2A instantaneous diffusion coefficient ± SEM. Control = 100 ± 3.5, n= 1388 trajectories (37 neurons). SCZ+ = 114 ± 2.5, n = 2658 trajectories (59 neurons). NT-HEK = 115 ± 2, n = 3867 trajectories (27 neurons). GluN1-HEK = 103 ± 1.6, n = 4799 trajectories (28 neurons). ***p < 0.001, Kruskal-Wallis followed by a Dunn's multiple comparison test.

Also see Figures S3 and S4.

Figure 4

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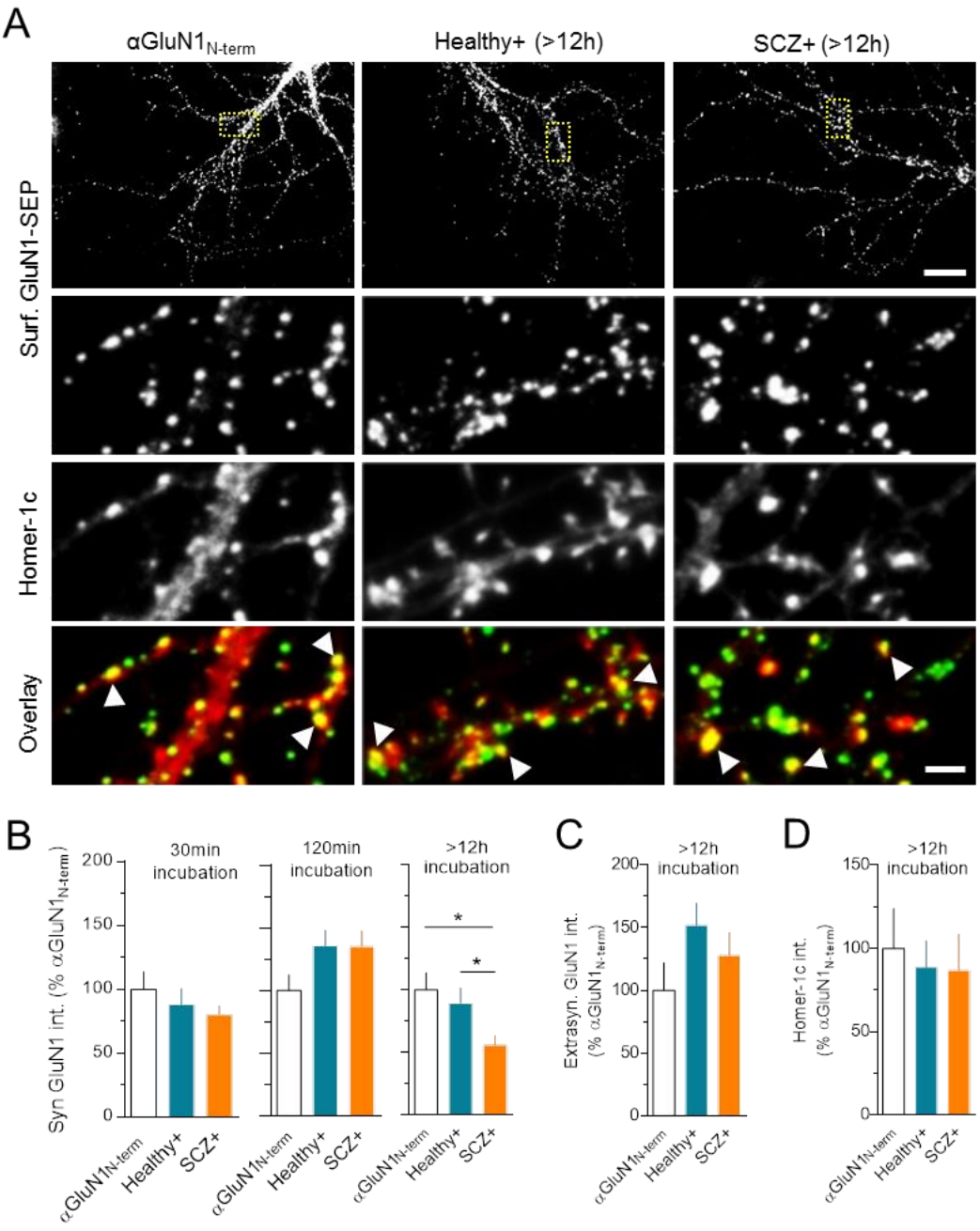

Figure 4. SCZ+ NMDAR-Ab specifically alter NMDAR synaptic content in a time-dependent manner

(A) Live staining of GluN1-SEP in hippocampal cultures treated overnight with either a control antibody ($\alpha GluN1_{N\text{-}term}$) or human purified IgG (Healthy+ or SCZ+ NMDAR-Ab). The synaptic localization of NMDAR clusters (green) was determined using Homer-1c (red). Colocalized staining was interpreted as synaptic GluN1-NMDAR clusters (arrow heads). Scale bars, 20 µm (upper panels), 2 µm (lower panels).

(B) Comparison of synaptic GluN1-NMDAR clusters fluorescence after different incubation times with NMDAR antibodies. Data are expressed as mean percent to $\alpha GluN1_{N\text{-}term}$ ± SEM. $\alpha GluN1_{N\text{-}term}$ 30min = 100 ± 14, n = 19 neurons. Healthy+ 30min = 88 ± 12.5, n = 36 neurons. SCZ+ 30 min = 80 ± 7, n = 55 neurons. $\alpha GluN1_{N\text{-}term}$ 2h = 100 ± 13, n = 24 neurons. Healthy+ 2h = 136 ± 12, n = 47 neurons. SCZ+ 2h = 135 ± 12, n = 77 neurons. $\alpha GluN1_{N\text{-}term}$ 12h = 100 ± 14, n = 22 neurons. Healthy+ 12h = 89.5 ± 12, n = 34 neurons. SCZ+ 12h = 56 ± 8, n = 28 neurons. *$p < 0.05$, One-way ANOVA followed by a Newman-Keuls multiple comparison test.

(C) Comparison of the extrasynaptic GluN1-NMDAR cluster fluorescence after 12h incubation with NMDAR antibodies. Data are expressed as mean percent to $\alpha GluN1_{N\text{-}term}$ ± SEM. *Healthy+* = 92 ± 3, n = 34 neurons. *SCZ+* = 91 ± 4, n = 30 neurons.

(D) Comparison of the Homer-1c DsRed fluorescence after 12h incubation with NMDAR antibodies. Data are expressed as mean percent to $\alpha GluN1_{N\text{-}term}$ ± SEM. G*luN1*$_{N\text{-}term}$ = 238 ± 56, n = 25 neurons. Healthy+ = 226 ± 38, n = 31 neurons. *SCZ+* = 185.5 ± 41, n = 16 neurons.

Also see Figure S5.

Figure 5

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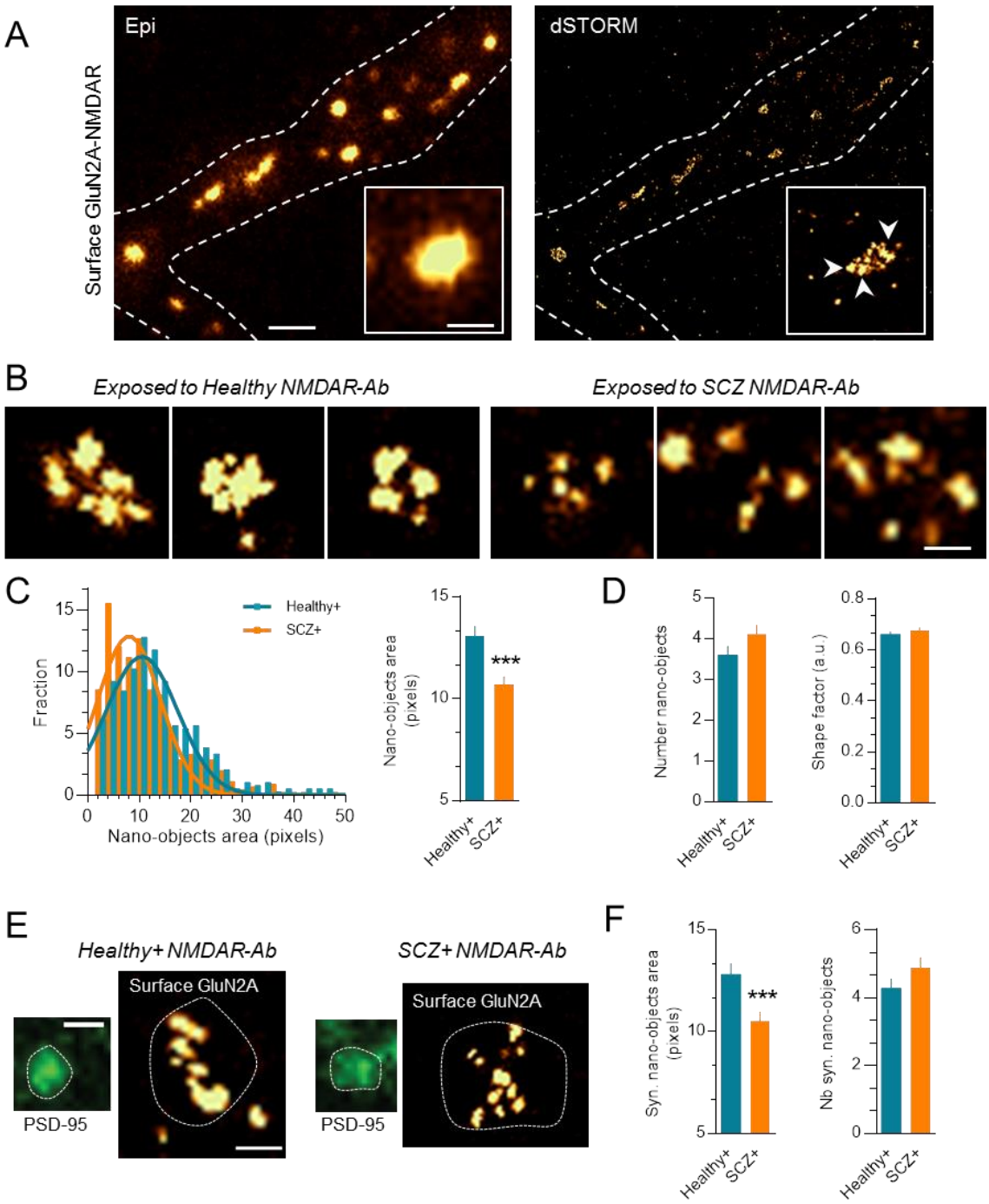

Figure 5. Super-resolved map of GluN2A-NMDAR surface organization in presence of Healthy+ and SCZ+ NMDAR-Ab

(A) Epifluorescence and dSTORM image of a dendritic segment with endogenous GluN2A-NMDAR staining. Scale bar, 1 µm. *Insert panels,* magnification of an isolated GluN2A-NMDAR cluster. Scale bar, 300 nm.

(B) Representative examples of GluN2A-NMDAR nano-objects imaged by dSTORM after incubation with either Healthy+ or SCZ+ NMDAR-Ab. Scale bar, 400 nm.

(C) Distribution of GluN2A nano-object area in either Healthy+ or SCZ+ NMDAR-Ab conditions (left panel). Comparison of Healthy+ or SCZ+ NMDAR-Ab conditions. Data are expressed as mean ± SEM. Healthy+ = 13.1 ± 0.47 pixels, n = 392 (4 neuronal fields). SCZ+ = 10.7 ± 0.37 pixels, n = 458 (5 neuronal fields). *** $p < 0.001$, Mann-Whitney test.

(D) Comparison of GluN2A-NMDAR nano-object numbers (Nb) and shape factors. Data are expressed as mean ± SEM. Healthy+ Nb = 3.6 ± 0.2, n = 109 nano-objects. SCZ+ Nb = 4.1 ± 0.2, n= 112. Healthy+ shape factor = 0.67 ± 0.01 a.u., n = 368 nano-objects. SCZ+ shape factor = 0.68 ± 0.01 a.u., n = 417 nano-objects. $P > 0.05$ for all comparisons, Mann-Whitney test.

(E) Epifluorescence image of PSD-95 staining and dSTORM image of GluN2A subunit in neurons exposed to Healthy+ or SCZ+ NMDAR-Ab. PSD-95 staining was used to delineate the synaptic area (white dotted line on GluN2A images). PSD-95 scale bar, 400 nm. GluN2A scale bar, 200 nm.

(F) Comparison of synaptic GluN2A-NMDAR nano-object area and number between Healthy+ and SCZ+ NMDAR-Ab conditions. Data are expressed as mean ± SEM. Healthy+ area = 12.8 pixels ± 0.52, n = 301 nano-objects. SCZ+ area = 10.5 pixels ± 0.45, n = 322 nano-objects. Healthy+ Nb = 4.3 ± 0.28, n = 70; SCZ+ = 4.9 ± 0.28, n = 66 nano-objects. ***$p < 0.001$, Mann-Whitney test.

Figure 6

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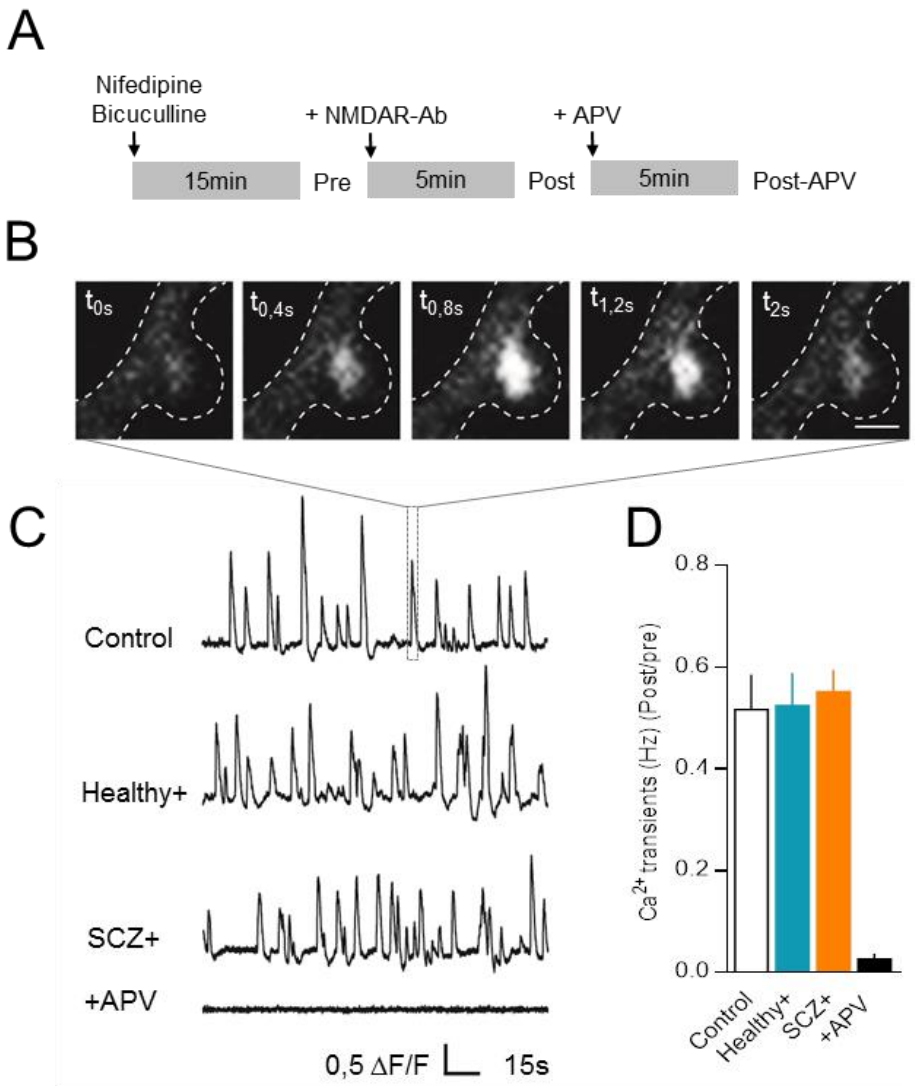

Figure 6. NMDAR-Ab from healthy+ subjects and SCZ+ patients do not affect NMDAR-mediated Ca^{2+} transients in spines of hippocampal neurons

(A) Representative time-lapse images of a spontaneous NMDAR-mediated Ca^{2+} transient in control condition (in presence of nifedipine (5 µM) and bicuculline (5 µM)). Scale bar, 2 µm. *Lower panel*, representative examples of NMDAR-mediated Ca^{2+} transient recorded in spines (expressed as $\Delta F/F$ ratio) exposed to Healthy+ or SCZ+ NMDAR-Ab. Note that all events were abolished by the NMDAR competitive antagonist APV (50 µM).

(B) Comparison of the frequency of spontaneous NMDAR-mediated Ca^{2+} transients between NMDAR-Ab conditions. Data are expressed as mean ± SEM. Control = 0.52 ± 0.07 Hz, n = 38 spines (4 neurons). Healthy+ = 0.52 ± 0.06, n = 68 spines (10 neurons). SCZ+ = 0.55 ± 0.04, n = 131 spines (15 neurons). APV = 0.02 ± 0.01, n = 38 spines (4 neurons).

Figure 7

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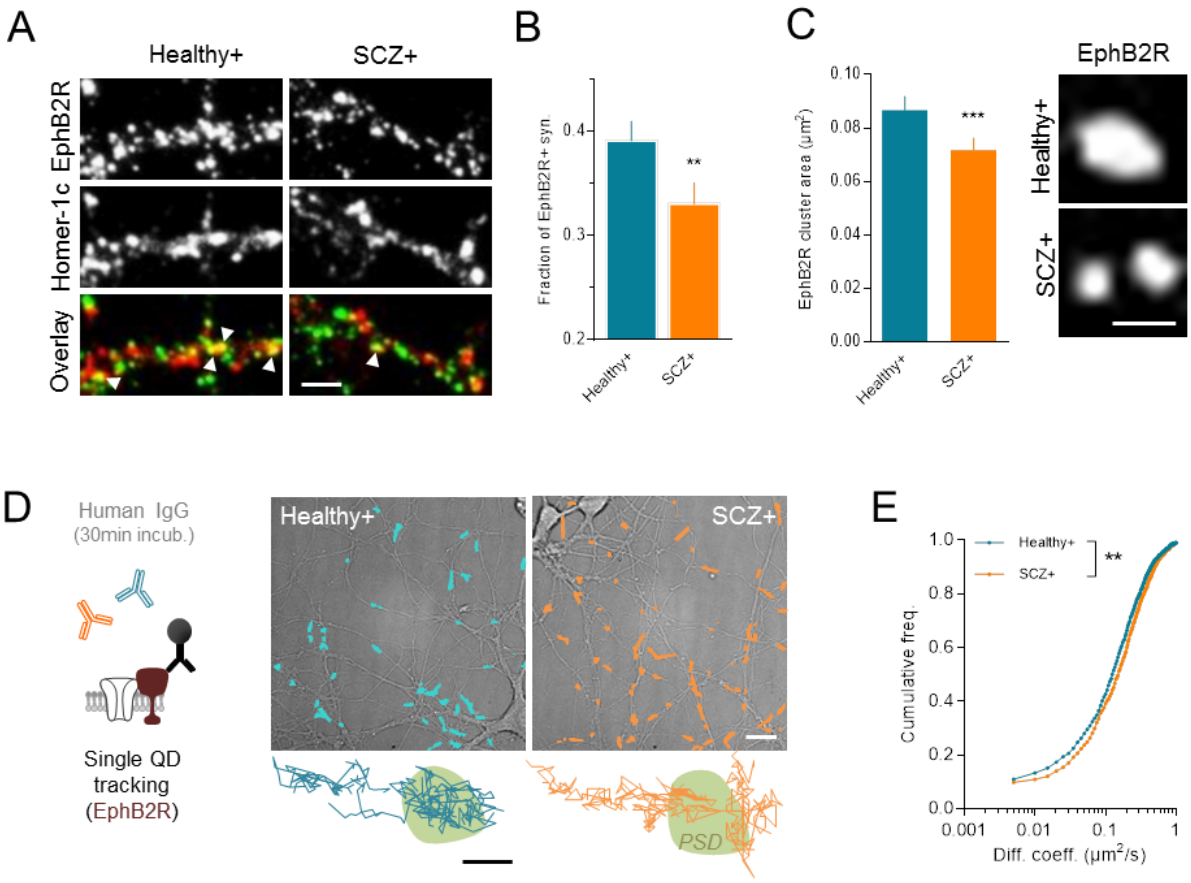

Figure 7. Synaptic EphB2R diffusion and distribution is specifically altered by SCZ+ NMDAR-Ab

(A) Immunostaining of EphB2R and Homer-1c in hippocampal neurons (12 *d.i.v.*) incubated overnight with either Healthy+ or SCZ+ IgG. Colocalized staining was interpreted as synaptic EphB2R clusters (arrow heads). Scale bar, 2 µm.

(B) Fraction of EphB2R-positive synapses after incubation with Healthy+ or SCZ+ NMDAR-Ab. Data were expressed as mean ± SEM. Healthy+ = 0.39 ± 0.019, n = 144 clusters (49 neurons). SCZ+ = 0.33 ± 0.205, n = 113 clusters (42 neurons). **p < 0.01, Mann-Whitney test.

(C) Comparison of the EphB2R cluster area between Healthy+ or SCZ+ NMDAR-Ab conditions. Examples of EphB2R and Homer-1c clusters in presence of either Healthy+ or SCZ+ NMDAR-Ab. Scale bar, 500 nm. Data were expressed as mean ± SEM. *Healthy+* = 0.09 ± 0.005 μm^2, n = 144 dendritic regions. *SCZ+* = 0.07 ± 0.005 μm^2, n = 114 dendritic regions. ***p < 0.001, Mann-Whitney test.

(D) Surface live tracking of endogenous EphB2R in hippocampal neurons (15 *d.i.v.*) exposed to Healthy+ or SCZ+ NMDAR-Ab for 30 min. Scale bar, 20 µm. Representative examples of single trajectories within PSD areas (lower panels). Scale bar, 500 nm.

(E) Cumulative distributions of the instantaneous diffusion coefficient of synaptic EphB2R. In presence of SCZ+ NMDAR-Ab, the distribution was shifted toward the right. **p < 0.01, Kolmogorov-Smirnov test.

Figure 8

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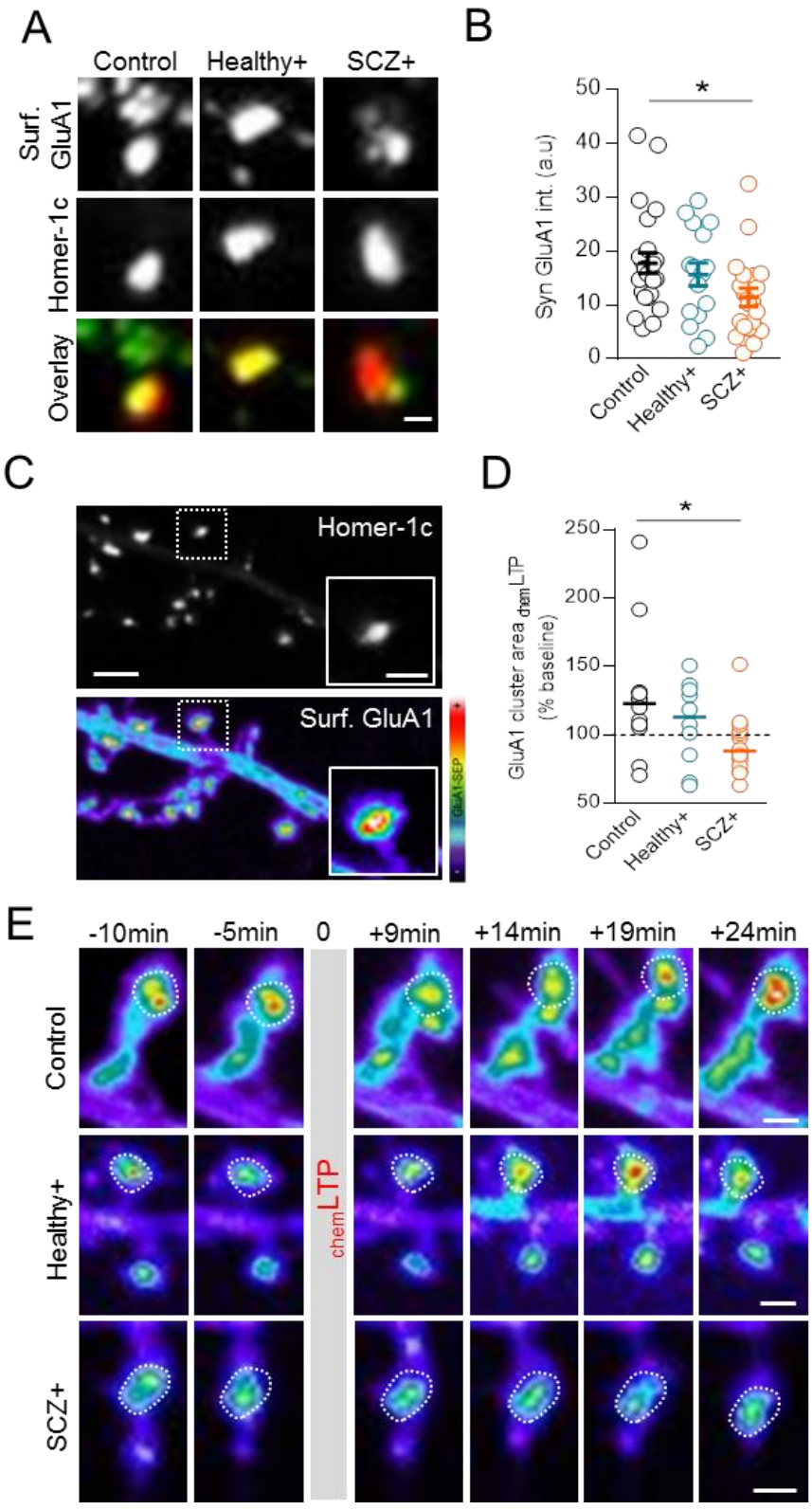

Figure 8. NMDAR-Ab from SCZ+ patients alter basal and activity-dependent recruitment of synaptic AMPAR

(A) Live immunostaining of surface GluA1-SEP clusters in presence of Healthy+ or SCZ+ NMDAR-Ab. GluA1-AMPAR clusters were defined as synaptic when their signal overlap with Homer-1c labeling. Scale bar, 500 nm.

(B) Comparison of the synaptic GluA1-AMPAR fluorescence intensity between Healthy+ or SCZ+ NMDAR-Ab conditions. Each plotted dot represents the mean intensity of synaptic GluA1-AMPAR clusters from a single neuron. Mean ± SEM are also expressed. Control = 18 ± 1.9 a.u., n = 24 neurons. Healthy+ = 16 ± 2.2 a.u., n = 16 neurons. SCZ+ = 11 ± 1.7 a.u., n = 20 neurons. *$p < 0.05$, One-way ANOVA followed by a Newman-Keuls multiple comparison test.

(C) Impact of NMDAR-Ab on chemically-induced long-term potentiation of synaptic AMPA receptor (cLTP). Representative dendritic areas of hippocampal neurons expressing GluA1-SEP and the synaptic marker Homer-1c (top panel). The pseudocolor representation codes for the GluA1-SEP fluorescence intensity levels. Scale bar top panel, 20 µm; scale bar inset, 1 µm. Neurons were first incubated overnight with NMDAR-Ab (5 µg/ml) from either Healthy+ subjects or SCZ+ patients and then challenged with a cLTP protocol. Scale bar, 1 µm.

(D) Comparison of GluA1-AMPAR clusters area between Healthy+ or SCZ+ NMDAR-Ab conditions. Each plotted dot corresponds to a single cell and represents the GluA1 cluster mean area value after cLTP normalized to its respective baseline. Data are expressed as mean ± SEM. Control = 128 ± 13, n = 12 neurons. Healthy+ = 109 ± 9.5, n = 10 neurons. SCZ+ = 92 ± 5, n = 17 neurons. *$p < 0.05$, one-way ANOVA followed by a Newman-Keuls multiple comparison test.

Supplemental table and figure legends

Table S1

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	Healthy	Healthy -	Healthy +	**SCZ**	SCZ-	SCZ+
Number	104	101	3 (2.9%)	48	39	9 (18.7%) (p = 0.01 vs healthy+)
Gender (F)	55.7%	58.4%	100%	21.3%	20.5%	33.3 %
Age	37.42 (13)	36.7 (14.7)	35.6 (3.3)	36.0 (11.5)	36 (12)	34.6 (9.8)
Years of education	13.04 (2.6)			11.39 (2.6)		
Anti-psychotic treatment	NA	NA	NA	35 (74.5%)		
Age at onset of principal disease	NA	NA	NA	24.5 (7.2)		
First episode	NA	NA	NA	10 (24.4%)	12.5%	26.5%
PANSS_tot	NA	NA	NA	68.63 (20)	64.71 (17.4)	84.63 (22.3)
PANSS_pos	NA	NA	NA	15.83 (6.7)	14.6 (5.8)	20.11 (8.2)
PANSS_Neg	NA	NA	NA	18.91 (8.2)	18.29 (8.1)	20.67 (8.8)
PANSS_G	NA	NA	NA	34.07 (9.9)	32.11 (8.6)	43.25 (10.2)
Other Antibodies	ANA 42/101 DNA 7/101 ANCA 8/101 SM 8/101 TPO 11/101	ANA 40/101 (39.6%) DNA 6/101 (5.9%) ANCA 8/101 (7.9%) SM 8/101 (7.9%) TPO 11/101 (10.9%)	ANA 2/3 (66.7%) DNA 1/3 (33.3%) ANCA 0/3 (0%) SM 0/3 (0%) TPO 0/3 (0%)	ANA 20/39 DNA 9/39 ANCA 5/39 SM 5/39 TPO 2/38	ANA 16/39 (41%) DNA 6/39 (15,4%) ANCA 4/39 (10,2%) SM 2/39 (5,1%) TPO 1/38 (2,6%)	ANA 4/9 (44.4%) DNA 3/9 (33.3%) ANCA 1/9 (11.1%) SM 3/9 (33.3%) TPO 1/9 (11.1%)
Infectious screening	HSV1 67/101 CMV 69/101 Toxo 53/101	HSV1 65/101 (64.3%) CMV 67/101 (66.3%) Toxo 53/101 (52.5%)	HSV1 2/3 (66.7%) CMV 2/3 (66.7%) Toxo 0/3 (0%)	HSV1 26/39 CMV 27/39 Toxo 33/39	HSV1 22/39 (56.4%) CMV 20/39 (51.3%) Toxo 27/39 (69.2%)	HSV1 4/9 (44.4%) CMV 7/9 (77.8%) Toxo 6/9 (66.7%)

Table S1. (Related to Figure 1) Demographic and clinical features of the studied population

PANSS_tot = Positive and Negative Syndrome Scale Total Score; PANNS_pos = Positive and Negative Syndrome Scale Positive Score; PANSS_Neg = Positive and Negative Syndrome Scale Negative Score; PANSS_G = Positive and Negative Syndrome Scale General Score.

ANA = anti-nuclear; ANCA = anti-neutrophilic cytoplasmic; CMV = Cytomegalovirus; DNA = anti double-stranded DNA; HSV1 = Herpes simplex virus 1; SM = anti-smooth muscle; TPO = anti-thyroid peroxidase; Toxo = Toxoplasmosis.

Table S2

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	SCZ+ (n = 9)	*SCZ- (n = 39)*	*P value*
	n (%)		
Sex (% men)	6 (67%)	31 (79%)	*0.773*
Acute state (% acute)	7 (78%)	24 (61.5%)	*0.305*
Cannabis (% positive)	2 (22%)	7 (18%)	*0.593*
First Episode	1	9	*0.659*
Season of birth	1	13	*0.453*
	Mean (SD)		
Age	36 (12)	35.6 (12)	*0.978*
BMI	28 (9.9)	24.28 (5.1)	*0.599*
Number of episodes	5.0 (5.6)	4.76 (4.7)	*0.938*
Age at onset of principal dis	24.4 (6.9)	24.52 (7.3)	*0.951*
Panss_tot	87.71 (22.2)	64.71 (17.4)	*0.01*
Panss_pos	21.25 (8)	14.6 (5.8)	*0.026*
Panss_Neg	21.63 (8.9)	18.29 (8.1)	*0.332*
Panss_G	43.85 (10.9)	32.11 (8.6)	*0.0075*
Duration of illness	11.62 (8.2)	11.08 (9.4)	*0.737*

Table S2. (Related to Figure 1) Comparative demographic and clinical features of seropositive versus seronegative schizophrenic patients

BMI = Body Mass Index; PANSS_tot = Positive and Negative Syndrome Scale Total Score; PANNS_pos = Positive and Negative Syndrome Scale Positive Score; PANSS_Neg = Positive and Negative Syndrome Scale Negative Score; PANSS_G = Positive and Negative Syndrome Scale General Score.

Table S3

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Pts	Age/ Gender	Age at onset	PANSS total	Current Treatment	Residual / active symptoms	CSF	EEG	Brain MRI	Other medical history
1	36/M	18	79	Clozapine 500mg/d Valproic acid 2000mg/d Biperidene 4mg/d	Dissociation Cognitive impairment	< 10 cells/mm3 Prot 636 mg/l Elevated Alb ratio	Normal	Nle	None
2	44/M	22	66	Risperidone 2mg/d Cyamemazine 25mg/d Diazepam 5mg/d	Persistant dissociation Cognitive impairment	< 10 cells/mm3 Prot 328 mg/l Nle	Left temporo-occipital theta slow waves with few spike-waves	Nle	Diabetes mellitus Type 2
3	28/M	25	132	Clozapine 500mg/d Levomepromazine 50mg/d Tropatepine 10mg/d	Negative symptoms: blunted affect Cognitive impairment	< 10 cells/mm3 Prot 543mg/l Elevated Alb ratio	Bi-frontal theta slow waves with few spike-waves	Nle	None
4	47/F	30	99	Aripiprazole 10mg/d Tropatepine 10mg/d	Persistantcoenesthetic hallucination, persecutive delusion Dissociation Cognitive impairment	< 10 cells/mm3 Prot 520mg/l OCB +	Normal	Nle	Multiple ovarian cysts
5	21/F	16	63	Clozapine 350 mg/d Tropatepine 10mg/d Propanolol 40 mg/d	Negative symptoms: blunted affect Cognitive impairment	< 10 cells/mm3 Prot 718mg/l Elevated Alb ratio	diffuse sporadic theta slow waves	Nle	Multinodulargoiter Ovarian Sertoli-Leydig tumour Willebrand disease type 1 Multiple ovarian cysts
6	31/M	17	73	NA	NA	NA	NA	NA	NA
7	46/F	38	64	NA	NA	NA	NA	NA	NA
8	24/M	21	87	NA	NA	NA	NA	NA	NA
9	27/M	24	78	NA	NA	NA	NA	NA	NA

ElevatedAlb ratio = Elevated Albumine CSF/serum ratio; OCB = oligoclonal bands; Nle = Normal

Table S3. (Related to Figure 1) Characteristics of schizophrenic patients with serum NMDAR-Ab at the second time of assessment

Elevated Alb ratio = Elevated Albumine CSF/serum ratio; OCB = oligoclonal bands; Nle = Normal.

Figure S1

Jezequel et al.

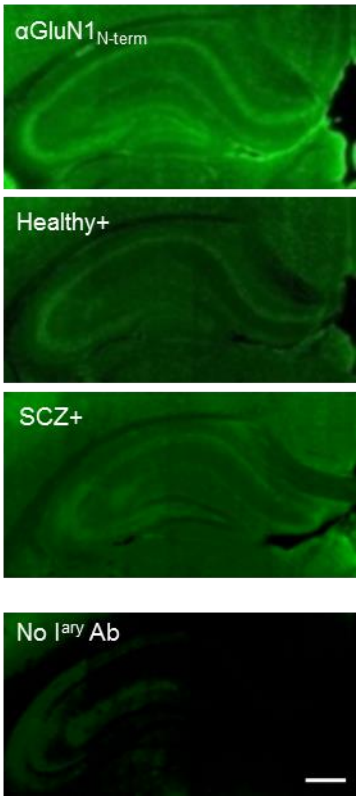

Figure S1. (Related to Figure 1) Detection of NMDAR-like staining with Healthy+ and SCZ+ samples in hippocampal slices

Immunostaining of mice hippocampal slices (postnatal day 22) incubated with a commercial GluN1N-term antibody (αGluN1N-term, 20 µg/ml, 3h), purified IgG from a healthy subject (Healthy+, 20 µg/ml), purified IgG from a schizophrenic patient (SCZ+, 20 µg/ml) or an anti-human Alexa 488 without any primary antibody (no primary Ab). Note the similar staining pattern between all conditions in presence of primary antibodies. Scale bar, 50 µm.

Figure S2

Jezequel et al.

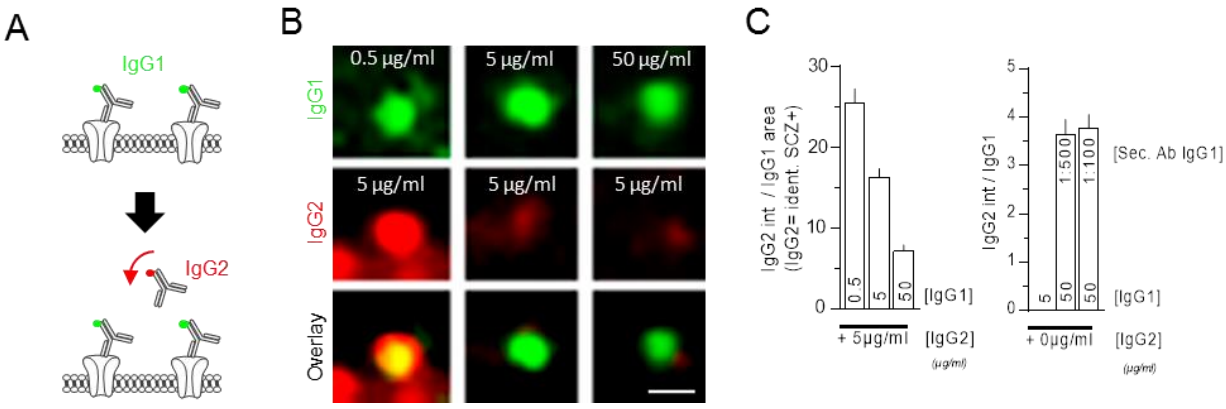

Figure S2. (Related to Figure 2) Optimization of immuno-competition test

(A) Experimental design of the immune-competition assay. Hippocampal neurons were first incubated with SCZ+ NMDAR-Ab (IgG1) labeled in green, followed by a secondary exposure (IgG2, red). The IgG2 was either identical or different from IgG1.

(B) Representative fluorescence staining after incubation with a SCZ+ NMDAR-Ab (IgG1, green) at different concentrations (0.5, 5, or 50 µg/ml, overnight, 4°C) followed by incubation with the same IgG (IgG2 red, 5 µg/ml, overnight, 4°C). Scale bar, 400 nm. Note that IgG1 were first coupled with a secondary anti-human Alexa 488 and then remaining antigen binding sites were blocked using anti-human Fab fragments to reduce unspecific labelling. The decrease in IgG2 staining indicates the lack of binding sites for IgG2.

(C) Quantification of mean fluorescence intensity of IgG2 within IgG1 cluster areas and comparison using different concentrations of IgG1: 0.5 µg/ml (n = 25 cluster fields, 14 neurons), 5 µg/ml (n = 30 cluster fields, 16 neurons) and 50 µg/ml (n = 33 cluster fields, 14 neurons).

(D) A high concentration of IgG1 produces unspecific red staining. The red staining (no IgG2 with 1:500 or 1:100 secondary IgG2) was measured and compared after 5 and 50 µg/ml IgG1 concentrations. Note that 50 µg/ml IgG1 produce an unspecific red staining, irrespective of the IgG2 secondary antibody dilution. Data are expressed as mean fluorescence intensity ± SEM. 5µg/ml IgG1: n = 14 cluster fields, 8 neurons. 50 µg/ml IgG1 / 1:500: n = 20 cluster fields, 8 neurons. 50 µg/ml IgG1 / 1:100: n = 19 cluster fields; n = 8 neurons. The IgG1 concentration of 5µg/ml was then selected as the best concentration for immuno-competition experiments.

Figure S3

Jezequel et al.

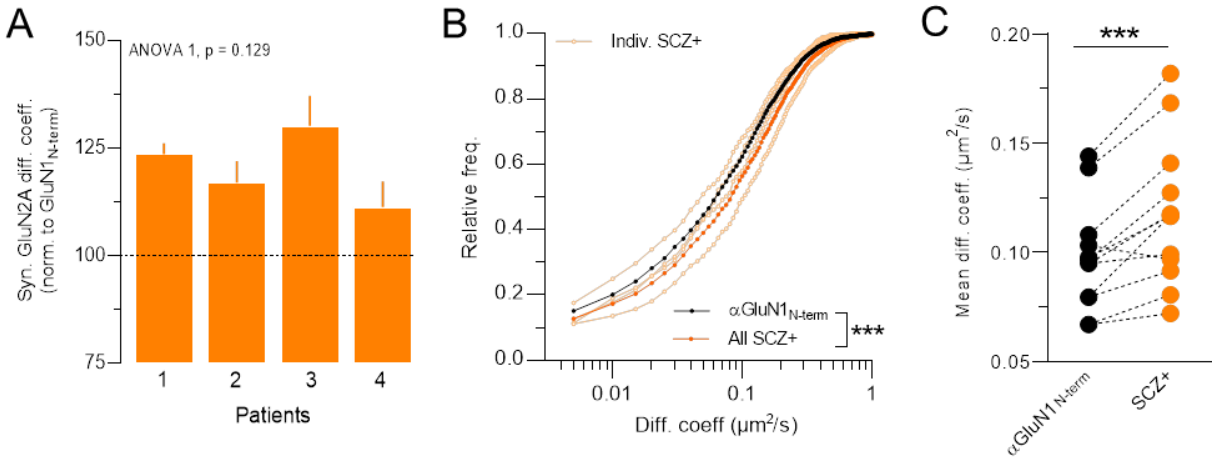

Figure S3. (Related to Figure 3) Intra-variability within the SCZ+ group

(A) NMDAR-Ab from different SCZ+ patients comparably affect synaptic GluN2A-NMDAR instantaneous diffusion coefficient. Data were expressed as the mean percent to αGluN1N-term ± SEM. Patient 1: 124 ± 2.5, n = 2641 trajectories (22 neurons). Patient 2: 117 ± 5.0, n = 928 trajectories (9 neurons). Patient 3: 130 ± 6.9, n = 517 trajectories (9 neurons). Patient 4: 111 ± 5.9, n = 369 trajectories (19 neurons). P = 0.129, One-way ANOVA.

(B) Cumulative distributions of instantaneous diffusion coefficient of synaptic GluN2A-NMDAR exposed to control IgG (αGluN1N-term) or SCZ+ NMDAR-Ab. Three out of 4 SCZ+ IgG induced a right shift in the distribution when compared to the control group. $\alpha GluN1_{N\text{-}term}$, n = 3975 trajectories from 66 neurons. All SCZ+ patients, n = 2959 trajectories from 59 neurons. ***p < 0.001, Kolmogorov-Smirnov test.

(C) Each patient and experiment with SCZ+ IgG increased the instantaneous diffusion coefficient of synaptic GluN2A-NMDAR. Each plotted dot-pair corresponds to the mean diffusion coefficient value. *** p < 0.001 two-tailed paired Student *t*-test. The mean ± SEM of the total values are: αGluN1N-term = 0.098 ± 0.007 $\mu m^2/s$, n= 12 experiments, and SCZ+ = 0.118 ± 0.010 $\mu m^2/s$, n = 12 experiments.

Figure S4

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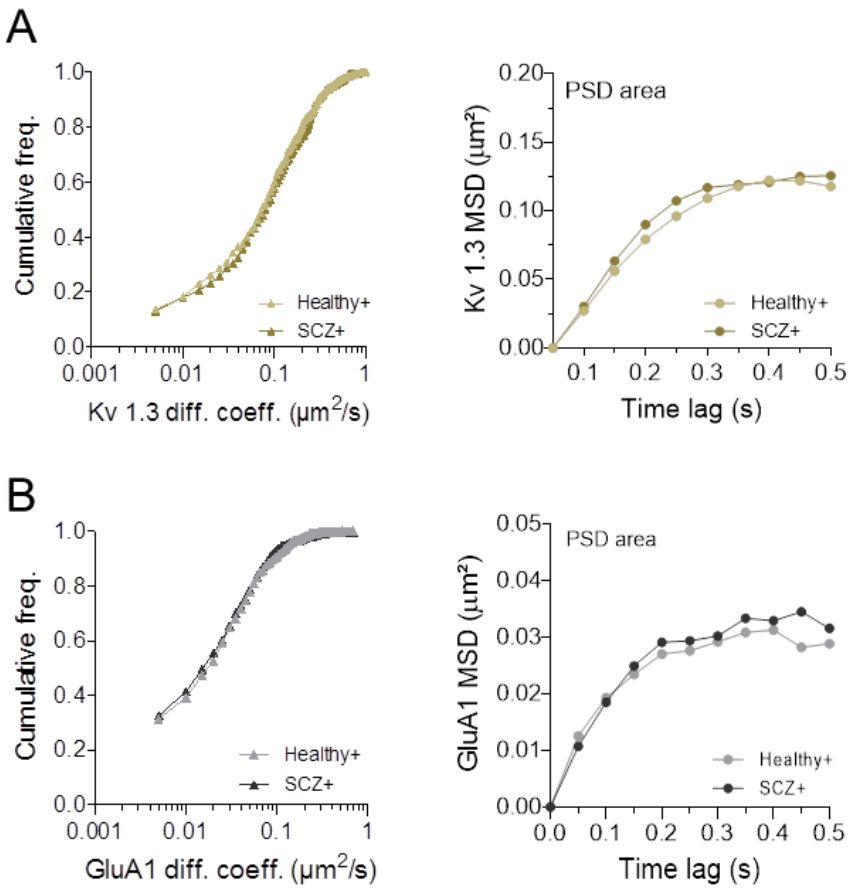

Figure S4. (Related to Figure 3) NMDAR-Ab from schizophrenic and healthy subjects do not alter potassium channel and AMPA receptor dynamics in synapses

(A) Cumulative distributions of the instantaneous diffusion coefficient of synaptic Kv1.3 (Median diffusion coefficient ± 25-75% IQR, Healthy+ = 0.075 µm^2/s, IQR= 0.021-0.166 µm^2/s, n = 580 trajectories from 6 neurons; SCZ+ = 0.084 µm^2/s, IQR= 0.026-0.190 µm^2/s, n = 598 trajectories from 7 neurons; p = 0.311, Mann-Whitney test) and MSD curves (p = 0.996, Kolmogorov-Smirnov test).

(B) Cumulative distributions of the instantaneous diffusion coefficient of synaptic GluA1-AMPAR (Median diffusion coefficient ± 25-75% IQR, Healthy+ = 0.018 µm^2/s, IQR= 0.037–0.049 µm^2/s, n = 699 trajectories from 10 neurons; SCZ+ = 0.015 µm^2/s, IQR= 0.004-0.041 µm^2/s, n = 1616 trajectories from 14 neurons; p = 0.0505, Mann-Whitney test) and MSD curves (p = 0.249, Kolmogorov-Smirnov test).

Figure S5

Jezequel et al.

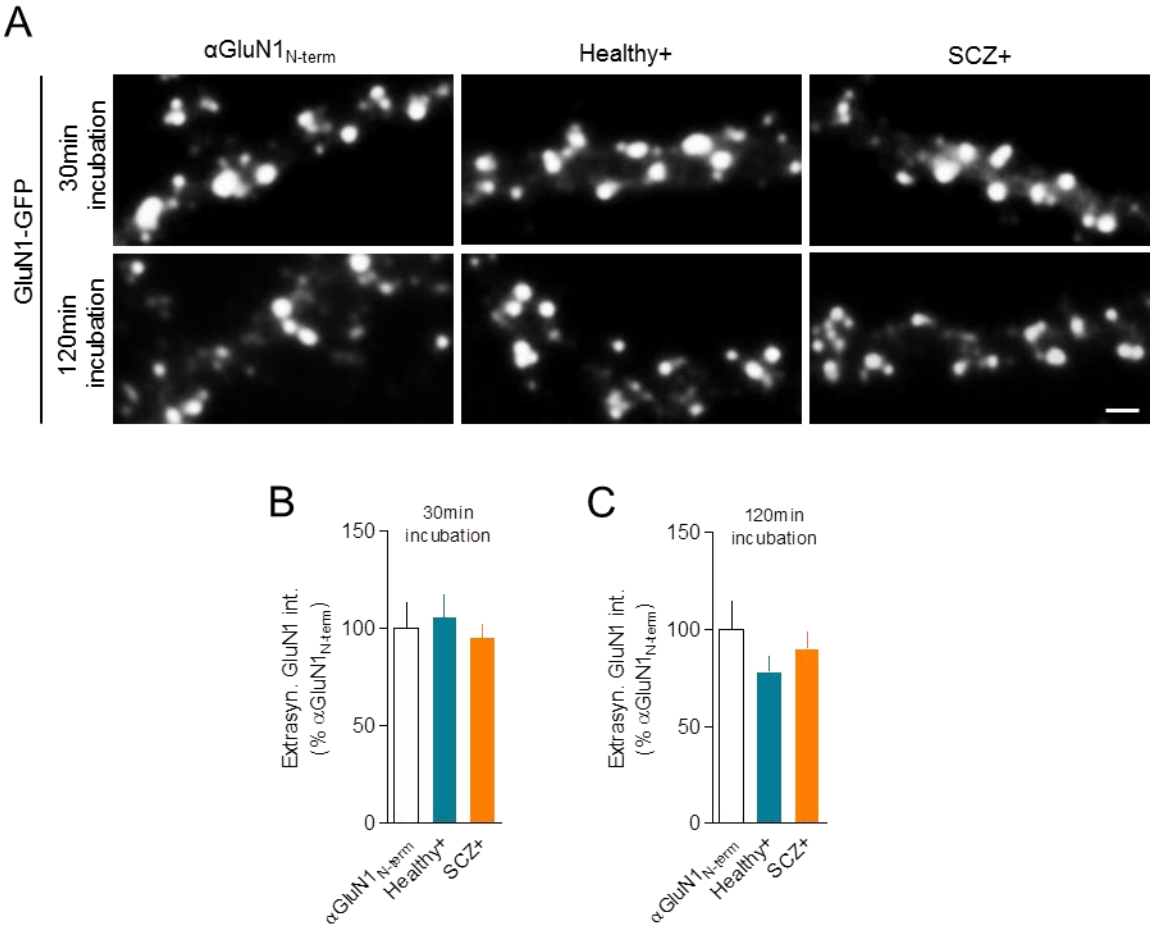

Figure S5. (Related to Figure 5) NMDAR-Ab does not affect NMDAR extrasynaptic population

(A) Surface live staining of GluN1-GFP clusters in hippocampal neurons exposed 30 or 120 min with either a control antibody (αGluN1N-term) or NMDAR-Ab (Healthy+ or SCZ+ NMDAR-Ab). Scale bar, 1 µm.

(B) Comparison of the extrasynaptic GluN1-GFP fluorescence intensity after 30 min incubation with Healthy+ or SCZ+ NMDAR-Ab. For each experiment, data were normalized to the respective control condition ($\alpha GluN1_{N\text{-}term}$) and expressed as mean ± SEM. $\alpha GluN1_{N\text{-}term}$ = 100 ± 13, n = 18 neurons. Healthy+ = 106 ± 12, n = 36 neurons. SCZ+ = 95 ± 6, n = 55 neurons. $P > 0.05$, one-way ANOVA followed by a Newman-Keuls multiple comparison test.

(C) Comparison of the extrasynaptic GluN1-GFP fluorescence intensity after 2h incubation with Healthy+ or SCZ+ NMDAR-Ab. $\alpha GluN1_{N\text{-}term}$= 100 ± 15, n = 23 neurons. Healthy+ = 78.5 ± 8, n = 48 neurons. SCZ+ = 91 ± 8, n = 76 neurons. $P > 0.05$, one-way ANOVA followed by a Newman-Keuls multiple comparison test.

Supplemental Experimental Procedures

Participants

Patients (both in and outpatients) with schizophrenia (n= 48) meeting the DSM-IV criteria (APA, 1994), consecutively consulted/admitted to two university-affiliated psychiatric departments, (Mondor Hospital, Créteil, University of Paris-Est and Fernand Widal Hospital, Paris, University of Diderot, France) were included in the present study after approval by a French ethical committee and written informed consent from the participants. Healthy subjects (n= 104) were enrolled through a clinical investigation center (Center for Biological Resources, Mondor Hospital, Créteil, France). The control group was matched with the schizophrenic sample for age, gender and years of education. Only individuals without a personal or first degree family history of psychotic disorders, affective disorders, addictive or suicidal behavior, as measured by the Family Interview for Genetic Studies (FIGS) and also without a personal or family history of autoimmune diseases (information obtained either from controls/patients or from the first degree relatives or from medical records) were included. Other exclusion criteria were: i) current or past immunosuppressive treatment, ii) recent infection or ongoing inflammatory disease *viz* arthritis, ankylosing spondylitis, Crohn's disease, asthma, systemic lupus erythematous, iii) a positive serology for HIV1/2; Hepatitis A, B and C prior to enrollment, and iv) neurological disorder with cognitive impairment *viz* multiple sclerosis, Parkinson's disease, head injury, cerebrovascular accident, Alzheimer's disease. Five patients (two females and three males) were re-hospitalized for thorough clinical investigation (Suppl. table 3).

Clinical evaluation

Patients were interviewed with the French version of the "Diagnostic Interview for Genetic Studies" (DIGS) for the assessment of lifetime clinical characteristics of schizophrenia as well as for demographic characteristics (*i.e.* number of years of education, working status, season of birth). Current medications as well as hospitalization status were recorded. Manic symptoms were assessed with the Young Mania Rating Scale (YMRS) and depressive symptoms with the Montgomery and Asberg Depression Rating Scale (MADRS) for bipolar disorder and the Calgary Depression Scale for schizophrenia (CDS). Positive and negative symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS). Current smoking status using the Fagerström scale and recent or past alcohol or drug abuse were recorded for all the participants.

Cognitive evaluation

All participants were evaluated for episodic verbal memory using the California Verbal Learning Test (CVLT). Working memory (backward digit span and letter number sequencing) was evaluated using the Wechsler Adult Intelligence Scale III (WAIS). Premorbid IQ was assessed with the National Adult

Reading Test (NART), which estimates premorbid ability level from a word reading test which provides an estimate of vocabulary size.

Collection of blood samples and medical exam

Blood samples were collected from patient and control groups within one week of the clinical assessment. Sera were purified in order to extract IgG isotype antibodies (Manto et al., 2007). Samples were then dialyzed against phosphate-buffered saline and solutions were used at pH 7, 4. Subjects who were seropositive for NMDAR-Ab were contacted for additional medical and biological evaluations. These patients underwent: i) a lumbar puncture to identify the presence of NMDAR-Ab in the cerebrospinal fluid (CSF), measure cell counts, CSF proteins, glucose, and CSF/serum albumin ratio, which is an indicator of blood-brain barrier impairment; ii) a tumor screening (females had a pelvic MRI and males had a thoracic-abdominal-pelvic CT scan (TAP CT scan)); iii) an additional blood test to measure and confirm the presence of NMDAR-Ab as well as antibodies to neurotropic pathogens, such as Toxoplasma Gondii (*T.gondii*), herpes simplex virus I (HSV I) and cytomegalovirus (CMV), and to measure cytokine concentrations; and iv) an electroencephalograph (EEG) to identify spikes indicative of seizures or other abnormalities.

Infectious antibody analysis

The reactivity of immunoglobulin G (IgG) and immunoglobulin M (IgM) classes antibodies to *T.gondii*, HSV I and CMV were measured using solid phase-enzyme microplate immunoassay methods (IBL America, USA). The results were quantified by calculating the ratio of the reactivity of the samples to a standard sample run on each microplate. *T. gondii* IgG seropositivity was defined as a *T. gondii*/IgG ratio ≥ 0.8, equivalent to ≥ 10 international units. The Stanley Laboratory of Developmental Neurovirology (USA) conducted blindly the antibody measurements.

Cell-based assay

Serum samples were tested for the presence of NMDAR-Ab using a cell-based assay on human embryonic kidney cells (HEK293) ectopically expressing both GluN1 and GluN2B-NMDAR subunits, as previously described (Mikasova et al., 2012; Viaccoz et al., 2014). Briefly, HEK293 were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). 24h later, cells were co-transfected (Lipofectamine LTX, Invitrogen) with plasmids coding for GluN1 and GluN2B subunits of the NMDAR. To visualize transfected cells, GluN1 was fused to a GFP (Green Fluorescent Protein). Cells were grown in the presence of NMDAR antagonists (500µM ketamine) for 42h and then incubated for 1h in a saturation buffer (DMEM, 25mM HEPES, 1% BSA, 5% normal goat serum). Live cells were incubated with human's serum (1/10 in saturation buffer) as a primary

antibody for 3h at room temperature, subsequently washed in DMEM-HEPES and fixed in 1% paraformaldehyde (PFA) for 15min. Cells were eventually incubated with a Cy3-conjugated anti-human antibody for 1h. Bound antibodies were visualized using an epifluorescence microscope (Axiophot, Zeiss). The observed labeling was scored from 0 to 4 by three independent blinded observers, the final score representing the median of the scores from 3 independent assays. Samples were considered as positive when the final score exceeded a threshold of 2-3.

Immuno-absorption

HEK293 were prepared as described above (see "Cell-based assay" paragraph). 24h after plating (150 000 cells/ml), cells were transfected with GluN1-SEP and GluN2B (Lipofectamine LTX, Invitrogen). After 4h incubation with the DNA mix, medium was replaced by fresh, equilibrated and heated supplemented DMEM medium. The following day, either transfected or non-transfected HEK cells were incubated for 1h with NMDAR-Ab (5µg/ml). This step was repeated 6 times and the resulting absorbed fraction was kept at 4°C (maximum 24h) for further live experiments.

Primary cell culture and protein expression

Cultures of hippocampal neurons were prepared from E18 Sprague-Dawley rats. Cells were plated at a density of 50 x 10^3 cells per ml on poly-lysine pre-coated coverslips. Coverslips were maintained in a 3% horse serum containing Neurobasal medium (Invitrogen). After a few days *in vitro* (*div*), the original plating medium was replaced by a serum-free medium. Cultures were maintained at 37°C in 5% CO_2 for 15*div* at maximum. For exogenous protein expression, 7-10*div* hippocampal cultured neurons were transfected at least 48h before each experiment using either the Effectene (Qiagen) or phosphate calcium transfection (Jiang et al. 2006).

Immunochemistry

Immunohistochemistry:

The pattern staining obtained with human IgG was assessed using immunohistochemistry on hippocampal slices. To do so, mice (P22) were perfused with 4% PFA. Coronal sections of 50µm were obtained on a vibratome (Leica) and incubated overnight at 4°C with either a polyclonal antibody against the N-terminal part of the GluN1 subunit ($\alpha GluN1_{N\text{-}term}$ Alomone Labs, 20µg/ml) or purified IgG from healthy (Healthy+, 20µg/ml) and schizophrenic (SCZ+, 20µg/ml) subjects. Fluorescent revelation was carried out with secondary anti-rabbit or anti-human Alexa 488 antibodies (Life Technologies, 1/1000) for 2h at room temperature. Images were obtained using a Nanozoomer and a confocal microscope (SP8, Leica).

Immunocytochemistry:

The pattern staining obtained with human IgG was also assessed in dissociated cells. Purified IgG were incubated as primary antibodies (5µg/ml) on fixed neurons (4% PFA, 15min, 10div). Cells were then carefully washed and incubated with a secondary anti-human Alexa 488 antibody (Life Technologies, 1/500, 30min).

To assess purified IgG's effect on NMDAR and EphB2R surface content, live neurons were incubated with either commercial or human IgG (overnight, 37°C). Surface exogenous GluN1-SEP receptors were then specifically stained using a monoclonal antibody against GFP (Roche, 1/500, 15min, 37°C) and Alexa 488-conjugated anti-mouse secondary antibody (Invitrogen, 1/500, 45min). Surface endogenous EphB2R were labeled with a polyclonal antibody against the extracellular part of EphrinB2R (R&D, 1/200, 3h, 37°C) and a secondary anti-goat Alexa 488 antibody (Invitrogen, 1/500, 30min). In order to label the post-synaptic density, neurons were fixed (4% PFA, 15min), permeabilized with Triton-BSA 1% (5min) and successively incubated with an anti-Homer-1c antibody (Synaptic systems, 1/500, 30min) and a secondary anti-guinea pig Alexa 594 antibody (Jackson ImmunoResearch, 1/500, 30min).

All imaging sessions were done on a video spinning-disk system (Leica DMI6000B, 63X) and quantification analysis was performed using MetaMorph software (Molecular Devices) and ImageJ (NIH).

Immunocompetition

Brain tissue:

Snap frozen, non-perfused 7µm-thick sagittal sections of Sprague-Dawley rat brains were used for this experiment. Sections of tissue were fixed (4% PFA, 15min), washed in PBS, and incubated with undiluted serum from patients with schizophrenia, anti-NMDAR encephalitis, or blood donors overnight at 4°C. Sections were then extensively washed with cold PBS and incubated for 1h at 4°C with biotinylated IgG from a representative patient with anti-NMDAR antibodies. After washing, the binding of biotinylated IgG was demonstrated with a standard avidin-biotin-peroxidase method (Vectastain ABC kit Elite, PK-6100, Vector). Slides were then mildly counterstained with hematoxylin, mounted, and results photographed with a digital camera (AxioCam MRc) adapted to a confocal microscope (Zeiss LSM710).

Hippocampal neurons:

After fixation (4% PFA, 15min), hippocampal cultures (12*div*) were treated with a blocking solution (PBS-BSA 1%, 30min) and successively incubated with SCZ+ NMDAR-Ab (purified IgG 5µg/ml, overnight, 4°C) and a secondary anti-human Alexa 488 antibody (Invitrogen, 1/500, 1h). Remaining antigen binding sites were blocked using anti-human Fab fragments (Jackson ImmunoResearch, 100µg/ml, 1h). Cells were then incubated with competing IgG from either SCZ+, Healthy+, Encep+ individuals (purified IgG 5µg/ml, overnight, 4°C), and bound antibodies were eventually stained with a secondary anti-

human Alexa 568 antibody (Invitrogen, 1/500, 1h). All imaging sessions were done on a video spinning-disk system (Leica DMI6000B, 63X) and quantification analysis was performed using ImageJ (NIH).

Direct stochastic optical reconstruction microscopy (dSTORM)

Live neurons were incubated with either Healthy+ or SCZ+ NMDAR-Ab for 2h at 37°C. Surface endogenous GluN2A-containing NMDAR were then specifically stained using an anti-GluN2A antibody (Agrobio, 0.1mg/ml, 15min). After fixation (4% PFA, 15min), neurons were permeabilized with 0.4% Triton X-100 (5min) and treated with a blocking solution containing 1.5% BSA/0.1% fish gel/0.1% Triton X-100 for 40min. Cells were then successively incubated with an anti-PSD-95 antibody (Thermo Scientific, 1/500, 45min) and secondary anti-rabbit Alexa 647 (Invitrogen, 0.1mg/ml, 30min) and anti-mouse Alexa 532 (Invitrogen, 0.1mg/ml, 30min) antibodies. A second fixation was performed after incubation with the secondary antibodies.

All imaging sessions were performed using a Leica SR GSD 3D microscope (Leica HC PL APO 160x 1.43 NA oil immersion TIRF objective) and an ANDOR EMCCD iXon camera. Samples were illuminated in TIRF mode and images were obtained with an exposure time of 10.85ms with up to 100,000 consecutive frames. Imaging was carried out at room temperature in a closed Ludin chamber (Life Imaging Services) using pH-adjusted extracellular solution containing oxygen scavengers and reducing agents. Image acquisition was controlled by the Leica LAS software. An initial high power 642nm laser was used to convert the fluorescence into the dark state in order to reach a desired density for single molecule detection. During acquisition, the 642nm laser power was kept at a constant low level while the 405nm laser power was adjusted to keep an optimal level of stochastically activated molecules per frame. Localization of single molecules and reconstruction of the super resolved image was performed by applying a fitting algorithm determining the centroid-coordinates of a single molecule and fitting the point-spread-function (PSF) of a distinct diffraction limited event to a Gaussian function. The final achieved spatial resolution was 40nm. Multicolor fluorescent microbeads (Tetraspeck, Invitrogen) were used as fiduciary markers to correct for lateral drifts.

GluN2A or PSD-95 clusters were identified on the respective epifluorescence images. Structures with a higher intensity than a respective value were identified as clusters. GluN2A nano-objects area and shape were quantified after segmentation of GluN2A dSTORM images (MetaMorph software, Molecular Devices). Morphological features, such as surface area, length and shape of each segmented structure, were exported to calculate their respective distributions. The dimensions were computed by 2D anisotropic Gaussian fitting, from which the principal and the auxiliary axes were extracted as 2.3σ long and 2.3σ short, respectively. The shape factor was calculated as a ratio between the auxiliary and the principal axes. The epifluorescence image of PSD-95 was superimposed on the GluN2A dSTORM image to identify the synaptic nano-objects.

Quantum dot (QD) tracking and surface diffusion calculation

QD labeling and microscopy were performed as previously described (Mikasova et al., 2012). Briefly, neurons were incubated (10min, 37°C) with primary antibodies against the desired target (see table below). Neurons were then washed and incubated for 10min with QDs. Non-specific binding was blocked by adding 1% BSA (Vector Laboratories) to the QD solution. Green Mitotracker (Life Technologies, 1/2000) was used as an endogenous synaptic marker. QDs were detected by using a mercury lamp, appropriate excitation/emission filters and an EM-CCD camera (Evolve, Photometrics). Images were obtained with an acquisition time of 50ms with up to 500 consecutive frames. QDs were followed on randomly selected dendritic regions for up to 20min. Recording sessions were processed with the Metamorph software (Universal Imaging Corp). The instantaneous diffusion coefficient, D, was calculated for each trajectory, from linear fits of the first 4 points of the mean square displacement versus time function using *MSD(t)= <r2>(t)= 4Dt.*

Targeted receptor	Primary Ab characteristics	Final concentration	QD characteristics	Final QD dilution
Human IgG's target	Healthy-, Healthy+, SCZ+ human	5µg/ml	655 rabbit F(ab')2 anti-human IgG	1/20 000
GluN2A-NMDAR	Alomone Labs rabbit	1/500 (1,6µg/ml)	655 goat F(ab')2 anti-rabbit IgG	1/10 000
EphrinB2R	R&D systems goat	1/200 (1µg/ml)	655 rabbit F(ab')2 anti-goat IgG	1/2000
GluA1-SEP	Invitrogen mouse	1/500 (0,4µg/ml)	655 goat F(ab')2 anti-mouse IgG	1/20 000
Kv1.3	Alomone Labs rabbit	1/200 (4µg/ml)	655 goat F(ab')2 anti-rabbit IgG	1/10 000

Calcium imaging

Dissociated neurons transfected with $GCaMP_3$ at 10div were transferred into a Tyrode solution containing (in mM): 110 NaCl, 5 KCl, 25 HEPES, 15 D-glucose, 2 $CaCl_2$ and 2 $MgCl_2$ (Sigma Aldrich) at 12-13div. 15min before imaging, cells were transferred to a Mg^{2+}-free Tyrode solution with 5µM Nifedipine (Tocris), and 5µM Bicuculline (Tocris). Time-lapse images were acquired at 20Hz. Three time-lapse movies (3000 frames) were successively recorded: 1) "Pre" (baseline period), 2) "Post" (5min after bath application of purified IgG, 5µg/ml) and 3) "APV" (5min after bath application, 50µM). Time-lapse movies were concatenated and realigned in ImageJ (PoorMan3DReg plugin, Michael Liebling). Fluorescence from calcium transients *vs.* time was measured within individual ROIs manually defined by the experimenter (ImageJ). All pixels within each ROI were averaged to give a single value time course associated to the ROI. Mean normalized fluorescence (ΔF/F) was calculated by subtracting each value with the mean of the previous 5s values lower than P_{50} (µ) and dividing the result by µ. Positive calcium transients were identified following a two-step procedure: initially, ΔF/F traces were smoothen by convoluting the raw signal with a 10s squared kernel. True positives (with

minimum 1s between transients) were then defined on an automated basis using custom-written MATLAB routines where the threshold was set at 5*SD of APV's average trace.

Chemically induced potentiation ($_{chem}$LTP)

Live hippocampal neurons transfected with GluA1-SEP were incubated overnight with human IgG (Healthy+ or SCZ+, 5µg/ml, 37°C). After washing thoroughly, chemically induced long-term potentiation ($_{chem}$LTP) was elicited by a bath co-application of glycine (200µM) and picrotoxin (5µM) for 4min (Dupuis et al., 2014). $_{chem}$LTP was always applied after a period of baseline acquisition and the medium was carefully replaced by fresh equilibrated and heated medium after induction. GluA1-SEP fluorescence signal was then recorded every 5min during the 30min following the stimulus. Synapses were defined using the synaptic protein Homer-1c DsRed. Homer-1c clusters were outlined and GluA1-SEP intensity was measured over time within the synaptic areas. Synaptic GluA1-SEP clusters intensity and area values were normalized to the baseline values. All images were collected on a video spinning-disk system (Leica DMI6000B, 63X) and a CoolSNAP HQ2 camera (Photometrics).

Statistical analysis

Univariate analyses were conducted to determine if there were significant differences between patients with schizophrenia and controls, as well as between seropositive and seronegative schizophrenic patients with respect to demographic, clinical, and biological characteristics. For categorical variables, χ^2 test and Fisher's exact test were used while Student's t-test and Mann-Whitney U test were used for continuous measures. Comparisons between groups were performed using parametric statistical tests, Student t-test (pair comparison) or ANOVA followed by a Newman-Keuls Multiple Comparison Test (group comparison). Comparisons between groups for instantaneous diffusion coefficients and calcium imaging were performed using Mann-Whitney test (pair comparison), Kruskal-Wallis followed by a Dunn's Multiple Comparison Test (group comparison). For distributions comparison a Kolmogorov-Smirnov test was used. Significance levels were defined as *p<0.05, **p<0.01, ***p<0.001. Statistical analysis was performed using STATA, release 12 (StataCorp. 2011. Stata Statistical Software: Release 12.College Station, TX: StataCorp LP) and Prism (GraphPad).

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Intermediate discussion

Source and brain access to NMDAR-Ab

In this work, we demonstrated *in vitro* the pathogenic potential of NMDAR-Ab isolated from the serum of schizophrenic patients. But how do NMDAR-Ab get into the central nervous system? Robust intrathecal antibody synthesis usually accompanies NMDAR encephalitis (Gresa-Arribas et al. 2013), even if a recent study demonstrated that GluN1 antibody-producing cells are relatively rare in the CSF, making up only 6% of antibody-secreting cells/memory B cells (Kreye et al. 2016). Contrary to classical NMDAR encephalitis, NMDAR-Ab are, except in very rare occasions (Steiner et al. 2013) never detected in the CSF of patients with schizophrenia, rather supporting the hypothesis of an altered BBB which would help systemic autoantibodies penetration within the brain tissue (Martinez-Martinez et al. 2013). NMDAR-Ab have been shown to cause NMDAR endocytosis in genetically-modified APOE4 mice associated with BBB leakage (Hammer et al. 2014). Several groups have used the ratio of CSF/serum albumin as a measure of the permeability of the BBB and have found increased permeability in 15–30% of people with schizophrenia (Amanda L. Jones et al. 2005). Consistently, in our study, 3 of the seropositive schizophrenic patients had elevated albumin CSF/ratio and one oligoclonal bands. NMDAR-Ab may also penetrate the brain parenchyma with unaltered BBB. It is a common misconception that the BBB is completely impermeable to antibodies, but in fact, antibodies cross to a limited extent the human BBB. The mechanisms by which antibodies pass the BBB under normal conditions are still poorly understood, but since NMDAR are present at the surface of epithelial barrier cells, they can favor receptor-antibody mediated transcytosis as shown for the neonatal Fc receptor (Brimberg et al. 2015). We tested this possibility by injecting intravenously NMDAR-Ab from a schizophrenic patient coupled to fluorescent nanoparticles (quantum dots, QD). Quantum dots are very bright nanoparticles which allow the detection of low concentrations of antibody into brain tissue. On the contrary, classical immunofluorescence approaches are far below the detection threshold necessary to image such molecules in tissue. This

qualitative experiment provided preliminary evidence that human NMDAR-Ab/QD complexes can cross the blood brain barrier and access to the brain without any volontary manipulation of BBB integrity. Future investigations will be needed to quantify the penetration of these complexes, comparing in particular NMDAR-Ab from patients and healthy subjects. In addition, it has long been documented that schizophrenic patients have history of infections, which may transiently induce breaches in the BBB and favour antibody infiltration into the CNS (Hammer et al., 2013; van Os et al., 2010). Noteworthy, all the schizophrenic patients included in our study with NMDAR-Ab were previously infected by Herpes simplex type1 virus (HSV1), cytomegalovirus or toxoplasma. It is now well known that HSV1 can be a trigger of NMDAR-Ab production (Armangue et al., 2014). However, there is no clear evidence of T-cell infiltration of the CNS or disruption of the BBB in schizophrenia (Amanda L Jones et al. 2005). The presence of a tumor that expresses NMDAR could also contribute to breaking immune tolerance. Systematic search for an underlying teratoma or at least, systematic report of existing tumors, has not been conducted in the majority of studies in patients with schizophrenia. It is thus impossible to evaluate the frequency of paraneoplastic cases of schizophrenia.

NMDAR-Ab epitopes

Using a classical immuno-competition assay, we tested whether NMDAR-Ab from a schizophrenic patient share the same epitope(s) than another patient with schizophrenia, NMDAR encephalitis or even a healthy control. Immuno-competition assay shows several limitations, intrinsic to any immunofluorescence technique and the subsequent use of antibodies. First, we cannot exclude that we overestimated the staining of the competing antibody (IgG 2), as saturation of all binding sites might not be full after the first antibody incubation step. To overcome this limitation, we added a saturating step using a human Fab fragment that binds to any free binding site, not already occupied by IgG 1. We performed control experiments in order to use the optimal and most saturating concentration of Fab fragment but even with this precaution, it is possible that some binding sites

were left free and thus accessible to the competing IgG2. We also tested whether the concentration of the secondary antibody (carrying the fluorophore for IgG1) could impact the binding of the competing antibody IgG2. We did not observe any difference between the dilutions we chose. Second, immuno-competition relies on competition between 2 comparable entities, *i.e.* comparable antibodies concentration and affinity. For the latter, we did not have access to IgG affinities and could not include this parameter to our conclusion. Regarding IgG concentrations, we do not control the pure amount of NMDAR-IgG present in our purified samples. Still, end-point dilutions provide us a good estimation of the concentration of IgG targeting NMDAR, even though this is a semi-quantitative measurement. To overcome these limitations (antibody concentration and affinity), the first antibody (IgG1) was not always incubated first, so that in some experiments IgG1 became IgG2, and the other way around. This way, we believe that we attenuated these potential heterogeneity factors so that they do not constitute limiting steps to our assay. Finally, we cannot exclude the possibility that fixation could modify antibody binding which is a dynamic process. Despite all these limitations that we are fully aware of, such an immuno-competition assay provided us first indications that NMDAR-Ab most likely bind to multiple epitopes. In addition, the immuno-competition assay on hippocampal slices was performed in another lab (Dalmau' group), with another protocol and still confirmed our observations on dissociated cultures.

Relevance of NMDAR-Ab in the etiology of schizophrenia

The pathogenic role of antibodies can be established using several criteria *in vitro* and *in vivo*, also called Kitebsky's criteria (Rose & Bona 1993; Moscato et al. 2010; Coutinho et al. 2014). Using a combination of classical immunostaining and high-resolution imaging techniques, we aimed at demonstrating that NMDAR-Ab from schizophrenic patients met most of the following criteria:

i) *Antibodies should have access to and bind extracellular antigenic epitopes in living cells and/or tissues.* The first and crucial step of this work was to identify the antigenic target of these circulating antibodies. To do so, live hippocampal slices and dissociated neurons were exposed to both healthy

and patients sera. Serum reactivity was observed in both conditions, and the resulting staining was comparable to the one obtained when using a commercial antibody against GluN1-NMDAR. We took advantage of the single particle tracking technique to confirm the nature of the target. By coupling quantum dots to the antigen-antibody complex most likely recognize NMDAR.

ii) *Antibodies recognize a particular antigen when it is overexpressed in heterologous cells*. Using a classical CBA, we detected the binding of NMDAR-Ab in live HEK cells ectopically expressing surface GluN1/2B heteromers. This binding was later duplicated in live hippocampal neurons overexpressing GluN1-NMDAR.

iii) *Antibodies cause structural and⁄or functional alterations of the target antigen.* We showed for the first time that NMDAR-Ab from SCZ patients rapidly alter NMDAR surface trafficking, and so does EphB2R, one of its main synaptic retention partner. This primary alteration, although it looks minimal, actually triggers a domino effect with functional consequences. Antibodies most likely mediate their effect through the loss of NMDAR synaptic pool rather than by a direct modulation of the receptor, as no antagonist-like effect was measured. Interestingly, none of these effects were observed in presence of NMDAR-Ab from healthy subjects, suggesting that all NMDAR-Ab do not share pathogenic characteristics.

iv) *The clinical syndrome should mirror some or all of the phenotypes of pharmacological or genetic manipulation of the antigen*. NMDAR-Ab have been detected in patients with schizophrenia. There is compelling evidence showing that NMDAR dysfunction leads to expression of schizophrenia. NMDAR antagonists like ketamine or PCP are able to induce psychotic symptoms in healthy individuals and to worsen existing symptoms in patients (Krystal 1994; Lahti 1995). Such observations have been reproduced in the animal using either pharmacological tools like NMDAR blockers (Manahan-Vaughan 2008; Newcomer 1999), anti-mitotic agents (MAM model, see Lodge and Grace 2009) or even genetic manipulations (Belforte 2010).

v) *Passive transfer of disease-specific antibodies to animals should reproduce the effects of the antibodies on the antigen as well as the clinical features of the disorder*. To our knowledge, there is

no animal model showing that NMDAR-Ab from SCZ patients can cause behavioral deficits. Like previously done for NMDAR encephalitis (Planagumà et al. 2015; Planagumà et al. 2016), it would be of great interest to inject NMDAR-Ab from schizophrenic patients (and other sources) either in the periphery or directly into the brain, and observe whether a schizophrenia-like phenotype emerges in the animal. More interestingly, we could imagine tagging antibodies with a potent fluorescent probe in order to follow their journey into the body and unravel the mechanisms involved to reach the CNS.

vi) *Antibody-mediated cellular and synaptic alterations, and clinical symptoms, should improve as antibody titer is reduced.* NMDAR-Ab from schizophrenic patients, but not healthy subjects, trigger many cellular and synaptic alterations. Using a classical immunoabsorption protocol, we assessed the effect of IgG depletion in a functional assay. Purified IgG were incubated on HEK cells expressing NMDAR, and the remaining depleted fraction was incubated on dissociated neurons. Doing so, NMDAR surface trafficking remained unchanged while it was still increased after absorption on non-transfected HEK cells. We showed that this effect is dose-dependent as high dilutions of NMDAR-Ab no longer affect NMDAR surface trafficking. By contrast, no matter the concentration, NMDAR-Ab from healthy individuals equally impacted NMDAR surface trafficking.

Altogether, we collected a series of evidence demonstrating that NMDAR-Ab do not represent a unique entity. They most likely target multiple epitopes and can induce distinct molecular impacts.

Detection of anti-NMDA receptor autoantibody in first episode psychosis patients from the OPTiMiSE project

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The detection of autoantibodies against neurotransmitter receptors in patients with neurological and psychiatric disorders has raised hopes for a better understanding of the molecular cascades underlying these pathologies and for treating appropriate patients with immunotherapy[1]. The link between psychotic disorders and autoimmunity is, in fact, an old concept that has regained strong support partly thanks to the discovery of anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis[2]. In this disorder, autoantibodies directed against the GluN1 subunit of NMDAR (NMDAR-Ab) are strongly associated with psychotic symptoms, sometimes catatonia, and profound reversible neurologic deterioration[2]. In patients with schizophrenia, the presence of circulating NMDAR-Ab is, however, highly debated, with detection prevalences ranging from 0 to less than 20% (reviewed in[3]). These discrepancies may reflect both different recruitment procedures and different sensitivities and specificities between the NMDAR antibody assays used[4]. In addition, most assays depend on demonstrating the presence of antibodies binding to the NMDAR, and do not address whether the antibodies affect the expression, localization or function of the NMDAR. We used three different European laboratories and an innovative single nanoparticle-imaging approach to test a large cohort of untreated patients with first episode psychosis.

Methods. Two hundred and 98 untreated first episode psychotic patients from the OPTIMISE cohort were examined (Table 1). We first used a cell-based assay (CBA) on human embryonic kidney cells (HEK293) expressing GluN1 and GluN2B subunits, as previously described[5]. The live cells were incubated with patients' sera (1/10 in saturation buffer), and bound antibodies were visualized using a fluorescence microscope in Lab A (Lyon). A similar live CBA was also performed on all positive samples in Lab B (Oxford), and compared with a CBA in Lab C (King's College, London). Finally, CBA was also performed on IgG purified from the positive sera (Lyon) (Figure 1A).

We previously demonstrated that antibodies directed against extracellular epitope of NMDAR have the capacity to alter their surface diffusion[5,6]. To determine whether the patient's IgG behaved similarly, we performed single nanoparticle tracking of the patients' IgG on live cultured hippocampal neurons

(see[5] for details). In this assay, the behaviour of an individual receptor, detected by antibody, is independent of the antibody concentration. Seropositive patients were examined by coupling purified human IgG to single nanoparticle (Quantum Dots, QD) and by imaging the behaviour of the autoantibody/membrane target(s) at video rate. QD complex surface dynamics parameters were calculated as previously described[5]. The surface dynamics of patient's IgG-QD was compared to that of endogenous GluN1-NMDAR (commercial anti-GluN1 subunit directed against an extracellular epitope). To ascertain further the identity of NMDAR-Ab in the seropositive patients, the diffusion coefficient of endogenous synaptic NMDAR was measured by tracking GluN2A-NMDAR in glutamate synapses (identified by the over-expression of Ds-Red Homer-1c) exposed to purified IgG.

Results. Of the 298 tested sera from untreated first episode psychotic patients, 14 bound to NMDAR colocalizing with GFP-positive HEK cells in the CBA (Figure 1B) in Lab A, with three only binding weakly. Independent evaluation in Lab B of these samples using a similar CBA found 9 were positive, and considered the remaining 5 below the threshold for positivity. In other respects, there was a reasonable correlation between the scores from the two labs (Fig 1C). Of concern, however, only 2 sera were rated positive when using the commercial CBA in Lab C. Purified IgG from 8/14 patients were positive on live CBA, corresponding to the highest serum scores.

To confirm that these samples contained NMDAR-Abs we first compared the surface diffusion of their membrane targets in live neurons using single QD tracking (Figure 1D), providing the live "signature" of the IgG membrane target. The mean square displacement (MSD) curve, an indicator of the surface explored by a given membrane receptor, and the instantaneous diffusion coefficients were calculated for all conditions and compared. Both the MSD curves and the instantaneous diffusion coefficients from the positive and weakly positive patients were not distinguishable from one another, and also not different to those of endogenous GluN1-NMDAR (Figure 1E, F). In addition, synaptic NMDARs (tracking of GluN2A subunit) were destabilized, i.e. increased dynamics, when neurons were exposed to purified IgGs from positive patients as compared to the healthy seronegative donor IgG condition (Figure 1G),

consistent with the previously-described effect of antibody directed against extracellular epitope of the NMDAR[5,6].

Discussion. In this research study, less than 5% of first episode psychotic patients were seropositive for autoantibodies against the NMDAR. Nevertheless, by taking advantage of the single molecule imaging, we were able to demonstrate that these NMDAR-Abs are not only present but have effects on NMDAR organization that could be pathogenically relevant *in vivo*. The single molecule imaging approach relies on individual antibody binding to their target and is therefore independent of the number of antibodies in the samples. Thus this test is suitable even for samples with low antibody levels (weak positives). Key challenges for the future will then to investigate whether the circulating NMDAR-Ab, present in a subgroup of psychotic patients, may play a direct pathological role in a particular subtype of psychosis, as only demonstrated so far in patients with anti-NMDAR encephalitis.

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Conflict of Interest Disclosures: AV and the University of Oxford hold patents for antibody assays and receive royalties.

Figure 1

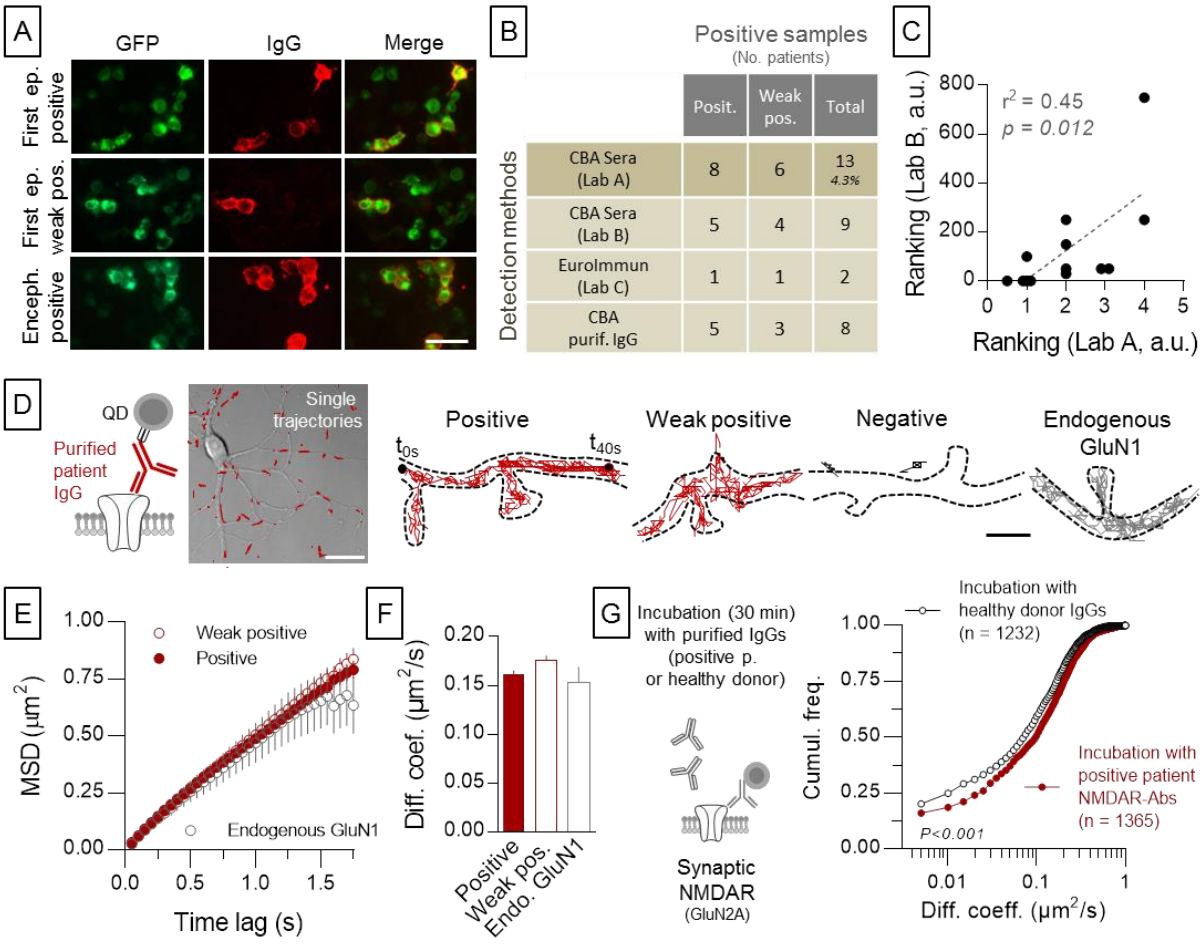

Figure 1. Multi-approach detection of circulating NMDAR-Ab in first episode psychotic patients.

(A) Representative images of HEK cells stained with first episode of psychosis (first ep.) and anti-NMDAR encephalitis (enceph.) patients' sera, illustrating positive and weak positive IgG staining. (B) The t results of patients' sera or purified IgG as performed in three independent European laboratories. (C) Comparison and correlation of the test scores between Lab A and B. AU = arbitrary units (A, binding score; B dilution beyond which binding was no longer positive) (D) Schematic representation of the single nanoparticle (Quantum Dot, QD) tracking design (left panel). Representative single trajectories (30-40 second duration) of the surface receptors targeted by IgG from seropositive patient ("positive p."), weak seropositive patient ("Weak positive p."), seronegative donor ("negative"), and endogenous surface GluN1-NMDAR (anti-GluN1 subunit

antibody, Alomone labs #AGC-001). (E) Comparison of the mean square displacement (MSD) curves of patients' IgGs (positive and weakly positive) and anti-GluN1 subunit antibody. (F) Comparison of the mean instantaneous diffusion coefficients (expressed in $\mu m^2/s$) between positive, weakly positive and endogenous GluN1-NMDAR surface trajectories. (G) Schematic representation of the experimental design used to test the impact of seropositive patients' IgGs when compared to healthy seronegative donor IgGs. Cultured hippocampal neurons were first incubated 30 min with IgGs (5 µg/ml), and then synaptic NMDAR surface dynamics was measured through single nanoparticle tracking of GluN2A-NMDAR (enriched in synapses). *Left panel*, Cumulative distributions of synaptic NMDAR instantaneous diffusion coefficients exposed to healthy seronegative donor or seropositive patients (N = 2 healthy donors, n = 1232 individual trajectories; N = 5 positive patients, n = 1365 individual trajectories; Mann-Whitney test, $p < 0.001$).

Table. Demographics, Diagnostic, and comparison between seropositive and -negative patients

	Cohort	Seropositive	Seronegative
Patients, No.	298	14 (4.7%)	284 (95.3%)
Age, mean (SD)	26.06 (5.97)	26.77 (6.61)	26.02 (5.95)
Sex, No. (percent)	Male 196 (65.8%) Female 102 (34.2%)	Male 8 (57.1%) Female 6 (42.9%)	Male 188 (66.2%) Female 96 (33.8%)
Duration of psychosis (mo.), mean (SD)	5.91 (5.89)	4.23 (6.44)	5.99 (5.86)
DSM-IV diagnosis, No. (percent)			
Schizophreniform disorder	131 (44 %)	6 (42.9%)	125 (44%)
Schizophrenia undiff. type	28 (9.4%)	2 (14.3%)	26 (9.2%)
Schizophrenia disorganised type	6 (2%)	1 (7.1 %)	5 (1.8%)
Schizophrenia paranoid type	110 (36.9%)	5 (35.7 %)	105 (37%)
Schizophrenia residual type	1 (0.3%)	0 (0%)	1 (0.4 %)
Schizoaffective disorder	21 (7.0 %)	0 (0 %)	21 (7.4 %)

Psychotomimetic uncompetitive NMDA receptor antagonists strongly alter the receptor membrane dynamics and distribution

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Abstract

The NMDA receptor (NMDAR) uncompetitive antagonists, MK-801 and ketamine, induce psychotic-like symptoms that have been used to model psychosis and other features of schizophrenia. However, the molecular mechanisms underlying these behavioral alterations, not observed with competitive NMDAR antagonists, remain poorly understood. Using a combination of single nanoparticle imaging and biochemical approaches, we here investigated the impact of competitive and uncompetitive NMDAR antagonists on the behavior of single membrane NMDAR in live hippocampal networks. The GluN1-NMDAR surface dynamics and synaptic content were unaltered by the competitive NMDAR antagonist AP5. Strikingly, MK-801 and ketamine strongly reduced GluN1-NMDAR surface dynamics, favoring its synaptic anchoring and content. Although the transmembrane EphrinB2 receptor plays a crucial in dynamically anchoring NMDAR in synapses, MK-801 and ketamine did not alter EphrinB2 receptor synaptic behavior. However, knocking-down the expression of two major MAGUK proteins (PSD-95 and SAP102), which are anchoring NMDAR in synapses, abolishes the synaptic content change induced by the NMDAR channel blockers. Together, our data provide the first evidence that competitive and uncompetitive NMDAR antagonists differentially alter the NMDAR surface dynamics, supporting the possibility that antagonists with psychotomimetic properties alter the receptor interaction with direct environment and overall distribution in hippocampal neurons.

Introduction

NMDA receptors (NMDAR) are constantly trafficked to and from the glutamatergic synapse in order to ensure the stability of the pool in basal condition (Lau & Zukin 2007). In addition to the exocytotic/endocytotic cycle, membrane NMDAR laterally diffuse between synaptic and extrasynaptic areas in a regulated and subunit-dependent manner (Groc et al. 2004b; Groc et al. 2006; Tovar & Westbrook 2002). Abnormal NMDAR trafficking and alteration of its surface distribution have been proposed to contribute to the emergence of severe psychiatric disorders (Lau & Zukin 2007; Paoletti et al. 2013). Of particular relevance, the glutamatergic model of psychosis and schizophrenia has been increasingly accepted as part of the etiopathology since the discovery that some NMDAR blockers induce schizophrenia-like psychosis, reproducing both positive and negative symptoms (Kantrowitz & Javitt 2012). The NMDAR uncompetitive antagonists with psychotomimetic properties in humans and rodents have thus been widely used to model psychosis and schizophrenia. In humans, a single dose of ketamine or phencyclidine induces psychosis in healthy volunteers (Javitt & Zukin 1991; Krystal et al. 1994; Malhotra et al. 1996; Lahti et al. 2001), and worsens the mental status of schizophrenic patients (Luby et al. 1959; Javitt & Zukin 1991; Krystal et al. 1994; Lahti et al. 1995; Lahti et al. 2001; Malhotra et al. 1997). The molecular mechanism underlying these behavioral alterations remains however poorly understood although changes in NMDAR expression have been reported in rodents after acute (Linden et al. 1997; Lindén et al. 2001; Wiseman Harris et al. 2003; Anastasio & Johnson 2008; Carty et al. 2012; F. Liu et al. 2013) or chronic (Sircar et al. 1996; Matthews et al. 2000; Oh et al. 2001; Rujescu et al. 2006; Mouri et al. 2007; Anastasio & Johnson 2008; Anastasio et al. 2009; Owczarek et al. 2011b; Chatterjee et al. 2012) treatments with psychotomimetic uncompetitive NMDAR antagonists. As competitive antagonists alter the behavioral repertoire but fail to induce psychotomimetic features (Morris et al. 1986; Koek & Colpaert 1990; Chiamulera et al. 1990; Chizh et al. 2001), it has been proposed that psychogenesis mostly relies on ion flux blockade (Petrovic et al. 2005). However, neither the low-affinity uncompetitive antagonist memantine nor the natural channel blocker magnesium produce similar behavioral effects (Emnett et al. 2013). Blocking the NMDAR channel is thus not

sufficient to explain the emergence of psychotic-like symptoms in humans and animal models, suggesting the presence of additional molecular deficits induced by psychotomimetic uncompetitive NMDAR antagonists.

The anti-NMDAR encephalitis is an autoimmune disorder associated with major psychiatric features, such as psychosis that are directly correlated with the presence of autoantibodies directed against an extracellular epitope of the obligatory GluN1 subunit of the NMDAR (Dalmau et al. 2007; Graus et al. 2008; Dalmau et al. 2011). These NMDAR autoantibodies decrease the NMDAR signaling mostly through changes in the receptor surface dynamics and synaptic retention, without any major antagonism action by themselves (Mikasova et al. 2012; Moscato et al. 2014). Together with non-ionotropic functions of the NMDAR (Dore et al. 2016; Weilinger et al. 2016; Stein et al. 2015; Nabavi et al. 2013; Kessels et al. 2013), one may hypothesize that psychotomimetic NMDAR antagonists alter the receptor surface trafficking in addition to the channel blockade. Here, we directly tackled this question using a combination of single nanoparticle tracking, immunocytochemistry and biochemistry approaches in cultured hippocampal neurons and hippocampal brain tissue. We compared the molecular effects of ketamine and MK-801, two psychotomimetic NMDAR uncompetitive antagonists, with the competitive antagonist AP5 on the NMDAR surface trafficking and synaptic content.

Results

NMDAR antagonists differentially alter NMDAR surface dynamics

To investigate the acute effect of NMDAR antagonists on the receptor surface diffusion, we tracked membrane GluN1 subunit using single molecule imaging approach in spontaneously-active cultured hippocampal networks (Figure 1). Based on their psychotomimetic properties, neuronal networks were exposed to either AP5 (a competitive antagonist), ketamine, or MK-801 (two channel blockers that induce psychosis)(Moghaddam & Krystal 2012). One hour incubation with AP5 (50 µM) had no effect on the instantaneous diffusion coefficient of surface GluN1-NMDAR as compared to the buffer condition (Figure 1A, B). In contrast, the same incubation with MK-801 (20 µM) or ketamine (1 µM) decreased GluN1-NMDAR surface diffusion (Figure 1A, B). As MK-801 and ketamine only block activated NMDAR and not resting receptors (Macdonald et al. 1991), these data indicate that during this incubation period NMDAR were spontaneously activated and blocked by the antagonists (Suppl Figure 1). Within synaptic areas, AP5 had no effect on GluN1-NMDAR surface diffusion (3% reduction) whereas MK-801 and ketamine decreased diffusion by 55 and 95%, respectively (Figure 1C, D). Consistently, the synaptic dwell times of GluN1-NMDAR exposed to ketamine and MK-801 were significantly increased (Figure 1E). The curves of the mean square displacement over time lag were compared between conditions (Figure 1F). Incubation with MK-801 or ketamine shifted the curves toward lower values, indicating a higher confinement of diffusing GluN1-NMDAR in presence of the channel blockers. It could be noted that AP5 incubation tend to decrease the confinement of GluN1-NMDAR, in contract to the increase observed with channel blockers. Altogether, these data indicate that MK-801 and ketamine acutely restrain the diffusive properties of NMDAR by favoring their synaptic anchoring and confinement.

These unexpected observations could, at first sight, be explained by an overall reduction of the spontaneous neuronal network activity induced by the antagonists, as previously reported (Sinner et al. 2005; Huang et al. 2013; Miller et al. 2014; Yuan et al. 2016). To discriminate a network *versus* a

more direct effect on the receptor itself, we tracked synaptic GluN1-NMDAR in neurons exposed to tetrodotoxin (TTX), which blocks the action potential-driven synaptic activity, and to the various NMDAR antagonists for at least 5 min. As the spontaneous activity of the neuronal network was abolished, NMDAR were artificially activated by the application of NMDA (Figure 2A). Neither the acute application of TTX nor NMDA altered the surface dynamics of NMDAR (Figure 2B, C). Likewise, AP5 was without significant effect (Figure 2B, C). Strikingly, ketamine effect on the receptor diffusion was maintained in this condition, i.e. absence of neuronal network activity (Figure 2B, C). MK-801 effect was however attenuated as it only tends to decrease the surface dynamics. The difference between these two conditions is possibly due to the fact that MK-801 and ketamine have different use- and voltage-dependent sensitivities to block NMDAR, MK-801 being putatively more sensitive than ketamine to the suppression of network activity (Davies et al. 1988; Macdonald et al. 1991; Paoletti & Neyton 2007). Altogether, these data demonstrate that ketamine and MK-801, two NMDAR channel blockers strongly impact the surface trafficking of NMDAR at the surface of hippocampal neurons whereas AP5, a competitive NMDAR antagonist was without obvious effect.

Psychotomimetic NMDAR blockers upregulate NMDAR synaptic content *in vitro* and *in vivo*

Alteration of the surface trafficking of NMDAR often results in changes of NMDAR synaptic content, and thus NMDAR-dependent synaptic processes. To address this question, we first investigated whether AP5, MK-801, or ketamine acutely change NMDAR synaptic content in spontaneously active hippocampal neuronal networks (Figure 3, S1). Neurons expressing a postsynaptic marker (Homer 1c) and flag-GluN1 subunit were used to label surface NMDAR in glutamate synapses. The incubation with AP5 increased the intensity of synaptic GluN1-NMDAR without any change in the cluster area (Figure 3A-C). However, MK-801 and ketamine treatment drastically altered the synaptic GluN1-NMDAR content. Indeed, MK-801 and ketamine upregulated GluN1-NMDAR cluster intensity (to ~350% and 200% of the control value, respectively) and decreased the cluster area (to ~80% and 75%

of the control value, respectively) (Figure 3C), reflecting a major reorganization of NMDAR in synapses exposed to the NMDAR channel blockers.

These *in vitro* observations were then tested *in vivo* by injecting these antagonists to adult rats and quantifying the synaptic content of NMDAR subunit levels (i.e. GluN1, GluN2A and GluN2B) from hippocampal synaptosomal preparations (Figure 3D). Because the competitive antagonist AP5 has a poor blood-brain barrier penetration, we used CPP instead, an analog of AP5 and AP7 and potent competitive antagonist of NMDAR (Davies et al. 1986). Quantitative immunoblot analyses revealed that NMDAR subunit expressions were upregulated in synaptic-enriched fractions extracted from the hippocampus of animals acutely injected with MK-801 and ketamine, but not with CPP (Figure 3D, E). Indeed, MK-801, and to a lesser extent ketamine, strongly increased GluN1, GluN2A and GluN2B subunit synaptic content (Figure 3D, E). However, CPP injection did not alter the content of GluN1, GluN2A and GluN2B subunits. Interestingly, the effects of the channel blockers were specific to the synaptic compartment since the subunits expression remained stable in the membrane-enriched fraction (Figure 3F). It could finally be noted that MK-801 and ketamine injections upregulated the synaptic content of the post-synaptic density protein, PSD-95 (Figure 3D), suggesting that MK-801/ketamine effects on NMDAR content can have further consequences on the glutamate synapse organization. Thus, these data demonstrate that both *in vitro* and *in vivo,* NMDAR synaptic distribution is robustly altered by the channel blockers whereas a competitive antagonist had a limited effect.

EphrinB2R surface trafficking and distribution are insensitive to NMDAR antagonism

NMDAR are part of a large multiprotein complex at the glutamatergic synapse (Husi & Grant 2001; Grant et al. 2005; Gold 2012; Frank et al. 2016). Their interactions with other membrane proteins provide multiple regulatory levels of the receptor surface dynamics (Ladépêche, Julien Pierre Dupuis, et al. 2013). The psychotomimetic NMDAR blockers reduce NMDAR synaptic diffusion, favoring their synaptic anchoring. One may propose that interaction changes between the receptor and synaptic

partners could explain the NMDAR channel blockers' effect. EphrinB2R (EphB2R) is one of the few direct interactor of the NMDAR at the plasma membrane (Dalva et al. 2000; Dalva et al. 2007; Nolt et al. 2011). The binding of the ephrinB2 ligand to EphB2R potentiates EphB2R-NMDAR interaction and stabilizes NMDAR at the synapse (Dalva et al. 2000; Dalva et al. 2007; Nolt et al. 2011). Remarkably, the EphB2R-NMDAR interaction can be dynamically prevented (e.g. with anti-NMDAR autoantibody), leading to a lateral dispersal of both NMDAR and EphB2R outside synapses (Mikasova et al. 2012). We thus tested the possibility that the alteration of NMDAR surface dynamics induced by the NMDAR channel blockers result from a change in the EphB2R-NMDAR complex. The surface dynamics and synaptic content of EphB2R was measured in presence of AP5, MK-801, or ketamine (Figure 5). In all conditions, the EphB2R surface diffusion remained equivalent to the control condition (Figure 4A-C). Neither the diffusion coefficient nor the confinement type of EphB2R was modified (Figure 4B, C), indicating that NMDAR antagonists do not disturb EphB2R surface dynamics. We next investigated the content of synaptic EphB2R. Consistently with the single molecule behavior, none of the antagonist disturbed EphB2R synaptic content (Figure 4D, E). Together, these data do not support the hypothesis that the EphB2R-NMDAR interaction plays a major role in the synaptic anchoring of NMDAR in presence of channel blockers.

Downregulation of PSD protein prevents channel blocker-induced NMDAR synaptic change

In the glutamate synapse, NMDAR mostly interact with PDZ domain-containing scaffold proteins of the MAGUK family (Yoshii et al. 2013; Elias & Nicoll 2007). Overall, MAGUKs play an important role in regulating synapse maturation and stabilization, as well as glutamatergic receptors trafficking (Elias et al. 2008). It has been well-established that this interaction takes place between the C-terminus part of the GluN subunit with the PDZ domains of the MAGUKs, regulating the surface dynamics and the synaptic content of NMDAR (van Zundert et al. 2004; Groc et al. 2009; Bard et al. 2010). Activation of NMDAR transiently disrupt the interaction between NMDAR and PSD-95 (Bard et al. 2010; Doré et al. 2014). Of particular interest, NMDAR inhibition by MK-801 favors the interaction

between NMDAR and PSD-95, preventing the activation-dependent disruption of the interaction (Doré et al. 2014). We thus investigated whether the effect of the NMDAR channel blockers MK-801 and ketamine on NMDAR surface trafficking requires the presence of MAGUKs. We used RNA silencing to downregulate the expression of PSD-95 and SAP102 proteins, two of the main scaffold interactors of the NMDAR at the glutamate synapse. To quantify the silencing efficiency, hippocampal cultured neurons were transfected with PSD-95/SAP102 shRNAs, a scramble inactive version, or GFP alone, and immunostaining of PSD-95 or SAP102 were performed between 2-7 days after transfection (Figure 5A). At the gross morphological level, the spine density was strongly reduced in neurons expressing PSD-95 and SAP102 shRNAs (Figure 5B). The remaining spines were more of the filopodial type (Figure 5B). In neurons transfected with PSD-95 and SAP102 shRNA, PSD-95 and SAP102 expression were equally reduced by 40% (Figure 5C), validating the efficiency of the shRNAs to downregulate their targeted proteins in these hippocampal cultured networks (Zhao et al. 2013; Murata & Constantine-Paton 2013).

We then tested *in vivo* whether the ketamine effect on NMDAR synaptic content requires the basal expression of these MAGUKs. At postnatal day 9, bilateral intra-hippocampal injections of PSD-95 or SAP102 shRNA lentiviruses were performed. Lentiviruses were left for 7 days to ensure a sufficient infection rate and efficiency *in vivo*. Animals were then injected intraperitoneally with saline or ketamine (100 mg/kg). After 1h, hippocampi were dissected for synaptosomal preparation and further quantification of NMDAR levels (Figure 5D). The NMDAR subunit levels in synaptic-enriched fractions were not affected in both shRNA-infected animals (not shown), consistent with previous studies showing that MAGUKs knockdown or knock-out mostly affect AMPAR synaptic content (Béïque et al. 2006; Elias et al. 2006; Elias et al. 2008; Bard et al. 2010; Murata & Constantine-Paton 2013). We then compared the different ScrRNA/shRNA conditions to the above data showing that ketamine injection in naïve rats increased both GluN1 and GluN2B subunit content in synaptic-enriched fractions (Figure E, F). Similar outcomes were observed in animals injected with both scramble shRNAs. Strikingly, the downregulation of either PSD-95 or SAP102 prevented the

ketamine-induced increase in GluN1 and GluN2B subunits in synapses (Figure 5E, F). These results then indicate that MAGUKs, PSD-95 and SAP102, are required to change the synaptic dynamics and content of NMDAR in presence of a channel blocker.

Psychotomimetic NMDAR blockers, but not competitive NMDAR antagonists, alter glutamate synapse density and morphology

We investigated whether the above molecular effect of the uncompetitive NMDAR blockers on the receptor surface dynamics and synaptic content functionally impact on the neuronal communication by estimating the density of glutamate synapses. The density of glutamate synapses was significantly decreased by MK801 and ketamine (1h incubation), whereas AP5 failed to produce an effect (Figure 6A, B). Consistently, the linear density of spines was reduced by ketamine (Figure 6A, C). Thus, the uncompetitive NMDAR blockers also impact over time on the density, and possibly morphology (spine versus shaft synapses), of glutamate synapses.

Discussion

Using a combination of high-resolution single-particle tracking, immunocytochemistry and biochemistry approaches, we here demonstrate that NMDAR uncompetitive channel blockers with psychotomimetic properties alter NMDAR surface trafficking and distribution. Whereas ketamine and MK-801 strongly reduced GluN1-NMDAR synaptic mobility and increased its retention in the synaptic area, the competitive antagonist AP5 was without any detectable effect on the receptor fast dynamics. Consistently, the two NMDAR uncompetitive antagonists, and not AP5, upregulated NMDAR synaptic content in hippocampal synapses. This differential impact of NMDAR antagonists on the NMDAR surface dynamics and synaptic content fuels thus the emerging view that the various behavioral consequences following NMDAR antagonism relies on alteration of the NMDAR trafficking and location in addition to its activity blockade *per se*. Psychotomimetic effects have been restricted to NMDAR uncompetitive channel blockers (Bubeníková-Valešová et al. 2008; Javitt et al. 2012). Indeed, these effects have not been described with glutamate and glycine site antagonists or ifenprodil-like molecules (Petrovic et al. 2005; Chizh et al. 2001). Furthermore, the uncompetitive channel blocker memantine, which exhibits indistinguishable pharmacodynamic properties with ketamine, fails to induce major psychotomimetic effects(Emnett et al. 2013). Thus, NMDAR antagonism, and even blockade, is not sufficient to induce psychotic symptoms, suggesting that psychotomimetic uncompetitive channel blockers bear additional molecular effects on the NMDAR function. The discovery of the anti-NMDAR encephalitis is, in that respect, of great interest. Indeed, the autoantibodies against the NMDAR are directly responsible for the expression of psychotic symptoms in patients and rodents (Planagumà et al. 2015) through major alteration of the NMDAR trafficking whithout evidence to date of a major antagonism effect (Mikasova et al. 2012; Moscato et al. 2014). Together, it emerges that part of the pathogenicity of psychotomimetic molecules is related to a selective impairment of the NMDAR fast trafficking.

Multiple regulatory levels have been shown to tune NMDAR fast surface dynamics and synaptic retention (Groc et al. 2009; Ladépêche, Julien Pierre Dupuis, et al. 2013). Two main interaction types

have been described: i) the interactions with transmembrane or extracellular partners that interact through extracellular domains of the NMDAR, and ii) interactions with PDZ domain-containing intracellular partners mostly at the C-terminus tail (Bard & Groc 2011). The absence of change in the EphB2R surface dynamics in presence of all tested NMDAR antagonists suggests that the interaction between the NMDAR and EphB2R is not involved in the channel blocker-induced change in NMDAR surface dynamics. This result contrasts with the molecular impact of the psychogenic NMDAR autoantibodies that disrupt the EphB2R-NMDAR interaction, unleach the receptor from the synapse, and lead consequently to a synaptic NMDAR hypofunction (Mikasova et al. 2012). Instead, NMDAR uncompetitive channel blockers lower surface dynamics, favor synaptic anchoring and increase the synaptic NMDAR content. Our data indicate that these effects require the presence of MAGUKs that are potent anchoring partners of the NMDAR at glutamate synapses. Indeed, NMDAR mostly interact with PDZ domain-containing scaffold proteins of the MAGUK family at glutamate synapses (van Zundert et al. 2004; Elias & Nicoll 2007). The intracellular C-terminus tail of NMDAR acts as a central hub in which several partner proteins converge (Husi & Grant 2001). Since PSD-95 expression was also increased after exposure to NMDAR channel blockers, we propose that the synaptic trapping of NMDAR strongly relies on a stronger interplay between NMDAR and MAGUKs in the postsynaptic density. Consistently, NMDAR inhibition by MK-801 favors the interaction between NMDAR and PSD-95, and prevents the receptor activation-dependent disruption of this interaction (Doré et al. 2014). Compared to competitive NMDAR antagonists, MK-801 and ketamine would thus change the interactions with MAGUKs, tuning NMDAR surface trafficking and synaptic retention. Direct evidences supporting this claim are surely needed. This could be assessed using FRET measurements between NMDAR and PSD proteins (Doré et al. 2014), or by using disrupting peptides that acutely and efficiently block the interaction between PDZ proteins and the NMDAR (Bard et al. 2010), in presence of various types of NMDAR antagonists. Of interest, a non-ionotropic role of NMDAR was recently proposed, based on the observation that NMDAR activation in presence of MK-801 or 7-CK leads to conformational changes of NMDAR cytoplasmic domains (Dore et al. 2015; Aow et al. 2015),

a process not observed following exposure to AP5. Thus, NMDAR uncompetitive channel blockers and competitive antagonists likely trigger distinct intracellular conformational changes of the receptor, NMDAR. Preliminary evidences acquired using FRET/FLIM measurements in hippocampal cultured neurons expressing GluN1-GFP and GluN1-mCherry constructs (Dore et al. 2015; Aow et al. 2015) support the claim that different NMDAR antagonists differentially impact on the intramolecular dynamics of the NMDAR (unpublished). Investigating the putative alterations of NMDAR conformation and surface trafficking in presence of psychotomimetic molecules, and testing the direct pathogenic role of such a change in receptor trafficking (in addition to the ion flux blockade), may shed new unsuspected lights on the molecular cascade underlying the NMDAR deficits in major psychotic disorders.

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Material and Methods

Primary cell culture and protein expression

Cultures of hippocampal neurons were prepared from E18 Sprague-Dawley rats. Cells were plated at a density of 50 x 10^3 cells per ml on poly-lysine pre-coated coverslips. Coverslips were maintained in a 3% horse serum containing Neurobasal medium (Invitrogen). After a few days *in vitro* (*div*), the original plating medium was replaced by a serum-free medium. Cultures were maintained at 37°C in 5% CO_2 for 15*div* at maximum. For exogenous GluN1-flag and Homer-GFP expression, 7-10*div* cultured hippocampal neurons were transfected at least 48h before each experiment using either the Effectene (Qiagen) or phosphate calcium transfection (Jiang et al. 2006).

Immunocytochemistry

Surface exogenous GluN1-flag receptors were specifically stained using a monoclonal antibody against flag (Sigma, 1/500, 20min, 37°C) and Alexa 568-conjugated anti-mouse secondary antibody (Invitrogen, 1/500, 30min). Endogenous EphB2R were labeled after fixation with a polyclonal antibody against the extracellular part of EphrinB2R (R&D, 1/200, overnight) and a secondary anti-goat Alexa 488 antibody (Invitrogen, 1/500, 30min). In order to label the post-synaptic density, neurons were fixed (4% PFA, 15min), permeabilized with Triton-BSA 1% (5min) and successively incubated with an anti-Homer-1c antibody (Synaptic systems, 1/500, 30min) and a secondary anti-guinea pig Alexa 594 antibody (Jackson ImmunoResearch, 1/500, 30min). All imaging sessions were done on a video spinning-disk system (Leica DMI6000B, 63X) and quantification analysis was performed using MetaMorph software (Molecular Devices) and ImageJ (NIH).

Quantum dot (QD) tracking and surface diffusion calculation

QD labeling and microscopy were performed as previously described (Mikasova et al. 2012). Briefly, live neurons were incubated with primary antibodies against exogenous GluN1-flag (Sigma, 1/500) or endogenous EphrinB2R (R&D, 1/200) for 10min at 37°C. Neurons were then washed and incubated

for 10min with QD 655 Goat F(ab')$_2$ anti-mouse or QD 655 rabbit F(ab')$_2$ anti-goat IgG, respectively. Non-specific binding was blocked by adding 1% BSA (Vector Laboratories) to the QD solution. Green Mitotracker (Life Technologies, 1/2000) was used as an endogenous synaptic marker. QDs were detected by using a mercury lamp, appropriate excitation/emission filters and an EM-CCD camera (Evolve, Photometrics). Images were obtained with an acquisition time of 50ms with up to 500 consecutive frames. QDs were followed on randomly selected dendritic regions for up to 20min. Recording sessions were processed with the Metamorph software (Universal Imaging Corp). The instantaneous diffusion coefficient, D, was calculated for each trajectory, from linear fits of the first 4 points of the mean square displacement versus time function using *MSD(t)= <r2>(t)= 4Dt.*

Evaluation of shRNA efficacy

Primary hippocampal neurons were transfected at 10-11*div* with PSD95-GFP and SAP102-GFP shRNA plasmids (Constantine-Paton lab) using phosphate calcium transfection. Scrambled shRNA sequences in which nucleotides were shuffled were used as negative controls. PSD95 and SAP102 proteins expression were analyzed by immunostaining after 3 days, 5 days or 7 days of shRNA expression.

***In vivo* drug treatment**

Ketamine hydrochloride (100mg, Virbac, France), (+)-MK-801 [(5*S*, 10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo [*a*,*d*]cyclohepten-5,10-imine maleate] (Tocris) and (RS)-CPP [(*RS*)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid] (Tocris) were dissolved in 0.9% NaCl. Ketamine hydrochloride (100mg/kg), (RS)-CPP (10mg/kg), MK-801 (5mg/kg), and saline 0.9% were injected intraperitoneally (i.p.) at a final volume of 2ml/kg on P60 Sprague-Dawley rats. The concentrations of NMDAR antagonists were chosen in accordance with previous studies (Manahan-vaughan et al. 2008; Chatterjee et al. 2012; Iafrati et al. 2014).

***In vivo* injection of lentivirus**

Briefly, P8-P10 Long Evans rat pups were anesthetized by inhalation of isoflurane. The stereotaxic coordinates for the injection of lentiviral solution in the hippocampus were the following:

coordinates relative to bregma, from AP: -2.5mm, AD: 2.9mm, DV: -2.9mm. Lentiviral solutions (100nl) of PSD95-GFP and SAP102-tdTomato shRNA or scramble (Zhao et al. 2013; Murata & Constantine-Paton 2013) were injected using Nanoject II injection system (Drummond). The needle was left *in situ* for 5 min to reduce reflux. After injection, the skin was sutured, and pups were placed in a warm cage until recovery from anesthesia, and then returned to their mother. For injection, litters were split in half, and one half of the pups were injected with the scrambled lentiviruses, the other half with the KD lentiviruses. After 7 days of lentiviral expression (P15-17), animals from each experimental group were injected with either saline 0.9% or ketamine hydrochloride 100mg/kg at a final volume of 1ml/kg, and sacrificed 1h after i.p. injection. All experiments were performed in accord with the guidelines of the Massachusetts Institute of Technology Institutional Animal Care and Use Committee.

Synaptosomes fractionation and quantitative immunoblotting

Synaptosomes were prepared from hippocampi of naive P60 rats or P17 rats injected with shRNA lentiviruses as previously described. Hippocampi were homogenized and dissociated in 1 ml of TPS (0.32M sucrose, 4mM HEPES pH 7.4, and a protease inhibitor cocktail (1:1000, Calbiochem)). After centrifugation at 1000g for 8min (4°C), the supernatant (S1) was saved and centrifuged at 12,500 g for 13min (4°C) to obtain a P2 crude membrane fraction. P2 pellet was resuspended in TPS. Synaptic fractions from P2 were separated on a sucrose gradient composed with 5ml of buffer B (1.2M sucrose, 4mM HEPES pH 7.4) and 5ml of buffer A (0.8M sucrose, 4mM HEPES pH 7.4). After ultracentrifugation at 50,000g for 1h10 (4°C), synaptic fractions were collected. Before loading on a gel, samples were boiled at 95°C for 5min. 0.5µg of samples were separated by SDS/PAGE (Mini-Protean TGX precast gels 4-20% STAINFREE, Biorad) for 40min at 200V, and blotted onto nitrocellulose membrane during 1h at 100V. After blocking 1h in 5% milk in Tris-saline - 0.05% tween 20 (TBST), membranes were hybridized overnight at 4°C with primary antibodies directed against GluN1-NMDAR subunit (BD Biosciences, 1/1000), GluN2A-NMDAR subunit (MERCK, 1/2000), GluN2B-NMDAR subunit (ThermoFisher Scientific, 1/2000) or PSD95 (ThermoFisher Scientific, 1/500).

Corresponding secondary antibodies were diluted at 1/10000 in TBST 0.5% milk. Detection was performed using the SuperSignal West Femto Maximum Sensitivity Substrate detection System (ThermoFisher Scientific), and revelation was done with a ChemiDoc system (Biorad). Quantification of band intensity was performed using Image Lab software (Biorad).

Statistical analysis

Comparisons between groups were performed using parametric statistical tests, Student t-test (pair comparison) or ANOVA followed by a Newman-Keuls Multiple Comparison Test (group comparison). Comparisons between groups for instantaneous diffusion coefficients and calcium imaging were performed using Mann-Whitney test (pair comparison), Kruskal-Wallis followed by a Dunn's Multiple Comparison Test (group comparison). For distributions comparison a Kolmogorov-Smirnov test was used. Significance levels were defined as *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical analysis was performed using Prism (GraphPad).

Figure 1

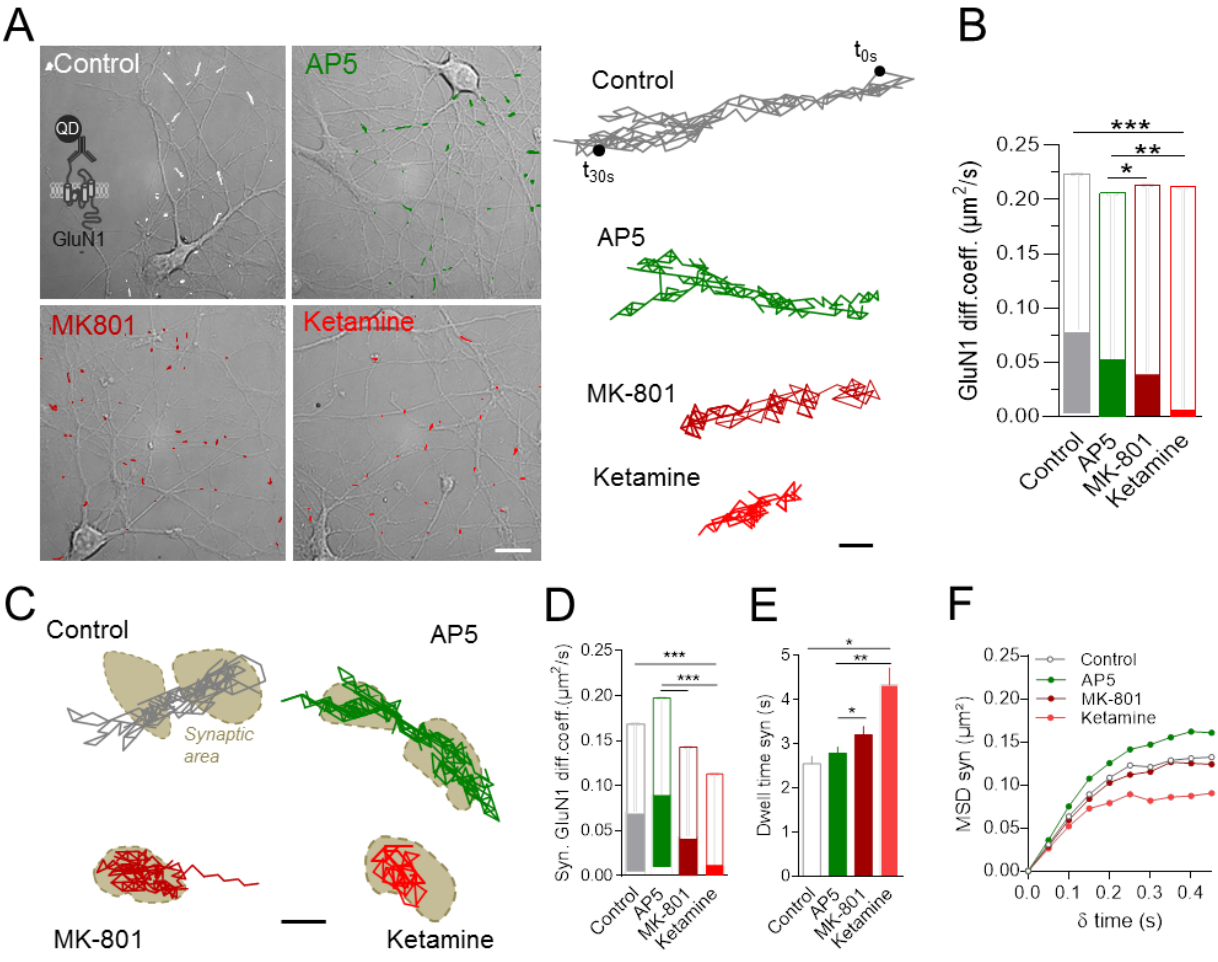

A
Control
AP5
MK801
Ketamine
QD
GluN1
Control
AP5
MK-801
Ketamine
t_{0s}
t_{30s}
B
GluN1 diff.coeff. (µm²/s)
0.25
0.20
0.15
0.10
0.05
0.00
***
**
*
Control
AP5
MK-801
Ketamine
C
Control
AP5
Synaptic area
MK-801
Ketamine
D
Syn. GluN1 diff.coeff.(µm²/s)
0.25
0.20
0.15
0.10
0.05
0.00
***
***
Control
AP5
MK-801
Ketamine
E
Dwell time syn (s)
5
4
3
2
1
0
*
**
*
Control
AP5
MK-801
Ketamine
F
MSD syn (µm²)
0.25
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
δ time (s)
Control
AP5
MK-801
Ketamine

Figure 1. NMDAR antagonists with psychotomimetic properties specifically alter NMDAR surface trafficking

(A) Left panel: Live detection of single GluN1-QD complexes, and representative trajectories of surface GluN1-NMDAR in dendritic fields of hippocampal neurons (9-11*div*) in control condition or after incubation for 1h with the competitive NMDAR antagonist AP5 (50µM), or the channel blockers MK-801 (20µM) and ketamine (1µM). Scale bar= 20µm. Right panel: Enlarged single GluN1-NMDAR trajectories from the corresponding dendritic fields. GluN1-QD complexes were tracked during 500 frames with a 20Hz acquisition frequency (First point of the trajectory (t_{0s}); last point of the trajectory (t_{25s})). Note the significant reduction of NMDAR mobility in presence of both MK-801 and ketamine. Scale bar= 500nm.

(B) Instantaneous diffusion coefficient of extrasynaptic GluN1-NMDAR in control condition or after treatment with the NMDAR antagonists AP5, MK-801 or ketamine for 1h (Median diffusion coefficient ± 25-75% IQR, *Control*= 0.0790µm^2/s, IQR= 0.0006 - 0.2230µm^2/s, n= 681 trajectories (from 14 neurons); *AP5*= 0.0518µm^2/s, IQR= 0.0004 - 0.2050µm^2/s, n= 1647 trajectories (27 neurons); *MK-801*= 0.0385µm^2/s, IQR= 0.0003-0.2130µm^2/s, n= 1155 (24 neurons); *Ketamine*= 0.0063µm^2/s, IQR= 0.0001 - 0.2113µm^2/s, n= 498 (14 neurons); Kruskal-Wallis followed by a Dunn's multiple comparison test *p<0.05 **p<0.01 ***p<0.001).

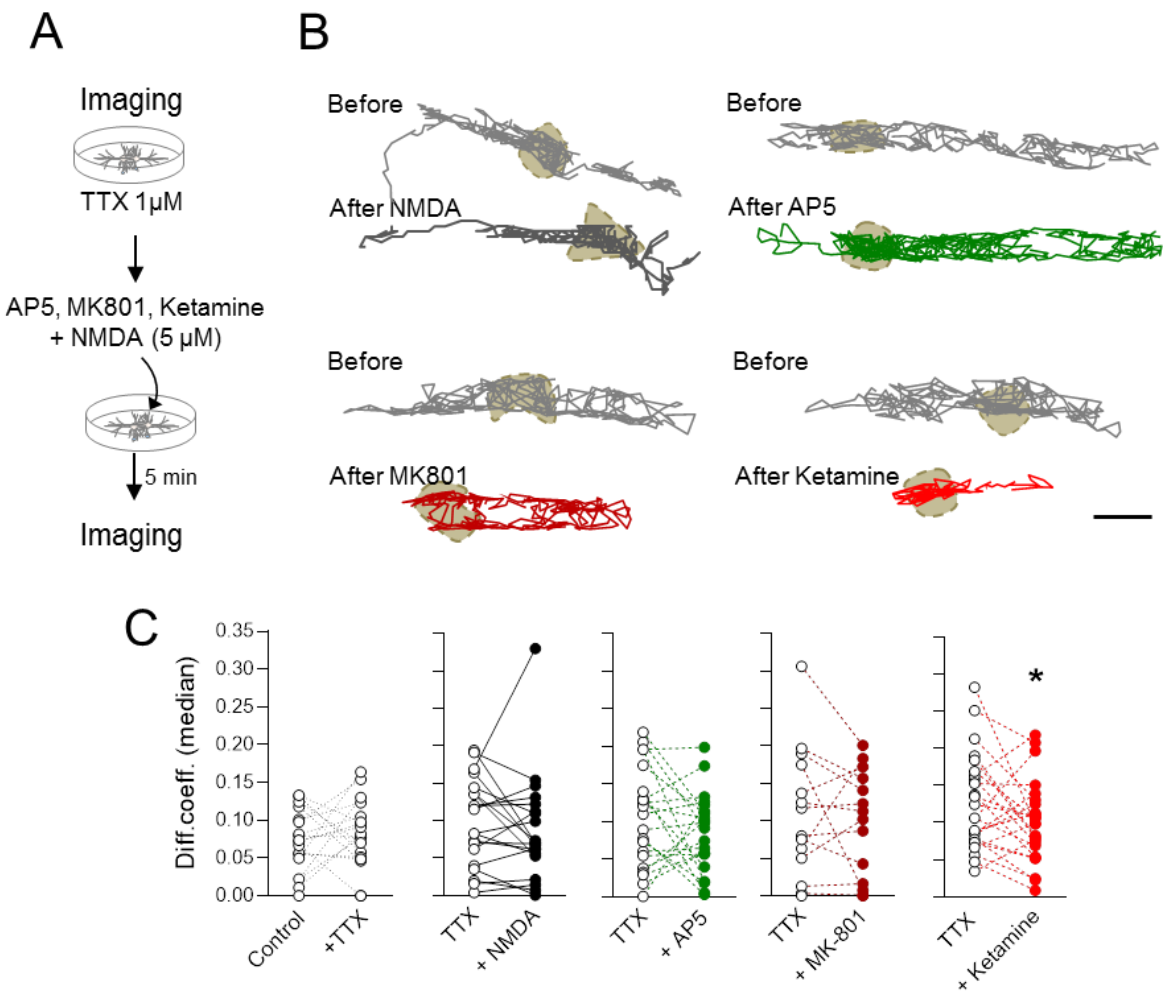
(C) Representative trajectories (500 frames, 20Hz acquisition rate) of synaptic GluN1-NMDAR in basal condition or in the presence of the different NMDAR antagonists. Scale bar= 500nm.

(D) Instantaneous diffusion coefficient of synaptic GluN1-NMDAR in control condition or after treatment with AP5, MK-801 or ketamine for 1h (Median diffusion coefficient ± 25-75% IQR, *Control*= 0.0698µm^2/s, IQR= 0.0057 - 0.1685µm^2/s, n= 341 trajectories (from 14 neurons); *AP5*= 0.0688µm^2/s, IQR= 0.0106 - 0.1970µm^2/s, n= 853 trajectories (27 neurons); *MK-801*= 0.0425µm^2/s, IQR= 0.0006 - 0.1428µm^2/s, n= 540 (24 neurons); *Ketamine*= 0.010µm^2/s, IQR= 0.0003 - 0.1130µm^2/s, n= 226 (14 neurons); Kruskal-Wallis followed by a Dunn's multiple comparison test ***p<0.001).

(E) Synaptic dwell-time of GluN1-NMDAR in the different experimental conditions (Mean ± SEM, *Control*= 2.551 ± 0.169 n=341 trajectories; *AP5*= 2.793s ± 0.142 n=835; *MK-801*= 3.209s ± 0.190 n=540; *Ketamine*= 4.335s ± 0.390 n=226; Kruskal-Wallis followed by a Dunn's multiple comparison test *p<0.05 **p<0.01).

(F) Mean Square Displacement (MSD) (Mean ± SEM) over time of synaptic GluN1-NMDAR in the different experimental conditions (*Control* n= 14 neurons; *AP5* n= 27; *MK-801* n= 24; *Ketamine* n= 14; Kolmogorov-Smirnov test, p>0.05).

Figure 2


A
Imaging
TTX 1µM
AP5, MK801, Ketamine
+ NMDA (5 µM)
5 min
Imaging
B
Before
After NMDA
Before
After AP5
Before
After MK801
Before
After Ketamine
C
Diff.coeff. (median)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
+TTX
TTX
+ NMDA
TTX
+ AP5
TTX
+ MK-801
TTX
+ Ketamine
*

F**igure 2. Network activity does not influence NMDAR antagonists' effect on surface trafficking**

(A) Primary hippocampal cultures were exposed to TTX (1µM) for ≈ 20min before imaging to ensure synaptic activity suppression, and was present all along the imaging session. GluN1-QD were tracked (500 frames, 20Hz acquisition rate) in this TTX-medium (condition "before"). NMDAR antagonists, together with NMDA (5µM) were then added into the bath, and after 5min incubation, a second measurement of GluN1-QD mobility was done (condition "after").

(B) Representative trajectories of GluN1-QD before and after addition of the different drugs into the bath. Dotted lines delimit the synaptic compartment (brownish area). Scale bar= 500nm.

(C) Instantaneous diffusion coefficients of synaptic GluN1-NMDAR before and after acute bath application of NMDAR antagonists. Paired data are plotted, each dot representing one neuronal field (Median, *TTX* n= 18 neuronal fields; *NMDA* n=20; *AP5* n= 21; *MK-801* n= 14; *Ketamine* n= 25; One-tailed paired t-test *p<0.05)

Figure 3

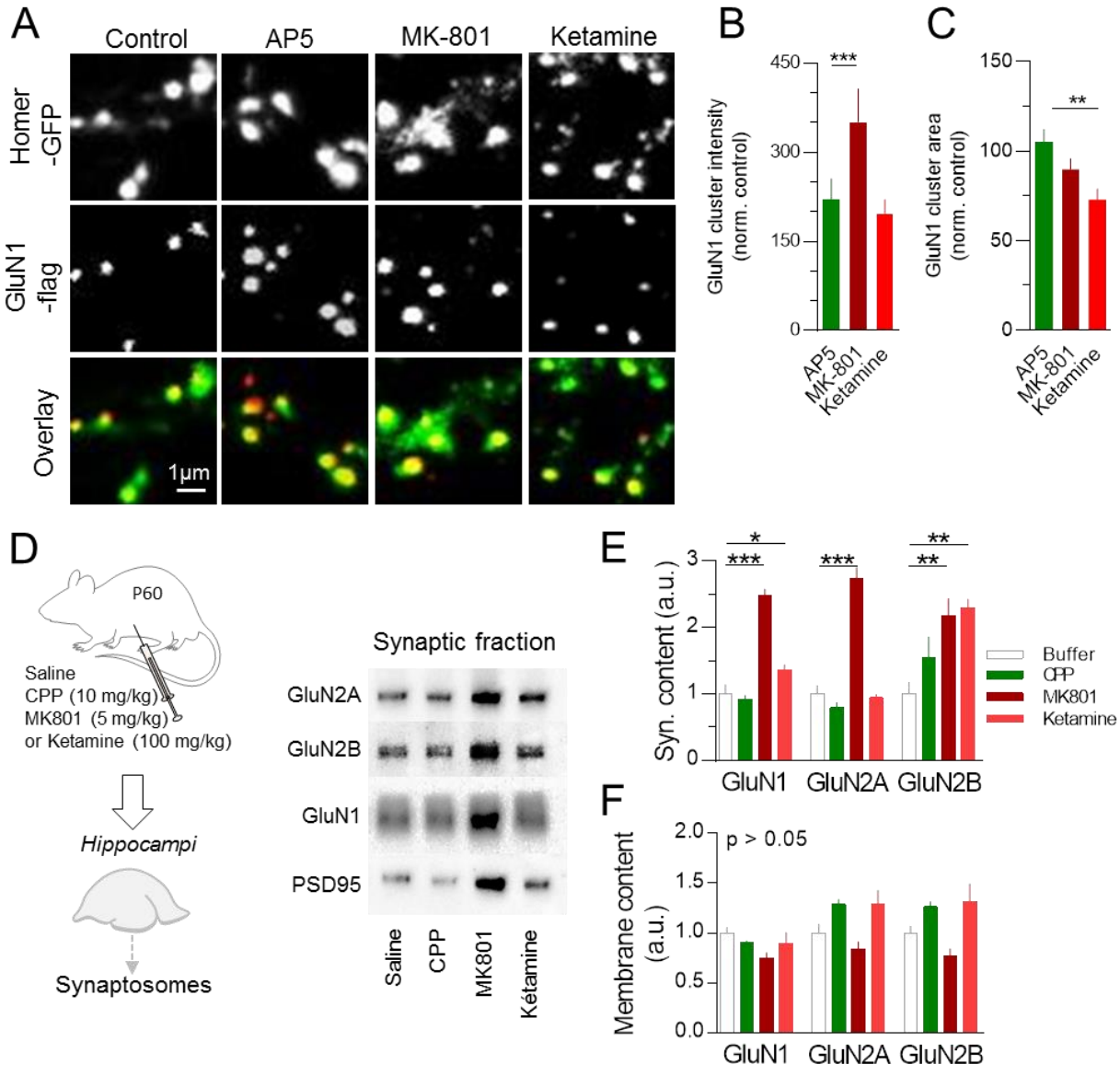

A
Control
AP5
MK-801
Ketamine
Homer -GFP
GluN1 -flag
Overlay
1µm
B
GluN1 cluster intensity (norm. control)
450
300
150
0
***
AP5
MK-801
Ketamine
C
GluN1 cluster area (norm. control)
150
100
50
0
**
AP5
MK-801
Ketamine
D
P60
Saline
CPP (10 mg/kg)
MK801 (5 mg/kg)
or Ketamine (100 mg/kg)
Hippocampi
Synaptosomes
Synaptic fraction
GluN2A
GluN2B
GluN1
PSD95
Saline
CPP
MK801
Kétamine
E
Syn. content (a.u.)
3
2
1
0
*
***
***
**
**
GluN1
GluN2A
GluN2B
Buffer
CPP
MK801
Ketamine
F
Membrane content (a.u.)
2.0
1.5
1.0
0.5
0.0
p > 0.05
GluN1
GluN2A
GluN2B

Figure 3. NMDAR synaptic content is specifically upregulated by psychotomimetic NMDAR antagonists

(A) Immunostaining of surface GluN1-flag clusters in basal condition or after incubation for 1h with the NMDAR antagonists AP5 (50µM), MK-801 (20µM) and ketamine (1µM). Synaptic NMDAR clusters (white arrowheads) were identified by colocalization with the synaptic marker, Homer-GFP. Scale bar= 1µm.

(B) Quantification of synaptic GluN1-NMDAR clusters fluorescence intensity in the different experimental conditions. Data were normalized to the control condition (Mean ± SEM; *AP5*= 221.1% ± 34.25, n= 54 dendritic regions (19 neurons); *MK-801*= 351.1% ± 55.64, n=52 (21 neurons); *Ketamine*= 197.4% ± 23.18, n= 39 (13 neurons); One-way ANOVA followed by a Newman-Keuls multiple comparison test, ***p<0.001).

(C) Quantification of synaptic GluN1-NMDAR clusters area in the different experimental conditions. Data were normalized to the control condition (Mean ± SEM; AP5= 105.3% ± 6.760 n= 54 dendritic regions (19 neurons); MK-801= 89.67% ± 77.51 n= 52 (21 neurons); Ketamine= 72.80% ± 6.004, n= 39 (13 neurons); One-way ANOVA followed by a Newman-Keuls multiple comparison test, **p<0.01).

(D) Representative immunoblots showing the expression of NMDAR subunits and PSD-95 in synaptic-enriched fractions from hippocampi of P60 rats injected with saline, or the NMDAR antagonists CPP (10mg/kg), MK-801 (5mg/kg) or ketamine (100mg/kg). 5 animals were included in each experimental group.

(E) Quantification of NMDAR subunit levels in synaptic-enriched fractions of rats hippocampi. Data were normalized to the saline condition (Mean ± SEM, GluN1 *CPP*=0.914a.u. ± 0.068; *MK-801*= 2.486a.u. ± 0.091; *Ketamine*= 1.366a.u. ± 0.074; GluN2A *CPP*=0.792a.u. ± 0.077; *MK-801*= 2.729a.u. ± 0.174; *Ketamine*= 0.942a.u. ± 0.053; GluN2B *CPP*=1.547a.u. ± 0.310; *MK-801*= 2.180a.u. ± 0.253; *Ketamine*= 2.294a.u. ± 0.130; One-way ANOVA followed by a Newman-Keuls multiple comparison test *p<0.05 **p<0.01 ***p<0.001). Note the significant upregulation of NMDAR subunits expression specifically induced by MK-801 and ketamine.

(F) Quantification of NMDAR subunit levels in membrane fractions of rats hippocampi. Data are normalized to the saline condition (Mean ± SEM, GluN1 *CPP*=0.906a.u. ± 0.017; *MK-801*= 0.746 ± 0.057; *Ketamine*= 0.901 ± 0.105; GluN2A *CPP*=1.291 ± 0.048; *MK-801*= 0.838 ± 0.076; *Ketamine*= 1.292 ± 0.137; GluN2B *CPP*=1.263 ± 0.053; *MK-801*= 0.776 ± 0.069; *Ketamine*= 1.318 ± 0.174; One-way ANOVA followed by a Newman-Keuls multiple comparison test p>0.05). None of the NMDAR antagonists modified NMDAR expression in membrane-enriched fractions.

Figure 4

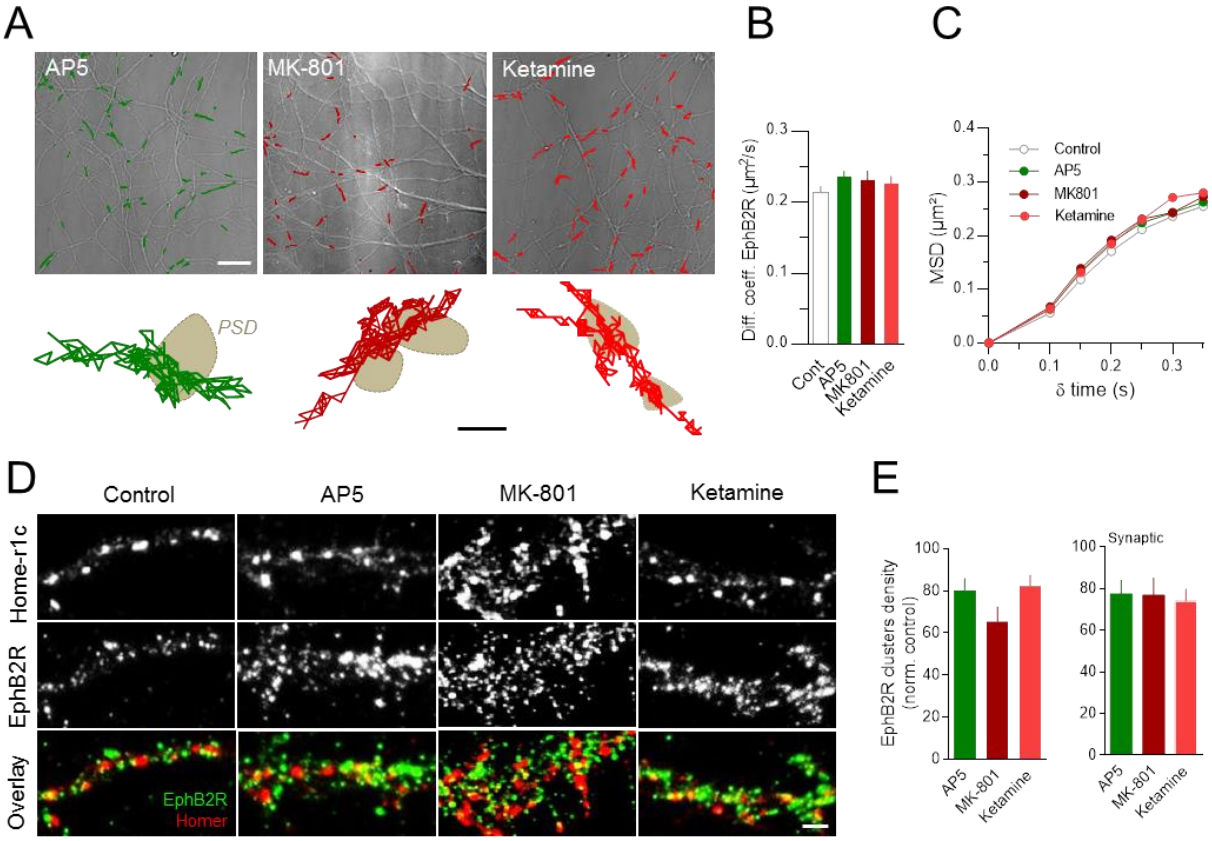

A
AP5
MK-801
Ketamine
PSD
B
Diff. coeff. EphB2R (μm²/s)
0.0
0.1
0.2
0.3
Cont
AP5
MK801
Ketamine
C
MSD (μm²)
0.0
0.1
0.2
0.3
0.4
Control
AP5
MK801
Ketamine
δ time (s)
D
Control
AP5
MK-801
Ketamine
Home-r1c
EphB2R
Overlay
EphB2R
Homer
E
EphB2R clusters density (norm. control)
0
20
40
60
80
100
AP5
MK-801
Ketamine
Synaptic

Figure 4. NMDAR inhibition does not impact EphB2R surface trafficking and distribution

(A) Surface live tracking of endogenous EphB2R in hippocampal neuronal fields (13-14*div*) pre-treated for 1h with NMDAR antagonists. Scale bar= 20µm. Surface trajectories depict the different diffusion behaviors of synaptic EphB2R depending on which NMDAR antagonist it has been exposed to. Scale bar= 500nm.

(B) Mean diffusion coefficient of perisynaptic EphB2R in control condition or after a 1h-treatment with AP5, MK-801 or ketamine (Mean diffusion coefficient ± 25-75% IQR, *Control*= 0.0698µm^2/s, IQR= 0.0057 - 0.1685µm^2/s, n= 341 trajectories (20 neurons); *APV*= 0.0688µm^2/s, IQR= 0.0106 - 0.1970µm^2/s, n= 853 trajectories (23 neurons); *MK-801*= 0.0425µm^2/s, IQR= 0.0006-0.1428µm^2/s, n= 540 (19 neurons); *Ketamine*= 0.010µm^2/s, IQR= 0.0003-0.1130µm^2/s, n= 226 (16 neurons); Kruskal-Wallis followed by a Dunn's multiple comparison test p>0.05).

(C) Mean Square Displacement (MSD) (Mean ± SEM) over time of perisynaptic EphB2R in basal condition or after treatment with NMDAR antagonists (*Control* n= 20 neurons; *AP5* n=23; *MK-801* n= 19; *Ketamine* n= 16; Kolmogorov-Smirnov test p>0.05).

(D) Immunolabeling of surface EphB2R in hippocampal cultures (13-14*div*) incubated for 1h with AP5, MK-801 or ketamine. EphB2R synaptic detection was performed using endogenous Homer-1c staining as a synaptic marker. Scale bar= 2µm.

(E) Quantification of surface EphB2R cluster density (data are normalized to the control condition) at the surface of hippocampal neurons (Mean ± SEM, *AP5*= 80.21% ± 5.627, n= 77 dendritic regions (23 neurons); *MK-801*= 65.32% ± 7.014, n= 37 (12 neurons); *Ketamine*= 82.12% ± 5.298, n=73 (23 neurons), One-way ANOVA p>0.05) and in synaptic areas (*AP5*= 77.34% ± 6.630, n= 77 dendritic regions (23 neurons); *MK-801*= 76.75% ± 8.334, n= 37 (12 neurons); *Ketamine*= 73.80% ± 5.894, n=73 (23 neurons), p>0.05).

Figure 5

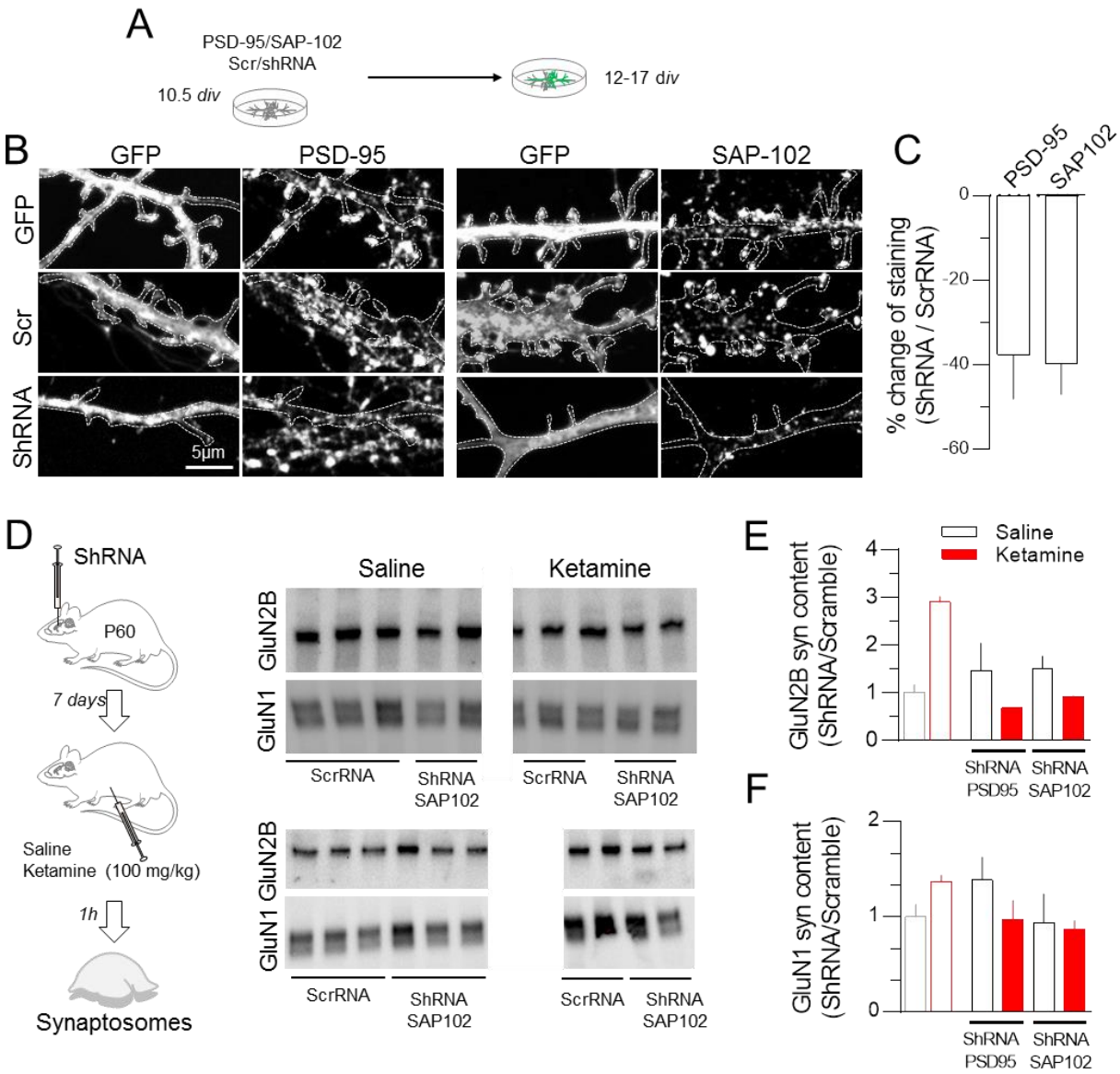


Figure 5. Down-regulation of PSD proteins expression prevents psychotomimetic NMDAR antagonists-induced deficits

(A) Primary hippocampal neurons (10-11*div*) were transfected with PSD95 or SAP102 shRNA/scramble, which were expressed for 2 days (12.5*div*), 4 days (14.5*div*) or 1 week (>17div). Knock-down efficiency was then assessed at the 3 time points by measuring endogenous levels of PSD95 and SAP102.

(B) Representative dendritic areas obtained by immunostaining of endogenous PSD95 and SAP102 levels after 4 days of expression (14.5*div*) with a GFP control plasmid, or with either shRNA or scramble plasmids targeting PSD95 and SAP102 respectively. Scale bar= 5µm. Note that dendritic spines almost disappeared in knock-down condition, while GFP and scramble conditions did not impact spines morphology.

(C) Quantification of PSD95 and SAP102 clusters density after shRNA or scramble transfection. Knock-down efficiency was measured at different time points, and was expressed as a ratio between shRNA and scramble effects (shRNA PSD95 *12.5div*= -20.21%, n= 3 independent experiments; *14.5div*= -49.20%, n=3; *>17div*= -55.01%, n= 1; shRNA SAP102 *12.5div*= -52.87%, n= 2; *14.5div*= -30.73%, n= 3).

(D) Left panel: P8-P10 rats were injected with either scramble or shRNA for PSD-95 and SAP-102. After 7 days of expression, animals from each experimental group received either a saline or a ketamine (100mg/kg) i.p. injection, and were sacrificed 1h after injection for hippocampus dissection and synaptosomes preparation. Right panel: Representative immunoblots showing the expression of GluN1 and GluN2B NMDAR subunits in synaptic-enriched fractions from hippocampi of P8-P10 rats injected as previously described.

(E) Quantification of GluN2B subunit levels in synaptic-enriched fractions of P15-17 rats hippocampi. Data are presented as a shRNA/scramble ratio (Mean ± SEM, *shRNA PSD95 Saline*= 1.516 ± 0.253, n=3 animals; *shRNA PSD95 Ketamine*= 0.9225 ± 0.0135, n=2; *shRNA SAP102 Saline*= 1.460 ± 0.580, n=4; *shRNA SAP102 Ketamine*= 0.680 ± 0.010, n=4, *Saline*= 1.000 ± 0.170, n=4; Ketamine= 2.900 ± 0.120, n=4).(F) Quantification of GluN1 subunit levels in synaptic-enriched fractions of P15-17 rats hippocampi. Data are presented as a shRNA/scramble ratio (Mean ± SEM, *shRNA PSD95 Saline*= 1.388 ± 0.242, n=3 animals; *shRNA PSD95 Ketamine*= 0.975 ± 0.198, n=2; *shRNA SAP102 Saline*= 0.943 ± 0.300, n=4; *shRNA SAP102 Ketamine*= 0.869 ± 0.092, n=4, *Saline*= 1.000 ± 0.130, n=4; Ketamine= 1.370 ± 0.07, n=4)

Figure 6

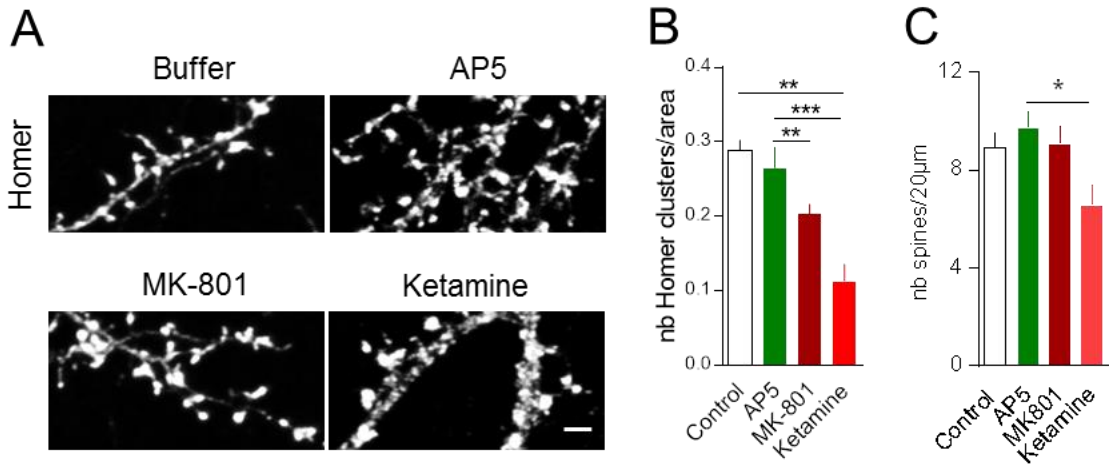


Figure 6. NMDAR channel blockers affect glutamate synapses and spines integrity

(A) Immunostaining of the synaptic protein Homer-GFP in basal condition or after incubation for 1h with the NMDAR antagonists AP5 (50µM), MK-801 (20µM) and ketamine (1µM). Scale bar= 2µm.

(B) Quantification of Homer cluster density (expressed as a number of clusters/area) in the different experimental conditions (Mean ± SEM; *Control*= 0.289 ± 0.014, n= 58 dendritic regions (18 neurons); *AP5*= 0.265 ± 0.028, n= 54 dendritic regions (19 neurons); *MK-801*= 0.204 ± 0.012, n=52 (21 neurons); *Ketamine*= 0.113 ± 0.023, n= 39 (13 neurons); One-way ANOVA followed by a Newman-Keuls multiple comparison test, **p<0.01 ***p<0.001).

(C) Quantification of the spines density (expressed as a number of clusters/20µm) in the different experimental conditions. (Mean ± SEM; *Control*= 8.927 ± 0.600, n= 23 dendritic regions (12 neurons); *AP5*= 9.738 ± 0.648, n= 21 dendritic regions (14 neurons); MK-801= 9.060 ± 0.736, n= 18 (15 neurons); Ketamine= 6.548 ± 0.832, n= 17 (10 neurons); One-way ANOVA followed by a Newman-Keuls multiple comparison test, *p<0.05).

Suppl. Figure 1

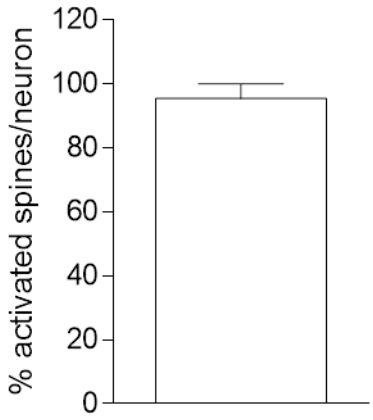


Figure S1. Dissociated hippocampal cultures are spontaneously active

Estimated proportion of activated spines per neuron in dissociated hippocampal cultures (Mean ± SEM= 95.37% ± 4.630, n= 6 neurons). The spontaneous activity of dissociated hippocampal cultures (>15*div*) previously transfected with the calcium indicator GCaMP3 was estimated using calcium imaging (3000 frames, 20Hz acquisition). Fluorescence from calcium transients *vs.* time was measured within individual ROIs manually defined by the experimenter (ImageJ). All pixels within each ROI were averaged to give a single value time course associated to the ROI. Positive calcium transients were identified as values above 2*SD of the background fluorescence value.

Suppl. Figure 2

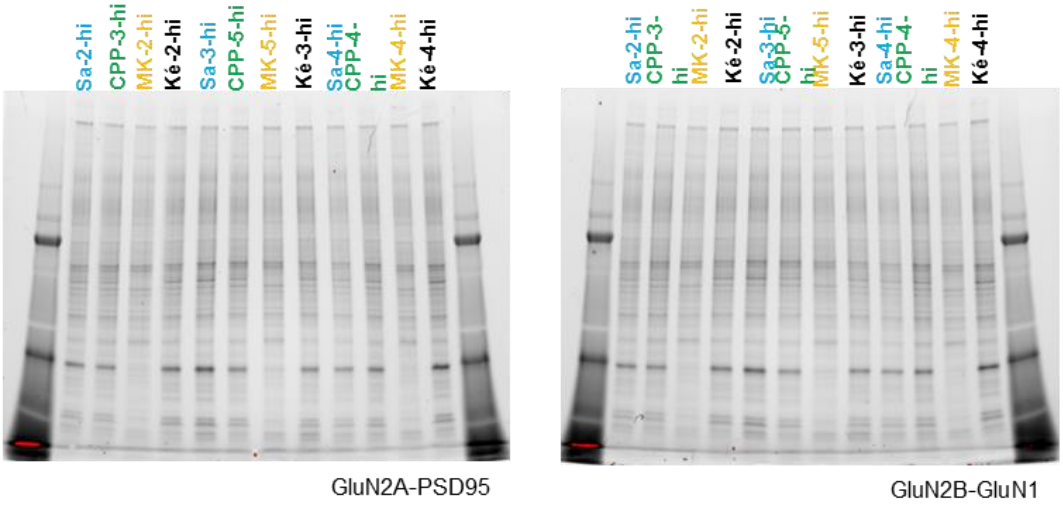


Figure S2. Quantitative immunoblots of NMDAR subunits and PSD-95 using the STAINFREE method

Discussion & Perspectives

Alteration of NMDAR surface trafficking: the molecular signature of psychotomimetic molecules?

The first evidence that NMDAR surface trafficking can be altered in a context of psychosis arose from investigations of NMDAR-Ab encephalitis (Mikasova et al. 2012), an autoimmune disorder associated with major psychiatric features that are directly correlated with the presence of NMDAR-Ab (Dalmau et al. 2007; Graus et al. 2008; Dalmau et al. 2011). Using single-nanoparticle tracking, early work from our group revealed that NMDAR-Ab induce a rapid dispersal of synaptic NMDAR while the surface dynamics of extrasynaptic NMDAR is highly reduced, favoring their internalization and degradation (Hughes et al. 2010; Mikasova et al. 2012; Moscato et al. 2014). Consistently, such modifications of NMDAR surface diffusion are accompanied by functional impairments. NMDAR-Ab induce a decrease in synaptic NMDAR currents (Hughes et al. 2010) and an acute suppression of global neuronal network activity (Jantzen et al. 2013), and also abolish synaptic plasticity processes (Mikasova et al. 2012; Zhang et al. 2012; Dupuis et al. 2014; Planagumà et al. 2015; Würdemann et al. 2016). Remarkably, similar observations were made in presence of NMDAR-Ab from patients with SCZ. We demonstrated that these autoantibodies indeed provoke a rapid increase of NMDAR mobility within the synaptic area and lead to a reduction of NMDAR synaptic content, associated with the suppression of synaptic LTP induction. Whether these functional impairments are caused by a direct action of NMDAR-Ab on the receptor function itself is unclear, although no obvious antagonist-like effect of these antibodies has been reported so far (Mikasova et al. 2012; Moscato et al. 2014). Indeed, we did not observe any change of NMDAR-dependent calcium activity in presence of NMDAR-Ab from SCZ patients, confirming previous observations with NMDAR-Ab from encephalitis patients (Mikasova et al. 2012). In addition, no change in the amplitude of AMPAR/NMDAR-mediated mEPSC was reported after treatment with NMDAR-Ab (Moscato et al. 2014). However, none of these methods provide direct information regarding the action of NMDAR-Ab on the receptor properties. Only one study properly tackled this question by performing single-channel recordings in heterologous cells expressing NMDAR, and reported no major effect of NMDAR-Ab other than a slight increase of NMDAR channel open time duration (Gleichman et al.

2012). Further studies are thus needed to provide a clear conclusion regarding the effect of NMDAR-Ab on NMDAR function, and would greatly help us to better understand the mechanistic behind NMDAR-Ab binding. Investigating whether NMDAR-Ab from different clinical conditions (healthy, encephalitis, SCZ, etc.) provide different outcomes would also be of great interest.

It thus appears from this work that a psychotomimetic molecule such as an NMDAR-Ab can trigger profound alterations of NMDAR surface trafficking, leading to further functional impairments. Whether such alterations of NMDAR surface diffusion are specific to NMDAR-Ab or can be triggered by other molecules with a psychogenic potential is unknown. To further explore the link between psychosis and NMDAR surface trafficking, we assessed the effect of well-known psychotomimetic NMDAR antagonists on NMDAR surface trafficking. Contrary to NMDAR-Ab, an acute application of MK-801 and ketamine strongly reduces NMDAR synaptic diffusion, producing an overexpression of NMDAR at the synapse. In the meantime, surface dynamics of extrasynaptic NMDAR is significantly decreased. Whether LTP is impaired after an acute application of NMDAR channel blockers remains to be tested, but based on previous *ex vivo* and *in vivo* studies, one can expect MK-801 and ketamine to alter synaptic plasticity (Abraham & Mason 1988; Wöhrl et al. 2007; Manahan-vaughan et al. 2008; Wiescholleck & Manahan-Vaughan 2013; Izumi & Zorumski 2014). At first sight, these results can contrast with the ones obtained with NMDAR-Ab. We indeed showed that NMDAR-Ab increase NMDAR synaptic diffusion, while NMDAR antagonists decrease it. Yet, a strong alteration of NMDAR surface trafficking and a subsequent disorganization of NMDAR surface distribution appear as a common feature between both types of psychotomimetic molecules. Furthermore, neither NMDAR-Ab from healthy subjects nor competitive NMDAR antagonists without psychogenic properties showed any effect on NMDAR surface diffusion, further supporting the view that different psychotomimetic molecules may share common pathophysiological pathways.

Non-ionotropic role of NMDAR: a possible influence on NMDAR surface trafficking and its dysregulation by psychotomimetic molecules

Until recently, psychotomimetic effects were thought to be the exclusive property of NMDAR channel blockers (Petrovic et al. 2005). The discovery of psychogenic molecules such as NMDAR-Ab without obvious antagonistic action has strengthened the view that NMDAR inhibition might not be sufficient to explain the emergence of psychosis. Recent studies have reported that NMDAR activation can trigger synaptic plasticity in the absence of ion flow, supporting a non-ionotropic role of the NMDAR. Indeed, low-frequency stimulation produces LTD in presence of the NMDAR antagonists MK-801 or 7-chloro-kynurenate (7CK) whereas AP5 prevents LTD expression, indicating that glutamate binding is required for NMDAR-dependent LTD but not ion flux through the NMDAR (Nabavi et al. 2013; but see Babiec et al. 2014). In line with this, Aβ-induced synaptic depression is abolished in the presence of antagonists that inhibit NMDAR activation (such as AP5 and ifenprodil), but not channel blockers such as ketamine and MK-801 (Kessels et al. 2013). Another study showed that transient application of NMDA decreases spontaneous EPSCs in presence of 7-CK but not AP5 (Aow et al. 2015). Beyond the variable influence of NMDAR blockade on synaptic plasticity, all these studies describe opposite effects between AP5 and MK-801/7-CK. Similarly, we observed a robust differential effect of the competitive NMDAR antagonists AP5 and CPP and the channel blockers MK-801 and ketamine on NMDAR surface dynamics and distribution, indicating that NMDAR inhibition is not predictive of the molecular effects caused by NMDAR antagonists. But how NMDAR inhibition could trigger different molecular effects? Such differences might result from the intrinsic inhibition mechanism of these compounds. AP5 and CPP are competitive antagonists which bind to the glutamate agonist site of the NMDAR (Davies et al. 1986), whereas MK-801 and ketamine are channel blockers targeting the PCP site deep inside the channel (Macdonald et al. 1991). MK-801 and ketamine exhibit pronounced trapping block so that when glutamate has dissociated from its binding site, these drugs remain trapped in the closed channel (Bolshakov et al. 2003). In comparison with AP5 and CPP which show fast dissociation kinetics (Benveniste & Mayer 1991), channel blockers with

a slow off-rate (86% trapping for ketamine, almost 100% trapping for MK-801) cause a prolonged tonic blockade which could disrupt physiological function and explain their pathogenic effects (Sleigh et al. 2014). Indeed, while inhibiting NMDAR with similar affinities and kinetics than ketamine, memantine presents a fast off-rate and is associated with minimal psychotomimetic effects (Kotermanski et al. 2009). Besides, recent crystallographic studies discovered that competitive antagonists binding induces conformational changes of the ATD and LBD of the NMDAR (Jespersen et al. 2014; Zhu et al. 2016). Although the structural impact of channel blockers binding is still unknown, the different functional effects of AP5/CPP and MK-801/ketamine may result from different conformational changes of NMDAR extracellular domains following antagonist binding.

To go one step further, one may hypothesize that modifications of the LBD and ATD conformation can trigger a domino effect and induce conformational changes of intracellular domains of the NMDAR. Using FRET/FLIM imaging between GFP and mCherry fused to the C-terminal tails (C-tails) of GluN1 subunits, Malinow's group observed that agonist binding to the NMDAR in presence of MK-801 or 7-CK leads to conformational changes of NMDAR cytoplasmic domains (Dore et al. 2015). By contrast, in presence of AP5, no movement of NMDAR C-tails occurred following agonist stimulation, leading to the conclusion that extracellular agonist binding drives intracellular conformational changes of the NMDAR in absence of ion flow (Dore et al. 2015; Aow et al. 2015). Regrettably, the authors do not show the effect of agonist binding only, and all the FRET/FLIM measurements have been performed with a co-application of NMDA and the different NMDAR antagonists, which does not allow to differentiate the effect of agonist binding from the effect of antagonist binding. Whether the differences observed between AP5 and MK-801/7-CK result from antagonist binding rather than agonist binding thus appears as an alternative proposition. To tackle this point, we performed preliminary FRET/FLIM measurements of GluN1-GFP/GluN1-mCherry (Dore et al. 2015) in presence of AP5, MK-801 and ketamine in spontaneously activated hippocampal neurons *in vitro*. In opposition to Malinow's work, we observed a decrease of FRET in presence of AP5 but no effect with MK-801 and ketamine, providing the alternative conclusion that extracellular binding of a competitive

antagonist (namely AP5) may trigger intracellular conformational changes. But how can we explain that NMDAR channel blockers produce a different effect? One could argue that the absence of FRET in presence of MK-801 and ketamine is due to a low spontaneous activity of hippocampal neurons *in vitro* which does not allow a proper activation of NMDAR, and thus a proper binding of the drugs. To test this possibility, we measured the spontaneous activity present in cultured hippocampal neurons routinely used in the laboratory using calcium imaging. Within 5min, >90% of identified spines were activated at least once, indicating that MK-801 and ketamine have the potential to block activated NMDAR. Differences between competitive and uncompetitive NMDAR antagonists may then arise from different contributions of intracellular interactions. Within the synaptic area, NMDAR are anchored *via* their C-terminal tails to PDZ-containing scaffolding proteins such as PSD-95 and SAP102. Using FRET/FLIM imaging, one study reported that NMDAR activation decreases the interaction between NMDAR and PSD-95, whereas NMDAR inhibition by MK-801 prevents the transient disruption of NMDAR/PSD-95 complexes occurring upon NMDAR stimulation (Doré et al. 2014). Thus, one may hypothesize that the absence of FRET we observed in presence of uncompetitive NMDAR antagonists can result from abnormal intracellular interactions between NMDAR and MAGUK proteins, which may lock NMDAR C-tails in a rigid conformation, fully consistent with the reduction of NMDAR surface diffusion observed after exposition to MK-801 and ketamine. On the contrary, competitive NMDAR antagonists may not modify intracellular interactions engaged with the NMDAR, thus preserving the dynamic movements of NMDAR C-tails and NMDAR surface trafficking. Intracellular cross-link using antibodies targeting NMDAR C-tails (Dore et al. 2015) followed by FRET/FLIM measurements and single nanoparticle tracking could be useful, though challenging, to test this hypothesis. Thus, although preliminary, our results suggest that NMDAR channel blockers lock NMDAR conformation, supporting the idea that ionotropic-independent functions of the NMDAR could promote abnormal NMDAR synaptic anchoring with consequent alterations of NMDAR surface trafficking and distribution. Accordingly, we showed that the downregulation of PSD-95 and SAP102 prevents the upregulation of synaptic NMDAR expression

induced by NMDAR channel blockers (see above). It would be interesting to further test this possibility by using competing peptides that disrupt the interaction between NMDAR and PSD-95 (Bard et al. 2010), and observe if NMDAR channel blockers still cause molecular deficits. Further experiments in presence of the agonist NMDA will also be crucial to better understand the mechanistic behind NMDAR inhibition induced by the different types of antagonists, and put our results in perspective with the current literature.

Is NMDAR dysfunction receptor and/or cell specific?

NMDAR are widely distributed over the CNS (Monyer et al. 1992; Monyer et al. 1994). Theoretically, if NMDAR antagonists or NMDAR-Ab would indistinctly target all NMDAR, patients would not only exhibit psychotic symptoms but would suffer from severe autonomous disorders. Thus, one may hypothesize that psychotomimetic molecules target a subpopulation of NMDAR. But what would determine these receptors as targets? Glycosylation and deamidation of the N368 region located on the extracellular part of the GluN1 subunit of the NMDAR have been proposed to contribute to the epitope formation of NMDAR-Ab in the eponymous encephalitis (Gleichman et al. 2012; Gresa-Arribas et al. 2013). Deamidation and glycosylation likely play a role in immune mechanisms (Skovbjerg et al. 2008; Ostankovitch et al. 2010). Thus, post-translational processes might contribute to the formation of immunogenic epitopes in SCZ. Evidences of epigenetic dysregulation have also been found in SCZ, and even constitute a target for drug therapy (Hasan et al. 2013). In addition, DNA methylation has been shown to induce immunopathogenicity (Thabet et al. 2012). Epigenetic modifications might thus create a “tag”, identifying the receptor as a target. It would be interesting to explore whether epigenetic modifications occur specifically in the subset of patients producing NMDAR-Ab. *Post-mortem* studies might be helpful to explore some of these processes, but fixation *per se* can modify receptor conformation and does not allow to explore receptor dynamics. A better possibility would be to use iPSC cells from patients with SCZ and examine whether NMDAR expressed at the surface of these cells intrinsically differ from those of healthy subjects. In addition to targeting

a specific population of NMDAR*,* specific cell populations may exhibit a hypersensitivity to NMDAR-Ab binding or NMDAR antagonist inhibition. CSF from patients with NMDAR-Ab encephalitis causes a significant decrease of surface NMDAR cluster density both in excitatory and inhibitory neurons, suggesting broad actions of NMDAR-Ab on cell types (Moscato et al. 2014). Of note, the specific effect of NMDAR-Ab from SCZ subjects on inhibitory cells has not been explored yet. Nevertheless, several studies propose that GABAergic interneurons, especially PV-positive interneurons, are preferentially affected in SCZ (Nakazawa et al. 2012). Indeed, NMDAR expressed on hippocampal GABAergic interneurons might be more sensitive to NMDAR antagonists than pyramidal neurons. NMDAR inhibition on pyramidal neurons results in a profound excitatory activity whereas NMDAR blockade on GABAergic neurons generates a disinhibition of GABAergic neurons, mainly contributing to the aberrant excitatory activity of pyramidal neurons (Homayoun & Moghaddam 2007). It would thus be of particular interest to investigate the effects of NMDAR-Ab on PV interneurons, and examine whether NMDAR-Ab and NMDAR channel blockers equally alter NMDAR surface trafficking in glutamatergic and GABAergic cell types.

NMDAR-Ab: virtually the same but all different

To date, the evidence for a direct pathological role of NMDAR-Ab has only been clearly demonstrated in NMDAR-Ab encephalitis (Hughes et al. 2010; Planagumà et al. 2015; Planagumà et al. 2016). Still, recent clinical and experimental evidences point towards a possible involvement of NMDAR-Ab in the pathogenesis of, at least, a subset of SCZ patients (Lennox et al. 2012; Pariante 2015; Leboyer et al. 2016). However, the detection of NMDAR-Ab in healthy subjects is intriguing, and calls into question the pathogenic relevance of NMDAR-Ab in SCZ. To further clarify this point, we compared the molecular effect of NMDAR-Ab purified from the serum of patients with SCZ and healthy subjects. Strikingly, our results revealed that NMDAR-Ab from both origins display different molecular pathogenicity while targeting the same receptor, *i.e.* the NMDAR. While NMDAR-Ab from SCZ patients induce strong impairments of the NMDAR in the glutamatergic synapse, NMDAR-Ab from

healthy subjects are without any effect. But, why some NMDAR-Ab would be pathogenic but not some others? The question of the epitope appears as a crucial one. Using a classical immuno-competition assay, we tested whether NMDAR-Ab from different clinical conditions (healthy, SCZ and NMDAR-Ab encephalitis subjects) share (a) common epitope(s). Our results suggest the existence of several binding sites and support the idea that NMDAR-Ab do not share a unique epitope, even within the same group of individuals. Using site-directed mutagenesis/deletions for epitope mapping, the N368 residue located on the extracellular part of the GluN1 subunit of the NMDAR was identified as a necessary component for the epitope formation of NMDAR-Ab in NMDAR-Ab encephalitis (Gleichman et al. 2012). Among the various GluN1/GluN2 mutants generated, the N368Q mutation completely abolishes the staining of NMDAR-Ab, indicating that glycosylation of residue N368 may be a necessary component of the epitope. From our part, we did not observe any reduction of the staining of NMDAR-Ab from encephalitis patients, neither in HEK cells, nor in dissociated hippocampal neurons expressing the N368Q. This discrepancy can be easily explained by the fact that NMDAR-Ab are likely polyclonal and do not recognize a single epitope. In line with this, one study reported that mutation of the N368 region abrogates only ≈ 65% of NMDAR-Ab reactivity of samples from patients with NMDAR-Ab encephalitis, while it does not affect the binding of NMDAR-Ab in SCZ (Castillo-Gómez et al. 2016). Possibly due to the restricted amount of SCZ patients with NMDAR-Ab and to the tedious technique of epitope mapping, only one study explored NMDAR-Ab binding in SCZ and other clinical conditions (Castillo-Gómez et al. 2016). Although conceptually of great importance, this work does not provide conclusive elements, as i) only one SCZ patient was included and does not allow intra-group comparison, ii) epitope mapping was performed using variable concentrations of serum, which can either enhance false positive or false negative, and iii) no clear images of the staining resulting from the different mutations were provided. Hence, the search for NMDAR-Ab epitopes in SCZ represents a major objective to explore inter-individuals differences and elucidate the mechanisms underlying antibody pathogenicity.

Inter-individual differences could also reflect the presence of several pathogenic immunoglobulins (Ig) subtypes. Although the majority of studies focused on the IgG subclass, the pathogenicity of IgA and IgM NMDAR-Ab has also been explored *in vitro* and *in vivo.* To date, several studies have reported the presence of IgA and IgM NMDAR-Ab in the serum of patients with NMDAR-Ab encephalitis (Prüss et al. 2012), SCZ (Hammer et al. 2014; Johann Steiner et al. 2013; Steiner et al. 2014) and neuropsychiatric disorders (Prüss et al. 2012; Hammer et al. 2014; Choe et al. 2013; J Steiner et al. 2013; Steiner et al. 2014; Doss et al. 2014; Castillo-Gómez et al. 2016) but few of them have explored their potential cellular pathogenicity (Prüss et al. 2012; Choe et al. 2013; Hammer et al. 2014; Castillo-Gómez et al. 2016). Both experimental and clinical data suggest that NMDAR-Ab of IgA and IgM subclass share with IgG antibodies the potency to trigger pathogenicity. However, the results obtained so far should be interpreted with caution knowing that: 1) patients and healthy controls often exhibit similar IgA/IgM seroprevalence (Hammer et al. 2014; J Steiner et al. 2013; Steiner et al. 2014), 2) most of the cohorts were composed of aging populations, a factor linked with increased IgA/IgM prevalence, and 3) several studies mixed patients with various clinical conditions, which does not allow proper interpretations (Doss et al. 2014; Castillo-Gómez et al. 2016). It would thus be of great interest to conduct further and larger studies to define the clinical phenotype associated with the presence of IgA and IgM NMDAR-Ab. Longitudinal studies will also be essential to follow the clinical evolution of seropositive patients in order to understand whether an acute immune boost is correlated or not with the expression of a psychotic episode, and could potentially help to address these outstanding questions: why only a subset of schizophrenic patients presents pathogenic antibodies? Are first-episode psychotic patients with autoantibodies more prone to develop SCZ? Are healthy subjects with NMDAR-Ab really healthy? Are they healthy carriers or will they later develop SCZ or related psychotic disorders?

Alternative therapeutics to current antipsychotics

Since the fortuitous discovery of the neuroleptic properties of chlorpromazine, almost all the current antipsychotic pharmacopeia relies on dopamine D2 receptor blockade. For several decades, drug development has mainly focused on molecules modulating monoaminergic receptors activity in order to either reduce dopaminergic signaling and/or potentiate glutamatergic transmission. But one has to admit that such a strategy has shown some limitations, possibly due to the fact that antipsychotics target downstream modulatory processes. Although the importance of current antipsychotic treatments to alleviate positive symptoms and to improve negative and cognitive symptoms must be recognized, approximately one third of schizophrenic patients are resistant or partially responsive to the existing antipsychotic treatments and have reached a therapeutic impasse (Wimberley et al. 2016). The recent identification of autoantibodies in patients with SCZ, in addition to the chronic inflammatory state present in the disease, suggest that immunotherapy and anti-inflammatory treatments can have a beneficial impact, at least in a subset of patients (Khandaker et al. 2015). Immunotherapy indeed showed a beneficial impact in first-episode psychotic patients with circulating autoantibodies (Zandi et al. 2011; Zandi et al. 2014; Senda et al. 2016; Gungor et al. 2016) while anti-inflammatory molecules such as celecoxib, minocycline and aspirin efficiently improved the clinical condition of SCZ-resistant patients, although randomized controlled trials are few and studies have often involved small samples (Andrade 2015; Khandaker et al. 2015). However, like antipsychotics, anti-inflammatory drugs modulate many targets and elicit a global and indirect response, which can potentially trigger both positive and side effects. Promising strategies arise from the modulation of the glutamatergic system, according to its proposed involvement in SCZ pathogenesis. Yet, first trials with compounds such as D-cycloserine specifically acting on the NMDAR and promoting its activation have not provided convincing outcomes (De Bartolomeis et al. 2012). The possible role for ionotropic-independent functions of the NMDAR in the regulation of its signaling then supports alternative strategies such as the modulation of NMDAR surface trafficking. NMDAR is a central hub of the glutamatergic synapse (Ladépêche et al. 2013) and interacts with

various neuromodulators through multiple protein-protein interactions. Among them, the functional cross-talk between NMDAR and dopamine receptors could constitute a relevant therapeutic target, fully consistent with the pathophysiological implication of both neurotransmitter systems in SCZ. The non-negligible amount of patients with NMDAR-Ab encephalitis suspected for neuroleptic malignant syndrome, a side-effect syndrome mimicking some of the symptoms of SCZ (Lejuste et al. 2016), further strengthens the relevance of designing therapeutic tools that directly and specifically modulate the NMDAR/dopamine interplay.

Conclusion & future perspectives

My thesis project aimed at investigating the molecular impact of different psychotomimetic molecules on NMDAR surface trafficking, and assessing whether a dysfunction of NMDAR surface dynamics could constitute a "pathogenic step" to the emergence of psychotic disorders. From this work, several key messages emerge. First, the discovery that NMDAR-Ab exhibit different molecular pathogenicity calls for great caution in using autoantibodies as biomarkers for brain disorders. Our results clearly demonstrate that the presence of NMDAR-Ab is not predictive of a pathogenic effect. Second, NMDAR-Ab are detectable in the serum of SCZ patients and induce strong molecular and functional deficits in the glutamatergic synapse. Third, NMDAR antagonists differentially impact on NMDAR lateral diffusion, and that NMDAR alterations specifically caused by psychotomimetic NMDAR antagonists are likely mediated through intracellular interactions of the NMDAR with PSD proteins. Fourth, a dysregulation of NMDAR surface trafficking appears a common feature between different psychotomimetic molecules, as both NMDAR-Ab and psychotomimetic NMDAR antagonists significantly impair NMDAR surface dynamics. Fifth, dysregulation of NMDAR surface trafficking likely occurs through abnormal NMDAR membrane and intracellular interactions which may involve non-ionotropic functions of the NMDAR.

Overall, these results strengthen previous work from our group, and strongly support the view that NMDAR surface dynamics is a key regulatory mechanism, and an unsuspected potential mechanism contributing to the pathophysiology of SCZ and psychotic disorders. Future studies will need to strengthen this hypothesis by looking whether a dysregulation of NMDAR surface dynamics appears as a convergent molecular alteration between different models of SCZ. Future work should also explore the pathogenic relevance of NMDAR surface trafficking impairment *in vivo* and its potential role in the etiology of SCZ, and will likely open new avenues of research for the development of alternative antipsychotic treatments.

Annexes

Ultradian corticosterone pulses balance glutamatergic transmission and synaptic plasticity

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This work resulted from a collaboration with the laboratory of Dr. Marina Joels in Utrech. My contribution to this work consisted in the validation of the electrophysiological results by performing AMPAR immunostaining following a chemical LTP protocol.

Ultradian corticosterone pulses balance glutamatergic transmission and synaptic plasticity

Ratna Angela Sarabdjitsingh[a,1], Julie Jezequel[b,c], Natasha Pasricha[a,b,c], Lenka Mikasova[b,c], Amber Kerkhofs[a,b,c], Henk Karst[a], Laurent Groc[b,c,1,2], and Marian Joëls[a,1,2]

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The rodent adrenal hormone corticosterone (CORT) reaches the brain in hourly ultradian pulses, with a steep rise in amplitude before awakening. The impact of a single CORT pulse on glutamatergic transmission is well documented, but it remains poorly understood how consecutive pulses impact on glutamate receptor trafficking and synaptic plasticity. By using high-resolution imaging and electrophysiological approaches, we report that a single pulse of CORT to hippocampal networks causes synaptic enrichment of glutamate receptors and increased responses to spontaneously released glutamatergic vesicles, collectively abrogating the ability to subsequently induce synaptic long-term potentiation. Strikingly, a second pulse of CORT one hour after the first—mimicking ultradian pulses—completely normalizes all aspects of glutamate transmission investigated, restoring the plastic range of the synapse. The effect of the second pulse is precisely timed and depends on a nongenomic glucocorticoid receptor-dependent pathway. This normalizing effect through a sequence of CORT pulses—as seen around awakening—may ensure that hippocampal glutamatergic synapses remain fully responsive and able to encode new stress-related information when daily activities start.

hippocampus | AMPA receptor trafficking

The rodent stress hormone corticosterone (CORT) is synthesized in the adrenal glands. Through the circulation, it reaches the brain, where it binds to intracellular receptors, i.e., the high-affinity mineralocorticoid receptor (MR) and lower affinity glucocorticoid receptor (GR) (1). These receptors are enriched in the hippocampus and act as transcriptional regulators (2). Intracellular MRs are already considerably occupied with low (i.e., nonstress) levels of CORT, whereas GRs become substantially bound only when corticosteroid levels increase (1).

After stress, hormone levels slowly increase and normalize after 2 h as a result of negative feedback actions in the pituitary and hypothalamus (3). Corticosteroid levels also show an endogenous circadian variation, which in fact is carried by brief hourly (ultradian) pulses (4). Pulse amplitudes steeply rise some hours before awakening and then slowly diminish again (5). The functional relevance of ultradian pulses is only starting to be understood; recent evidence suggests that pulses are necessary to maintain optimal transcriptional activity of GRs (4–8), thus coordinating essential bodily functions in preparation of the active phase.

It is well known that, through DNA binding and transcriptional regulation, GRs slowly and persistently alter glutamatergic transmission in the hippocampus (9–11). A brief CORT pulse increases the surface expression and synaptic localization of AMPA receptor (AMPAR) subunits (10) and increases the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (11)—the postsynaptic response to a spontaneously released glutamate-containing vesicle. CORT-dependent signaling is thought to (partly) overlap with pathways giving rise to long-term potentiation (LTP) (12, 13). In agreement, administration of CORT occludes the subsequent induction of chemically (10) and electrically evoked LTP (9, 14). In line with GRs acting as transcriptional regulators, these effects become apparent ∼1 h after CORT administration and last for many hours. We here examined how glutamatergic transmission in the hippocampus is affected when cells are exposed to a second pulse of CORT 1 h after the first, mimicking the ultradian pattern of endogenous CORT release (6).

Results

Consecutive CORT Pulses Normalize AMPAR Surface Dynamics and Synaptic Content. Previously, we showed in hippocampal neurons that a single pulse of CORT drastically increases the surface diffusion and synaptic content of GluA2- and GluA1-containing AMPARs several hours later (10). This CORT-induced synaptic potentiation of AMPARs was prevented by selectively and artificially blocking AMPAR surface diffusion. Furthermore, the increased surface trafficking and synaptic accumulation of AMPARs after CORT exposure likely contributed to the occlusion of classical synaptic LTP (10, 13). To investigate the impact of CORT pulsatility, we first examined how two 10-min pulses of CORT (100 nM) with an interval of 1 h affect the surface dynamics of AMPARs. For this, we took advantage of the single particle tracking approach to monitor individual particle/receptor complexes in hippocampal cultured neurons (Fig. 1*A*). Hippocampal networks were exposed to one or two pulses, and single nanoparticle imaging was performed 120 min after onset of the first exposure (Fig. 1*B*). Consistent with previous results (10), a single CORT pulse significantly increased the surface diffusion of

Significance

A pulse of the adrenal hormone corticosterone (CORT) changes hippocampal glutamate transmission for many hours. CORT is normally released in hourly pulses, with a steeply rising amplitude just before awakening. How organisms can be prepared for imminent danger if the first high-amplitude pulse of CORT would lastingly change glutamate transmission—thus potentially deadlocking the system—has remained an enigma. We show that exposure of hippocampal cells to a second high-amplitude CORT pulse completely normalizes all aspects of glutamate transmission (including synaptic plasticity), thus lifting the potential deadlock caused by a first pulse. This ensures that the system remains fully responsive to any stressful event that requires encoding of information, an important principle that promotes survival of individuals.

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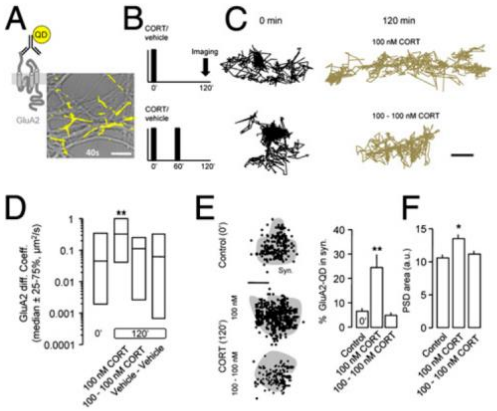

Fig. 1. Consecutive CORT pulses normalize AMPAR surface distribution and synaptic content. (*A*) Surface GluA2 was labeled with a single quantum dot (QD)-antibody complex (*Left*), allowing single particle tracking. (*Right*) Representative trajectories (yellow lines; 40-s duration imaging; 20-Hz acquisition rate) in cultured hippocampal neurons (15 d in vitro). (Scale bar: 5 μm.) (*B*) Schematic representation of the experimental protocol. Neurons were exposed to one pulse of 100 nM CORT (or vehicle) for 10 min or two consecutive 100 nM CORT pulses (or vehicle) with an interval of 60 min. In all experiments, surface AMPAR trafficking was assessed at 120 min relative to the onset of the first CORT application. (*C*) Representative single GluA2–AMPAR trajectories in the different experimental paradigms described in *B*. Note the increased lateral diffusion 120 min after a single pulse, an up-regulation not observed in presence of two pulses (100–100 nM CORT). (Scale bar: 500 nm.) (*D*) Distribution (median ± 25–75% interquartile range) of the instantaneous diffusion coefficients of surface GluA2–AMPAR, measured 120 min after one pulse of 100 nM CORT or two consecutive 100 nM CORT pulses (or vehicle). The diffusion coefficient was significantly increased after a single CORT pulse (0′; n = 2,790 trajectories; 100 nM CORT, n = 916; 100–100 nM CORT, n = 449; vehicle–vehicle, n = 452; Kruskal–Wallis test, $P < 0.05$; post hoc tests, $^{**}P < 0.01$, 0′ control vs. 100 nM CORT; $P > 0.05$, 100 nM CORT vs. other conditions). (*E*) Single QDs (black dots) can be located with a high pointing accuracy in a given membrane compartment, such as the synapse (syn., gray area). (*Left*) Examples of 500 frame stacks obtained while tracking down single GluA2–AMPAR/QD complexes. Those 500 locations are then projected on a single image, providing the successive positions of this receptor/particle complex in the various experimental paradigms. (*Right*) Relative fraction of synaptic GluA2–AMPAR/QD particles (control, n = 28 neuronal fields; 100 nM CORT, n = 19; 100–100 nM CORT, n = 27; Kruskal–Wallis test, $P < 0.05$; post hoc tests, $^{**}P < 0.01$, control vs. 100 nM CORT or 100 nM CORT vs. 100–100 nM CORT; $P > 0.05$, control vs. 100–100 nM CORT). (*F*) The PSD area was quantified by using immunocytochemical staining of Homer 1c or Shank proteins (control, n = 315 PSDs; 100 nM CORT, n = 259; 100–100 nM CORT, n = 212; one-way ANOVA test, $P < 0.05$; $^{*}P < 0.05$, control vs. 100–100 nM CORT or 100 nM CORT vs. 100–100 nM CORT).

GluA2–AMPAR (Fig. 1 *C* and *D*). Strikingly, the introduction of a second pulse of CORT—i.e., 60 min after the first—abolished this effect, yielding a surface diffusion comparable to the vehicle control situation (Fig. 1 *C* and *D*). Of note, the second pulse did not simply delay the CORT-induced AMPAR surface diffusion increase, as the dynamics measured 120 min after the second pulse (at t 180 min) were undistinguishable from the one measured 60 min after two pulses at t 120 min (value at t 180 min GluA2–AMPAR surface diffusion: median, 0.092 $\mu m^2/s$; interquartile range, 4.10^{-3} to 0.14 $\mu m^2/s$; n = 670 trajectories; $P > 0.05$ vs. CORT–CORT diffusion at 120 min; Fig. 1). The diffusion changes observed after one or two CORT pulses at t 120 min appear to be mostly caused by a change in the mobile fraction (diffusion > 0.005 $\mu m^2/s$), as control (54%) and two-pulse (47%) values were lower than the fraction seen after one pulse (76%). Together, to our knowledge, these data provide the first evidence that consecutive pulses of CORT restore the surface trafficking of AMPARs toward a basal range.

We next investigated the impact of consecutive CORT pulses on synaptic AMPAR signaling by using high-resolution imaging and electrophysiological means. First, the single particle tracking approach allows to point, with subwavelength accuracy (~20 nm resolution), the location of tagged-receptors in specific membrane compartments, such as the synapse. Based on that, we estimated that, on average, fewer than 10% of the labeled AMPARs are present in the synapse under control conditions. This value significantly increased after exposure to a single CORT pulse whereas it was comparable to baseline after two pulses (Fig. 1*E*), indicating that the second pulse reverses or normalizes the CORT-induced GluA2-AMPAR synaptic accumulation caused by the first pulse. A similar effect was observed when we quantified the area of the postsynaptic density (PSD) of synapses exposed to a single or two CORT pulses (Fig. 1*F*). Of note, the increase in GluA2–AMPAR synaptic fraction observed after one pulse (+304%) was larger than the increase of the PSD area (122%), suggesting that the altered AMPAR content is an active process that cannot solely be explained by a change in PSD area. Together, these data demonstrate that a second pulse of CORT (60 min after the first pulse) reverses or prevents the synaptic accumulation of glutamate synapses observed 120 min after a single pulse.

We predicted that functional indices of glutamatergic transmission would follow a similar pattern, i.e., that signals via AMPAR subunits would be enhanced 2 h after a single pulse but normalized when tested after two consecutive pulses. In CA1 pyramidal neurons, recorded in slices from 2-mo-old mice, we first replicated that, ~120 min after a single brief (10 min) pulse of CORT, the mEPSC amplitude was significantly increased (vehicle, 14.3 ± 0.6, n = 9; CORT, 18.4 ± 1.3, n = 7; $P < 0.01$; Fig. 2). As predicted, such increase was not seen in a second series of experiments in which the 2-h delay after the first pulse was interrupted by another CORT pulse (60-min interval between pulses; vehicle, 16.4 ± 1.0, n = 7; CORT, 16.9 ± 1.0, n = 8; $P = 0.73$; Fig. 2). In none of the conditions did CORT treatment affect mEPSC frequency (all $P > 0.05$; Table S1). Altogether, these data provide direct evidence that two consecutive pulses, 60 min apart, restore the AMPAR surface dynamics and synaptic content to values close to those seen under basal conditions.

CORT Pulses Restore the Plastic Range of Hippocampal Glutamate Synapses. Earlier studies showed that exposure of hippocampal slices to a single pulse of CORT impairs the induction of LTP in subsequent hours (9, 14). This was interpreted as a CORT-induced

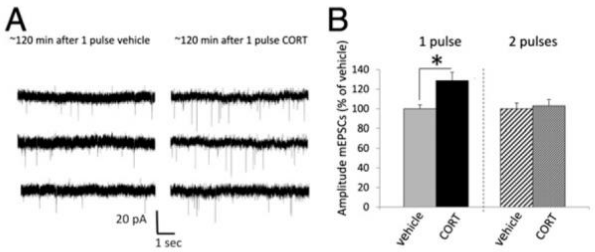

Fig. 2. A single CORT pulse increases mEPSC amplitude, whereas two pulses do not. (*A*) Typical traces showing mEPSCs of a mouse CA1 hippocampal cell ~120 min after exposure to a brief (10 min) pulse of vehicle (*Left*) or 100 nM CORT (*Right*). (*B*) The mEPSC amplitude was significantly enhanced in neurons recorded ~120 min after a single pulse of CORT as opposed to vehicle (*Left*). By contrast, two consecutive CORT pulses, with a 1-h interval, caused no change in mEPSC amplitude compared with two vehicle pulses when neurons were recorded ~120 min after onset of the first pulse. Data represent the mean ± SEM of the mEPSC amplitudes recorded 5–10 min after establishing the whole cell configuration. To allow easy comparison, we normalized the effects of CORT exposure to those of vehicle in both experiments (see main text for absolute values). $^{*}P < 0.01$, unpaired t test.

occlusion of pathways necessary for the development of LTP (12, 13). We therefore examined how synaptic plasticity is affected by consecutive pulses. First, we tested the impact of consecutive pulses on LTP expression in hippocampal cultured neurons by using a previously described chemical LTP (cLTP) induction protocol (10) and measuring the synaptic content (synapses were identified by Homer 1c detection) of AMPARs through live immunocytochemical staining (Fig. 3*A*). We focused our attention on GluA1–AMPAR because of their primary and well-characterized recruitment to synapses following LTP (15). Application of a single pulse of CORT prevented the cLTP-induced GluA1–AMPAR synaptic recruitment, as previously described (10) (Fig. 3 *B* and *C*). However, when neurons were exposed to two consecutive pulses, full-blown synaptic recruitment was observed 120 min after the first pulse (Fig. 3*B*), indicating that the second pulse restores the potentiation range of the glutamate synapses. Consistent with the aforementioned electrophysiological data, two consecutive pulses of CORT maintained the synaptic content of GluA1–AMPAR close to basal values (Fig. 3*C*). Together, these data indicate that a second pulse of CORT normalizes the potentiation range of glutamate synapses, which was occluded by the first pulse.

Hippocampal cultured cells are not connected to their normal afferent and efferent fibers as in the intact brain. We therefore switched to acutely prepared hippocampal slices from young adult mice and used a high-frequency paradigm that (in vehicle-treated slices) causes mild LTP in the CA1 area (Fig. 4 *A1* and *B*). Induction of LTP was fully prevented by a single 10-min pulse of CORT applied to the slices 120 min before high-frequency stimulation [$F_{(2, 21)} = 15.77$; $P < 0.001$; vehicle vs. one CORT pulse; $P < 0.001$; Fig. 4 *A1*, *B*, and *C*]. However, if we applied two CORT pulses (with a 1-h interpulse interval), synaptic potentiation caused by high-frequency stimulation 120 min after the first pulse was comparable to that seen in the vehicle group ($P > 0.1$ vs. vehicle; Fig. 4 *A2*, *B*, and *C*). As 100 nM CORT is most likely a concentration not reached under physiological conditions, we also tested two pulses of 30 nM CORT. This resulted in comparable results [$F_{(2, 21)} = 9.87$; $P < 0.01$], i.e., suppression of LTP 120 min after a single CORT pulse ($P < 0.01$ vs. vehicle), but efficient LTP after two pulses ($P > 0.1$ vs. vehicle; Fig. 4*D* and Fig. S1*A*). We also wondered to what extent the naturally occurring 1-h interval is relevant for the observed phenomenon. As is evident from Fig. 4*E* and Fig. S1*B*, the interval is indeed important [$F_{(4, 29)} = 12.52$; $P < 0.001$]: no normalization of LTP was seen when the second pulse was given 10 min after the first pulse ($P < 0.001$ vs. vehicle), and only partial normalization was found with an interpulse interval of 30 min ($P < 0.05$ vs. vehicle). With intervals of 60 or 90 min, LTP induced 120 min after onset of the first pulse was comparable to that in the vehicle control group ($P > 0.1$ vs. vehicle). Together, these results suggest that the CORT-induced restoration of LTP is physiologically relevant and depends on the timing of the second CORT pulse.

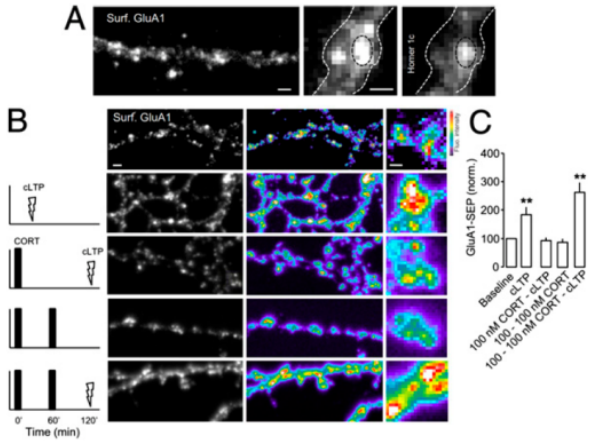

Fig. 3. Consecutive CORT pulses restore cLTP-induced AMPAR potentiation. (*A*) (*Left*) Representative dendritic fragment of hippocampal neurons expressing GluA1–SEP AMPAR. (Scale bar: 1 μm.) (*Right*) Enlargement of a synaptic GluA1–SEP cluster, with the postsynaptic area labeled by the detection of Homer-1c (dashed black line). (*B*) (*Left, Center*) GluA1–SEP dendritic fragments from neurons under basal condition or after the application of a cLTP protocol with or without the presence of CORT pulses [protocols (*Left*): control cLTP, 100 nM CORT cLTP, 100–100 nM CORT cLTP, or 100–100 nM CORT]. The pseudocolor GluA1–SEP subunit representation shows the different intensity levels of the GluA1–SEP staining. (Scale bar: 1 μm.) (*Right*) Enlargement of a GluA1–SEP cluster. (Scale bar: 500 nm.) Note that cLTP fails to potentiate GluA1–SEP fluorescence after a single CORT pulse. (*C*) Normalized measures of GluA1–SEP clusters intensity compared with baseline (n = 3,495 clusters). The cLTP protocol induced an increased GluA1–SEP expression in control condition (one-way ANOVA test, $P < 0.05$; control cLTP, n = 2,619 clusters; $^{**}P < 0.01$) and after consecutive CORT pulses (100–100 nM CORT cLTP, n = 1,458 clusters; $^{**}P < 0.01$), whereas GluA1–SEP fluorescence remained unchanged after a single pulse (100 nM CORT cLTP, n = 4,372 clusters).

CORT Pulses' Effects on the Plastic Range Are Nongenomic and GR-Dependent. To better understand the mechanism contributing to normalization of LTP as seen after two pulses of CORT, we first questioned whether the second pulse reversed or prevented the effect of the first pulse. Therefore, we tested if suppression of LTP after a single pulse was already seen after 60 min, i.e., at the time point at which the second CORT pulse normally would be delivered. As shown in Fig. 5*A* and Fig. S2*A*, this was indeed the case [$F_{(4, 30)} = 10.75$; $P < 0.001$; $P < 0.001$ vs. vehicle). Additionally, to exclude time-dependency of CORT effects, we tested for LTP 120 min after the second pulse (at t 180 min). At this time point, LTP was efficiently induced, suggesting that the second pulse lastingly reverses or compensates for the consequences of the first CORT application ($P < 0.05$ vs. 100 nM CORT; Fig. 5*A* and Fig. 2*A*). The results from the experiment in which two pulses were applied with a 90-min interval (leaving only 20 min between the end of the second pulse and LTP induction; Fig. 4*E*) suggest that this reversal or compensation develops rapidly, in a time frame that seems incompatible with the classic genomic pathway. Such nongenomic signaling by CORT has indeed been described in recent years (16–18). In the hippocampus, nongenomic signaling so far was found to involve MRs (16), but, in other areas of the brain (e.g., the hypothalamus or amygdala), rapid effects through GRs have been described (17, 18). To determine the receptor mediating the reversal or compensation, we applied the second pulse of CORT in the presence of an MR or GR antagonist. As is clear from Fig. 5*B* and Fig. S2*B*, we observed significant differences [$F_{(4, 34)} = 13.68$; $P < 0.001$] between the treatment groups. The MR antagonist spironolactone was entirely ineffective in blocking the effect of CORT ($P > 0.1$ vs. vehicle), whereas the GR antagonist mifepristone fully prevented the normalizing effect of the second CORT pulse ($P < 0.001$ vs. vehicle), supporting involvement of GRs. Nongenomic, as opposed to genomic, actions of CORT can be mimicked by the membrane impermeable conjugate of CORT to BSA (CORT–BSA). In agreement with a nongenomic pathway, normalization of LTP was replicated when slices were exposed to CORT–BSA, delivered 60 min after a first pulse of CORT [$F_{(4, 33)} = 4.16$; $P < 0.01$; $P > 0.1$ vs. vehicle or 100–100 nM CORT; Fig. 5*C* and Fig. S2*C*]; in agreement with the genomic pathway underlying the response to the first pulse, a single pulse of CORT–BSA was unable to suppress LTP induced 120 min later ($P > 0.1$ vs. vehicle). Finally, we reasoned that, if the second pulse of CORT would compensate for (i.e., act in the opposite direction) rather than reverse the response to the first pulse, a sequence of two pulses of CORT–BSA might result in enhanced LTP compared with the effect seen with two pulses of CORT. Although LTP levels were, on average, slightly increased after two pulses of

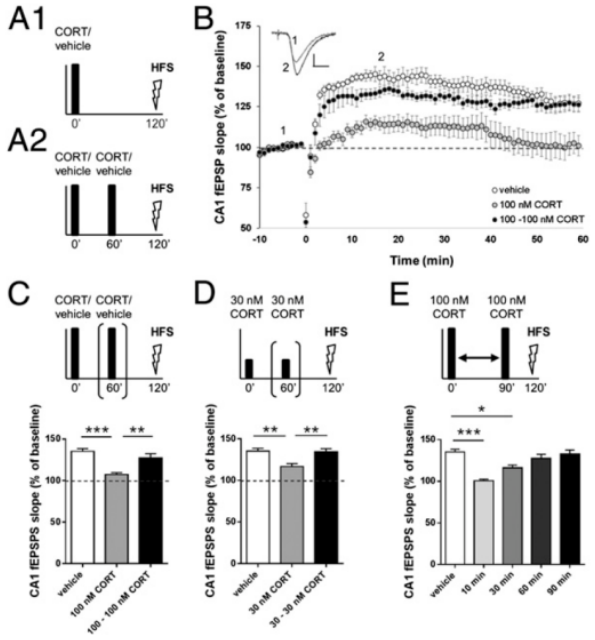

Fig. 4. Synaptic plasticity in hippocampal slices is restored by consecutive CORT pulses. (*A1* and *A2*) Schematic representation of the experimental protocol. (*A1*) Mouse brain slices were exposed to one pulse of 100 nM CORT (or vehicle) for 10 min. (*A2*) Brain slices were exposed to two consecutive 100 nM CORT pulses (or vehicle) with an interval of 60 min. In both experiments, high-frequency stimulation (HFS) was applied at 120 min relative to the onset of the first CORT application. (*B*) HFS (10 Hz, 90 s) resulted in significant potentiation of synaptic responses at CA1 synapses in vehicle-treated brain slices (open circles). LTP was attenuated by 100 nM CORT given 120 min before HFS (gray circles). A second application of 100 nM CORT 60 min after the first pulse reversed this effect (black circles). (*Upper*) Representative individual fEPSP traces for the vehicle group taken during baseline or ∼20 min after HFS. (Scale bars: horizontal, 5 ms; vertical, 0.5 mV.) (*C*) Averaged mean values during the 60-min posttetanic recording period indicate significantly lower LTP in slices treated 120 min earlier with one 100 nM CORT pulse (gray bar) compared with vehicle (open bar). A second 100-nM CORT pulse applied 60 min after the first yielded LTP comparable to that seen in the vehicle group (black bar). (*Upper*) Experimental paradigm (see also *A1* and *A2*).The second CORT pulse in between brackets was applied for only the group receiving two CORT pulses (100–100 nM CORT). All groups, $n = 8$. (*D*) Similar results were found with lower, physiologically more relevant CORT concentrations (30 nM). (*Upper*) Schematic representation of the experimental protocol in which slices were exposed to one or two 30 nM CORT pulses with an interval of 60 min. The second CORT pulse between brackets was applied for only the group receiving two CORT pulses (30–30 nM CORT). HFS was applied 120 min after the start of the first pulse (vehicle and 30 nM CORT, $n = 8$; 30–30 nM CORT, $n = 6$). (*E*) Variations in the duration of the interval between the two 100-nM CORT pulses. Normalization of LTP was seen with 60- and 90-min intervals but not with shorter (10 or 30 min) intervals between the two CORT pulses. (*Upper*) Schematic representation of the experimental protocol in which the delay between application of the two 100-nM CORT pulses was varied between 10 and 90 min. In all cases, HFS was applied 120 after the start of the first pulse. Vehicle and 60 min delay group (100–100 nM CORT), $n = 8$; all other groups, $n = 6$. Values indicate group means ± SEM (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

CORT–BSA, especially directly after high frequency stimulation, we did not observe a significant effect [$F_{(3, 27)} = 14.67$; $P < 0.001$; $P > 0.1$ for CORT–BSA/CORT–BSA vs. vehicle or 100 nM CORT–CORT; Fig. 5*D* and Fig. S2*C*]. Together, these experiments indicate that the normalizing effect of consecutive CORT pulses is mediated by a noncanonical membrane GR-dependent signaling pathway.

Discussion

A single pulse of CORT facilitates glutamatergic transmission in hippocampal cells (9–11, 13), a condition that is associated with an impaired ability to subsequently induce synaptic potentiation (12, 13). This has been interpreted earlier as a means to promote the encoding of stress-related information, by protecting the encoding process from interfering input reaching the same synapses at a later point in time (12). Although this may be beneficial when CORT is released as part of a stress response, it may deadlock the system when CORT is increased as a result of endogenous circadian variations: this would fully prevent encoding of information for many hours after the first high-amplitude ultradian CORT pulse before awakening.

We show that a second pulse of CORT, hitting the cells at a time point when the effects of the first pulse have already developed, can fully normalize all investigated aspects of glutamatergic transmission in the hippocampus and restore responsiveness to high-frequency dependent encoding. The system does so by triggering an entirely different signaling pathway after the second pulse than the one involved in the first pulse. Although, in the absence of CORT, surface expression of corticosteroid receptors is apparently low (because CORT–BSA was ineffective in mimicking the effect of CORT in the single-pulse paradigm), the first pulse of CORT may trigger surface localization of GRs, thus priming the system to a nongenomic effect that effectively reverses the genomic suppression of LTP.

The observation that limbic cells respond differently to a second pulse of CORT than to the first is not unprecedented. Recently, we reported that a single pulse of CORT quickly but persistently increases mEPSC frequency in amygdala cells (18); through this persistent effect, amygdala cell characteristics are slowly changed such that they respond in an opposite manner to a second pulse arriving >1 h after the first. Here we show that, in the hippocampus, metaplastic responses can be observed not only at the level of mEPSC amplitude, but also with respect to AMPAR trafficking and synaptic plasticity.

The selected CORT application paradigm was based on earlier studies (e.g., ref. 6) and mimics as closely as possible the natural pulsatile pattern with gradually rising/falling flanks and very low CORT levels during the interpulse interval (4) (Fig. S3). It therefore seems unlikely that our observations are a mere product of an artificial "on/off" paradigm. Although our reduced preparations—cultured cells and brain slices—allow a straightforward interpretation of the results, we are fully aware that they lack the complex connectivity of the brain and connections with peripheral organs. It would thus be interesting and important to see if similar phenomena occur in the intact organism. Unfortunately, this is currently not feasible at the single-cell level because of technical issues.

Most of the experiments described here were performed with 100 nM CORT because earlier studies have shown very clear actions with this concentration. Most likely, this is a supraphysiological concentration (19). The fact that we also saw normalization of LTP with two pulses of 30 nM CORT supports physiological relevance, but future experiments would need to explore the concentration dependency in greater detail. Similarly, it would be of interest to follow metaplastic changes for more than the current 2-h range. Finally, several studies have shown that nuclear GR signaling—e.g., translocation, binding, and transcription of target genes—normalizes to baseline levels between pulses (4, 6–8). It is therefore of great interest to resolve in detail if and particularly how a first pulse of CORT could lead to enhanced surface expression of GRs and which signaling pathways underlie the reversal exerted by the second pulse on processes activated by the first pulse. In that respect, additional experiments that interfere with transcriptional and/or translational regulation by CORT would strengthen the notion of nongenomic signaling regarding the latter, which is presently based only on our results with CORT–BSA.

In summary, we show that pulsatile exposure to CORT has the potential to maintain glutamatergic transmission in the hippocampal

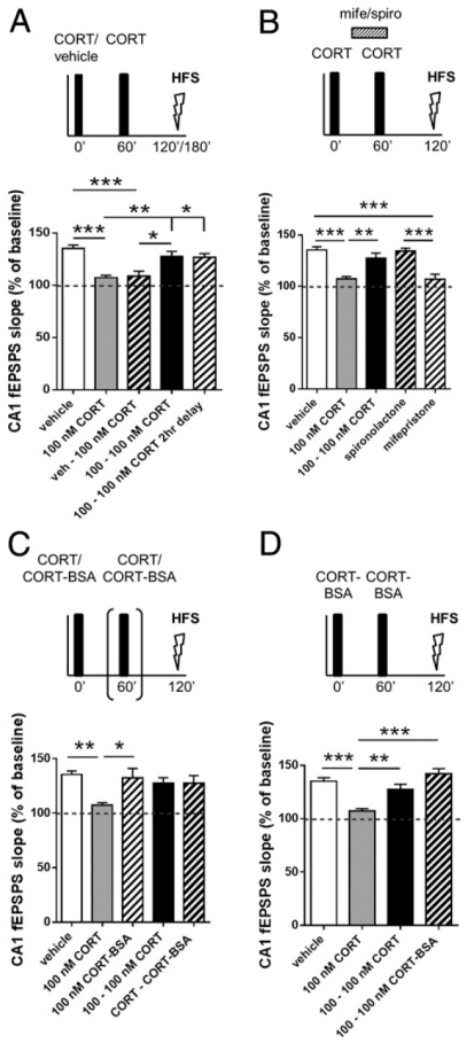

Fig. 5. Normalization of LTP depends on nongenomic GR-dependent pathway. (*A*) Averaged mean values during the 60-min posttetanic recording period. Application of one 100 nM CORT pulse at the moment of the second CORT pulse (bar with white dashed stripes) still attenuated LTP compared with vehicle-treated slices (open bar) in a similar way as 120 min after a single CORT pulse (gray bar). HFS applied 120 min after the onset of the second CORT pulse (at *t* 180 min) very effectively induced LTP (bar with black dashed stripes). (*Upper*) Experimental paradigm. All groups, $n = 8$; second pulse CORT group, $n = 6$; 2 h delay, $n = 5$. (*B*) The presence of spironolactone during the second CORT administration (black dashed bar) did not affect synaptic potentiation, whereas mifepristone (gray dashed bar, far right) significantly attenuated the level of LTP. (*Upper*) Schematic representation of the experimental protocol. Coincubation with 100 nM spironolactone or 500 nM mifepristone started 20 min before the application of the second 100-nM CORT pulse and coterminated with the second pulse. HFS was applied 120 min after the start of the first pulse. Spironolactone, $n = 7$; all other groups, $n = 8$. (*C*) When CORT–BSA (dashed black bar, far right) was delivered during the second pulse instead of CORT (black bar), synaptic plasticity was normalized, and fully comparable to what was seen in slices treated only with vehicle (open bar). A single pulse of CORT–BSA did not affect synaptic plasticity (dashed open bar), different from the reduction seen with a single CORT pulse (gray bar). (*Upper*) CORT–BSA was applied as a single pulse (100 nM CORT–BSA) or as a second pulse after a CORT pulse (second pulse CORT–BSA). HFS was applied 120 min after the start of the first pulse. Second pulse CORT-BSA and 100 nM CORT–BSA, $n = 7$; all other groups, $n = 8$. (*D*) Two consecutive pulses of 100 nM CORT–BSA (dashed black bar) did not significantly increase LTP levels compared with vehicle-treated slices (open bar). (*Upper*) Two pulses of 100 nM CORT–BSA were administered with an interval of 60 min. HFS was applied 120 min after the start of the first pulse (100–100 nM CORT–BSA, $n = 7$; all other groups, $n = 8$). Values indicate group means ± SEM (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). Note that the vehicle, single-pulse CORT, and double-pulse CORT groups shown in this figure are based on the same data as Fig. 4 to allow comparisons.

CA1 area at a stable level, preventing a "fixed" situation that may arise after a single pulse. This maintenance is in line with the observation that pulsatile CORT release is important to maintain transcriptional activity via GRs at an optimal level (4–8). If so, pulsatile CORT exposure would prepare the organism in a flexible manner to upcoming challenges at the start of the active period of the day.

Materials and Methods

Drugs. CORT (30 or 100 nM; first dissolved in 90% (vol/vol) ethanol and next diluted to the final concentration, containing a maximum of 0.009% ethanol) was administered in pulses of 10 min, which effectively cause an increasing concentration during this period, followed by a slow decrease in the subsequent 10-min period (Fig. S3). This paradigm was previously applied to mimic ultradian pulses in vitro (6). To determine the receptor involved, we applied the selective MR antagonist spironolactone (100 nM; first dissolved in chloroform and next diluted to the final concentration, containing 0.002% chloroform) or the GR antagonist mifepristone (500 nM; in H_2O to which 5 N HCl was added in an amount just sufficient to dissolve the antagonist, and then diluted to the final concentration). All drugs were obtained from Sigma-Aldrich. To study the involvement of putative membrane-bound corticosteroid receptors, we used CORT–BSA conjugate (100 nM; first dissolved in H_2O and then diluted to the final concentration; Sigma-Aldrich).

Hippocampal Cell Culture, Protein Expression, Immunocytochemistry, and cLTP. Cultures of hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rats following a previously described method (20). Briefly, cells were plated at a density of 200–300 × 10^3 cells per dish on poly-lysine–precoated coverslips. Mixed cultures of neurons and glial cells were layered on coverslips and maintained in a 3% (vol/vol) serum containing Neurobasal medium. This medium was replaced after 4 d in vitro by a serum-free Neurobasal medium, and cultures were kept at 37 °C in 5% (vol/vol) CO_2.

Pulses of CORT (20 min total; 10 min exposure and 10 min washout; comparable dynamics and kinetics as shown in Fig. S3) were applied to the neuronal coverslips through a peristaltic pump (2 mL/min for CORT pulse, 1 mL/min between pulses). For live immunocytochemical staining, neurons were transfected with GluA1–super ecliptic pHluorin at 8–10 d in vitro by using the Effecten transfection kit (Qiagen) following the provider's protocol. Surface GluA1–AMPARs were specifically stained by using a monoclonal anti-GFP antibody (1:500; Roche) for 15 min on live neurons at 37 °C in culture medium. Briefly, neurons were then fixed with 4% (wt/vol) paraformaldehyde/4% (wt/vol) sucrose for 15 min, washed, and incubated with secondary anti-mouse Alexa 488 antibodies (1:600, 30 min). To label postsynaptic areas, neurons were permeabilized by using 0.1% Triton X-100, incubated with a primary guinea pig polyclonal anti-Homer 1c antibody (1:500, 30 min; Synaptic Systems) or shank antibody (rabbit polyclonal antibody; 1:1,000, 1 h; Abcam), and finally incubated with secondary anti-guinea pig Alexa 568 antibodies (1:600, 30 min; Molecular Probes) or anti-rabbit Alexa 488 antibodies (1:600, 30 min; Molecular Probes). Neurons were washed, mounted, and preparations were kept at 4 °C until observation. For the quantification of surface AMPAR staining within individual synapses, Homer 1c staining served as a mask filter to isolate surface GluA1 subunit staining in individual Homer 1c clusters. The integrated fluorescence level over the Homer 1c cluster area was then measured for each cluster. The fluorescence analysis was performed by using imaging tools from Metamorph software (Universal Imaging), as previously described (21–23). The chemically induced potentiation protocol consisted of a bath coapplication of glycine (200 μM) and picrotoxin (5 μM) for 4 min. In all live experiments, cLTP was always applied after a period of baseline acquisition and the medium was carefully replaced by fresh equilibrated and heated medium after induction.

Single Quantum Dot Tracking. Single particle detection and imaging were performed as previously described in detail (24, 25). In-depth information is given in *SI Materials and Methods*.

Animals. Male C57BL/6 mice (~7–8 wk of age at arrival; Harlan) were group-housed at a 12-h light/dark schedule (lights on at 0700 hours) with food and water provided ad libitum. After an acclimatization period of ~1–2 wk, mice (one at a time) entered the experiment. Mice were decapitated early in the morning when endogenously circulating plasma levels are still low. All experiments were approved by the animal ethical commission of Utrecht University, and all efforts were made to minimize suffering of the animals.

Electrophysiology. Early in the morning, the mouse was decapitated and the brain was rapidly dissected and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) 120 NaCl, 3.5 KCl, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 10 glucose, and 25 $NaHCO_3$, and continuously gassed [mixture of 95% (vol/vol) O_2/5% (vol/vol) CO_2]. Next, dorsal hippocampal slices (350 μm) were made by using a Vibratome (VT 1000S; Leica) and stored in aCSF at room temperature for >1 h before recording commenced at a bath temperature of 30–32 °C (16).

For patch-clamp electrophysiology, one slice at a time was transferred to the recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss) with differential interference contrast and a water immersion objective (×40) to identify the cells. Slices were continuously perfused (flow rate, 1.5 mL/min) with warm aCSF (temperature, 30 °C; pH 7.4) containing TTX (0.5 μM; Latoxan) to block sodium channels and bicuculline (50 μM; Enzo) to block $GABA_a$ receptors. Slices were exposed to a single 10-min CORT (100 nM; or vehicle as control) pulse or two consecutive pulses (of CORT or vehicle) with a 60-min interval. Unless stated otherwise, all recordings were performed ∼2 h after onset of the first pulse.

Patch pipettes (borosilicate glass pipettes; inner diameter, 0.86 mm; outer diameter, 1.5 mm; Hilgenberg) were pulled on a Sutter micropipette puller (resistance; 3–6 MΩ).The intracellular solution contained (in mM): 120 Cs methane sulfonate, 17.5 CsCl, 10 Hepes, 2 MgATP, 0.1 NaGTP, 5 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), pH 7.4, adjusted with CsOH. BAPTA was obtained from Molecular Probes, and all other chemicals were obtained from Sigma-Aldrich. An Axopatch 200B amplifier (Axon Instruments) was used for whole-cell recordings, operating in the voltage-clamp mode. The patch-clamp amplifier was interfaced to a computer via a Digidata (type 1200; Axon Instruments) analog-to-digital converter. Data acquisition and storage was done by using PClamp (version 9.2).

Holding potential was −70 mV. The liquid junction potential caused a shift of 8 mV at most, for which we did not compensate. Recordings with an uncompensated series resistance of <2.5 times the pipette resistance were accepted for analysis. In view of the small current amplitudes, the recordings were not corrected for series resistance. Currents were identified as mEPSCs when the rise time was faster than the decay time; mEPSC frequency and peak amplitude were determined.

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the Schaffer collateral-CA1 pathway of coronal mouse brain slices as described previously (26). Briefly, a bipolar stimulation electrode (60 μm stainless-steel wires insulated except for the tip) was placed on the Schaffer collaterals, and glass recording pipettes (filled with aCSF; 2–5-MΩ impedance) were positioned in the CA1 stratum radiatum. At the start of each experiment, an input–output curve was established to record the slope of the fEPSP, from which maximal and half-maximal slope as well as the corresponding maximal and half-maximal stimulus intensity were determined. The half-maximal stimulus intensity that was calculated was used throughout the remainder of the recording session. For each experimental group, baseline synaptic transmission was recorded with a frequency of 0.033 Hz (0.15 ms) for 10 min. Thereafter, repetitive tetanic stimulations (10 Hz; 90 s) were applied, after which recordings proceeded for another 60 min at a frequency of 0.033 Hz; this stimulation paradigm is very sensitive to the effects of CORT (26). Two consecutive traces were averaged to represent the mean per minute. Data were acquired, stored, and analyzed by using Signal 2.16 (Cambridge 159 Electronic Design).

Data Analysis. Because data of diffusion coefficients are not normally distributed, comparisons between groups for instantaneous diffusion coefficients were performed by using a Mann–Whitney test (pair comparison) or Kruskal–Wallis test followed by Dunn multiple comparison test (group comparison). All other group comparisons for live cell imaging or single-cell electrophysiology were performed by using parametric statistical tests, Student *t* test (unpaired or paired comparison as appropriate), ANOVA followed by Newman–Keuls multiple comparison test (group comparison), or Kolmogorov–Smirnov test (distribution comparison). Significance levels were defined at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

For mEPSC properties, data for both series (a single CORT/vehicle pulse or two CORT/vehicle pulses) were compared with an unpaired Student *t* test. For the fEPSP recordings, all statistical analyses were done with SPSS 21.0. Group values are expressed as mean ± SEM. A one-way ANOVA was used to assess group differences in the level of potentiation. Where applicable, Tukey post hoc tests were used. Student paired *t* tests was used to compare within-group baseline values with the level of LTP. *P* values <0.05 were considered to indicate a significant difference.

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Supporting Information

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SI Materials and Methods

Single particle detection and imaging were performed as previously described in detail (1, 2). Schematically, nanoparticle quantum dots (QDs) 655 Goat F(ab′)2 anti-rabbit IgG (Invitrogen) were first incubated for 30 min with 1 μL of monoclonal antibodies directed against GluA2 (Chemicon). Nonspecific binding was blocked by additional casein (Vector Laboratories) added to the QDs 15 min before use. Neurons were then incubated for 10 min with precoated anti-GluA2 subunit QDs (final dilution, 1:10,000) and mounted on a heated chamber for observation. QDs were detected by using a mercury lamp, an oil immersion objective (×100 apochromatic, N.A. 1.45, working distance 0.13 mm), and appropriate excitation/emission filters (excitation 435/40–25 nm, emission 655/15–25 nm, dichroic 510 nm; Semrock) on a wide-field epifluorescent microscope (Nikon). Images were obtained with an acquisition time of 50 ms with up to 1–1,000 consecutive frames. Signals were detected by using an EM-CCD camera (Evolve; Roper Scientific) and a pointing accuracy of ∼20 nm (pixel size at imaging magnification, 160 nm).

QDs were followed on randomly selected dendritic regions; imaging sessions on a labeled neuronal preparation lasted to a maximum of 20 min. QD recording sessions were processed with MetaMorph software (Universal Imaging). All recording sessions were acquired within 30 min following primary antibody incubation to minimize receptor endocytosis. The instantaneous diffusion coefficient D was calculated for each trajectory, from linear fits of the first four points of the mean square displacement vs. time function using $\mathrm{MSD}(t) = \langle r^2 \rangle (t) = 4Dt$. The 2D trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images by using a Vogel algorithm. To determine the distribution and synaptic fraction (synapses labeled with MitoTracker; Molecular Probes) of single QD complexes, 500–1,000 frame stacks were performed while tracking down a single QD complex. The precise location of the receptor/particle complex was determined on each frame, and those 500–1,000 locations were then projected on a single image, providing a high-resolution map of the successive positions of the receptor/particle complex during the stack. Synaptic, perisynaptic, and extrasynaptic receptor/particle complex locations were then defined for all receptors of a given neuronal field with respect to MitoTracker labeling.

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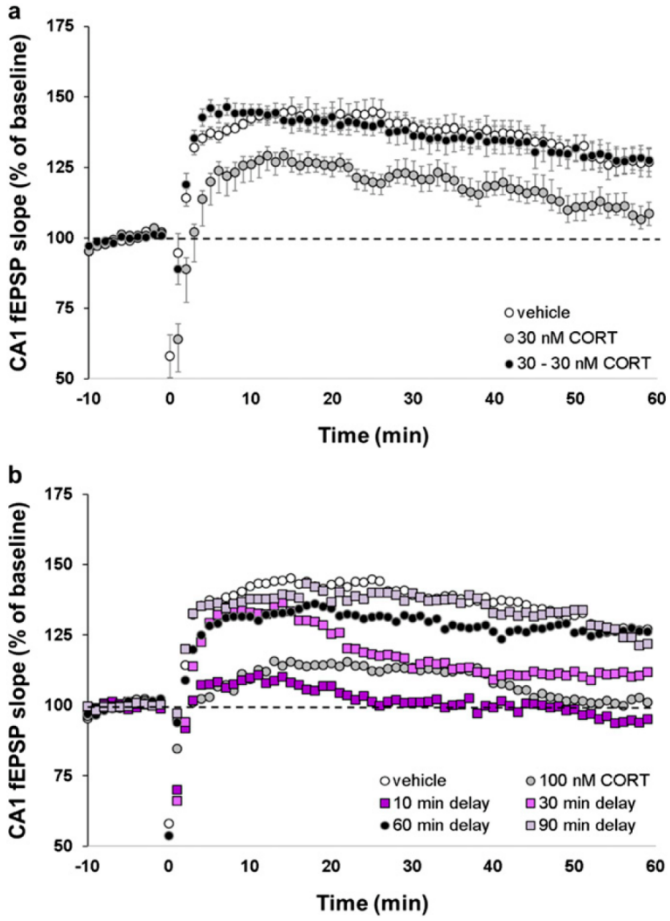

Fig. S1. Synaptic plasticity in hippocampal slices is restored by consecutive corticosterone (CORT) pulses. (*A*) High-frequency stimulation (HFS; 10 Hz, 90 s) resulted in significant potentiation at CA1 synapses in brain slices of vehicle-treated mice (open circles), which was significantly attenuated by 30 nM CORT treatment (gray circles). However, a second application of 30 nM CORT 60 min after the first reversed this effect (black circles). HFS was applied 120 min after the start of the first pulse. Vehicle and 30 nM CORT, $n = 8$; 30–30 nM CORT, $n = 6$. (*B*) The interval between the two 100-nM CORT pulses was varied from 10 to 90 min. Whereas 10-min (purple squares) and 30-min intervals (pink squares) significantly attenuated synaptic plasticity, 60-min (black circles) and 90-min intervals (gray squares) did not affect the level of long-term potentiation (LTP) compared with vehicle-treated slices (open circles). In all cases, HFS was applied 120 min after the start of the first pulse. Vehicle and 60-min delay group (100–100 nM CORT), $n = 8$; all other groups, $n = 6$.

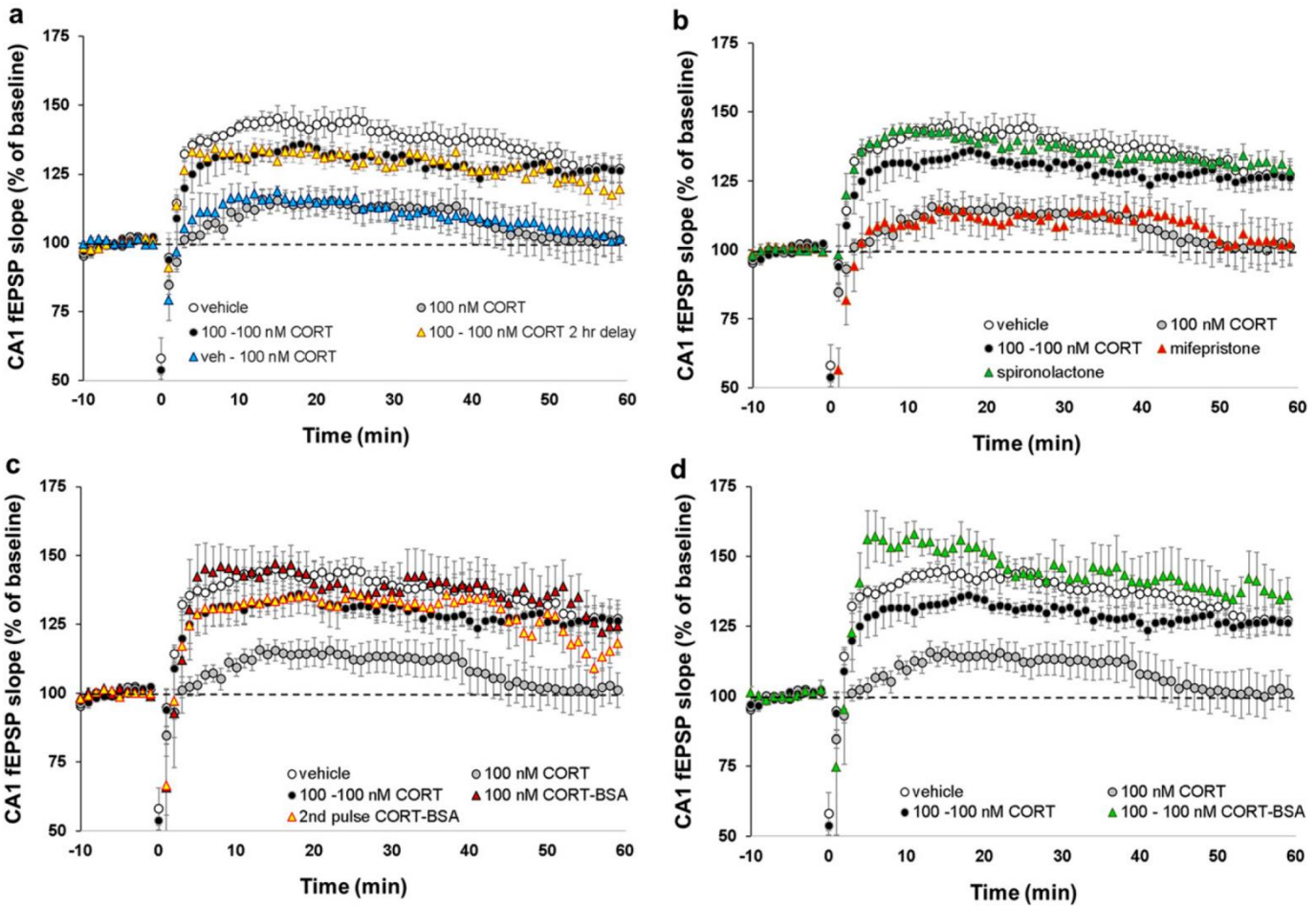

Fig. S2. Normalization of LTP depends on nongenomic glucocorticoid receptor-dependent pathway. (*A*) A single pulse of 100 nM CORT resulted in attenuated LTP 120 min later (gray circles) compared with the vehicle control group (open circles), whereas application of a second pulse at the intermediate time point of *t* 60 min resulted in effective LTP induction at *t* 120 min (black). The discrepancy between the two groups was not explained by a difference in delay between (the last) CORT application and LTP induction, because LTP was also comparable to the vehicle group when induced 120 min after the second CORT pulse (at *t* 180 min; yellow triangles). If the first pulse contained vehicle (and the second CORT), LTP was suppressed at *t* 120 min (blue), indicating that suppression was visible already 60 min after CORT exposure. On purpose, we introduced the first pulse of vehicle in this experiment to keep the overall incubation time of the slices comparable for the two groups receiving only one CORT pulse. All groups, $n = 8$, except the vehicle–CORT group ($n = 6$) and 120-min delay group with two CORT pulses ($n = 5$). (*B*) Administration of the mineralocorticoid receptor antagonist spironolactone during the second CORT pulse (green triangles) did not prevent its normalizing effect on LTP induction (black circles), whereas the glucocorticoid receptor antagonist mifepristone (red triangles) did prevent the normalizing effect of the second CORT pulse. Spironolactone, $n = 7$; all other groups, $n = 8$. (*C*) A CORT–BSA pulse 60 min after a single CORT pulse (yellow triangles) fully normalized LTP, comparable to the 100–100-nM CORT group (black circles). A single CORT–BSA pulse (brown triangles) yielded LTP levels comparable to those seen in the vehicle control group (open circles). CORT–BSA second pulse and a single 100-nM CORT–BSA pulse, $n = 7$; all other groups, $n = 8$. (*D*) Two consecutive pulses of 100 nM CORT–BSA (green triangles) did not significantly increase LTP levels compared with vehicle-treated slices (open circles); 100–100 nM CORT–BSA, $n = 7$; all other groups, $n = 8$. fEPSP, field excitatory postsynaptic potential.

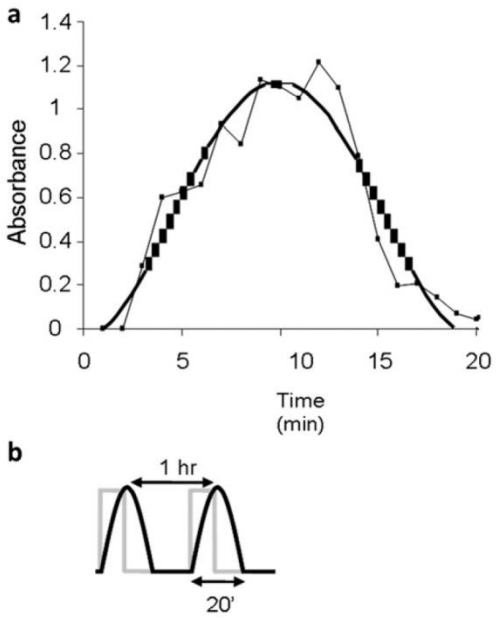

Fig. S3. Method of CORT pulse application. (*A*) Typical example of optical measurements showing the absorbance in samples of artificial cerebrospinal fluid containing a dye. Samples from the recording chamber were collected for each consecutive minute during a 20-min period (black dots). The bold black smooth line depicts the best fit between the data points indicating the gradual distribution of the perfusion solution in the recording chamber resulting in a natural pulse-like shape with a 20-min pulse width. (*B*) Schematic overview of the CORT application protocol. Two consecutive pulses of CORT (30 or 100 nM, first dissolved in 90% (vol/vol) ethanol and next diluted to the final concentration) or vehicle (artificial cerebrospinal fluid with a maximum of 0.009% ethanol) were applied according to the natural pattern resulting in a 20-min pulse width (black line) and an interpulse interval of 1 h. This protocol was based on ref. 1.

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Table S1. Effect of CORT pulses on mEPSC frequency

Experiment	mEPSC Amplitude	*n*	*P* value
Experiment 1			0.81
1-Pulse vehicle	0.46 ± 0.17	9	
1-Pulse CORT	0.52 ± 0.12	7	
Experiment 2			0.73
2-Pulse vehicle	0.25 ± 0.05	7	
2-Pulse CORT	0.27 ± 0.03	8	

The data show the mean ± SEM in *n* cells based on averaged recordings 5–10 min after achieving the whole cell configuration. In experiment 1, CORT (100 nM, 10 min) or vehicle was administered to hippocampal slices and mEPSC frequency was determined ~120 min after onset of the pulse. In experiment 2, cells were tested after two pulses of CORT or vehicle (60-min interpulse interval), again 120 min after onset of the first pulse. In both experiments, mEPSC frequency was unaffected by CORT vs. vehicle treatment. mEPSC, miniature excitatory postsynaptic current.

Tissue-type plasminogen activator controls neuronal death by raising surface dynamics of extrasynaptic NMDA receptors

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[6] These authors contributed equally to this work.

This work resulted from a long collaboration with the team of Dr. Denis Vivien in Caen and aimed at investigating the impact of tPA, an endogenous thrombolytic agent on the NMDAR-dependent glutamate signaling. I perfomed single nanoparticle tracking to investigate the effect of tPA and its selective blocking antibody Glunomab on the surface trafficking of NMDAR. I also performed immunostainings to characterize the localization of the Glunomab's target at the surface of hippocampal cultured neurons. My contribution to this study allowed the characterization of the effect of tPA on the NMDAR surface dynamics.

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Tissue-type plasminogen activator controls neuronal death by raising surface dynamics of extrasynaptic NMDA receptors

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***N*-methyl-D-aspartate receptors (NMDARs) are ion channels whose synaptic *versus* extrasynaptic localization critically influences their functions. This distribution of NMDARs is highly dependent on their lateral diffusion at the cell membrane. Each obligatory subunit of NMDARs (GluN1 and GluN2) contains two extracellular clamshell-like domains with an agonist-binding domain and a distal N-terminal domain (NTD). To date, the roles and dynamics of the NTD of the GluN1 subunit in NMDAR allosteric signaling remain poorly understood. Using single nanoparticle tracking in mouse neurons, we demonstrate that the extracellular neuronal protease tissue-type plasminogen activator (tPA), well known to have a role in the synaptic plasticity and neuronal survival, leads to a selective increase of the surface dynamics and subsequent diffusion of extrasynaptic NMDARs. This process explains the previously reported ability of tPA to promote NMDAR-mediated calcium influx. In parallel, we developed a monoclonal antibody capable of specifically blocking the interaction of tPA with the NTD of the GluN1 subunit of NMDAR. Using this original approach, we demonstrate that the tPA binds the NTD of the GluN1 subunit at a lysine in position 178. Accordingly, when applied to mouse neurons, our selected antibody (named Glunomab) leads to a selective reduction of the tPA-mediated surface dynamics of extrasynaptic NMDARs, subsequent signaling and neurotoxicity, both *in vitro* and *in vivo*. Altogether, we demonstrate that the tPA is a ligand of the NTD of the obligatory GluN1 subunit of NMDAR acting as a modulator of their dynamic distribution at the neuronal surface and subsequent signaling.**

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N-methyl-D-aspartate receptors (NMDARs) are tetrameric assemblies of dimers of GluN1 and GluN2 (GluN2A-D) subunits (possibly GluN3A-B). Their extracellular region forms a massive protrusion composed of eight clamshell-like domains arranged in two layers: a distal N-terminal domain (NTD) layer and a layer of four agonist-binding domains (ABDs) directly connected to the transmembrane domain (TMD).[1] Subunit composition and synaptic *versus* extrasynaptic localization critically influence NMDAR functions.[2] Synaptic *versus* extrasynaptic distribution of NMDARs is highly dependent on their lateral diffusion at the cell membrane.[3–5] It is interesting to note that this diffusion can be modulated by extracellular factors such as matrix metalloproteases or co-agonists.[6–8] In cortical and hippocampal areas, NMDARs are principally composed of GluN1 subunits that are associated with GluN2A and GluN2B.[9] In contrast to GluN2 subunit NTDs,[10] less is known about the obligatory role and dynamics of the NTD of the GluN1 subunit (GluN1 NTD) in NMDAR allosteric signaling. A recent work by Zhu *et al.*[10] show that GluN1 NTD is highly mobile and actively participates in defining the gating and pharmacological profile of NMDARs. These data suggested that any ligand that binds GluN1 NTD may stabilize its opened or closed conformations and thus act as a positive, or negative, allosteric modulator of NMDAR.

Tissue-type plasminogen activator (tPA), a serine protease of 69 kDa, is expressed in most organs, including the brain and the spinal cord.[11–14] It consists of five different functional domains through which it interacts with various substrates, binding proteins and receptors.[15,16] In the central nervous system (CNS), tPA can be synthesized and released by virtually all cell types. This neuromodulator displays an array of important functions, which are involved in synaptic plasticity,[17] learning and memory processes,[18,19] anxiety [20] and neuronal survival or death.[14,21–23] Although previous studies demonstrated that tPA was a modulator of NMDARs signaling through a possible interaction with the GluN1 NTD,[22,24] the exact molecular mechanism of this function remains under debate.[10]

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Abbreviations: Cter GluN1, C-terminal end of GluN1 subunit; DIV, day *in vitro*; GluN, NMDA receptor subunit; K2, kringle 2 domain of tPA; LBS, lysine-binding site; NMDAR, *N*-methyl-D-aspartate receptor; NTD, N-terminal domain; QD, quantum dot; TMD, transmembrane domain; tPA, tissue-type plasminogen activator; WT, wild-type.

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In this study, we postulated that tPA could alter NMDAR-evoked signaling and subsequent neurotoxicity through modifications of their surface dynamics and distribution. Thus, using nanoparticle tracking and antibody-based screening in living neurons, we demonstrate that the neuronal extracellular serine protease tPA can selectively increase neuronal extrasynaptic NMDARs surface diffusion. This selective diffusion of NMDAR is the consequence of a direct interaction of tPA with a functionally critical single amino acid (lysine 178) within the GluN1 NTD. By this mechanism, tPA promotes NMDAR-dependent calcium influx and excitotoxic neuronal death both *in vitro* and *in vivo*.

Results

tPA selectively increases extrasynaptic NMDAR surface dynamics, clustering and subsequent signaling. In living hippocampal culture, single nanoparticle tracking was used to image surface NMDARs and their response to exogenously applied tPA (see Materials and methods section; Figure 1). NMDAR surface diffusion was recorded in cultured neurons that were incubated with single nanoparticle complexes containing a quantum dot (QD) associated with polyclonal antibodies raised against GluN1 NTD (Figure 1a). Surface diffusion of GluN1-NMDAR was recorded under control conditions and in the presence of either a wild-type (WT) tPA (tPA, 300 nM; Figure 1a) or a non-proteolytic tPA (tPAm, 300 nM). Figures 1a to c illustrate, at different magnifications, the distinct diffusion patterns of representative trajectories of surface NMDAR following exposure to either WT tPA or tPAm. We then compared the diffusion NMDAR to discriminate potential compartment-specific effect of tPA. As shown previously, synaptic NMDAR are less diffusive than their extrasynaptic counterparts.[3,25] Although the diffusion of synaptic receptors remained unchanged, exposure to tPA increased the diffusion of extrasynaptic receptors (***$P<0.001$) (Figures 1c and d). In contrast to its WT form, inactive tPAm failed to influence the

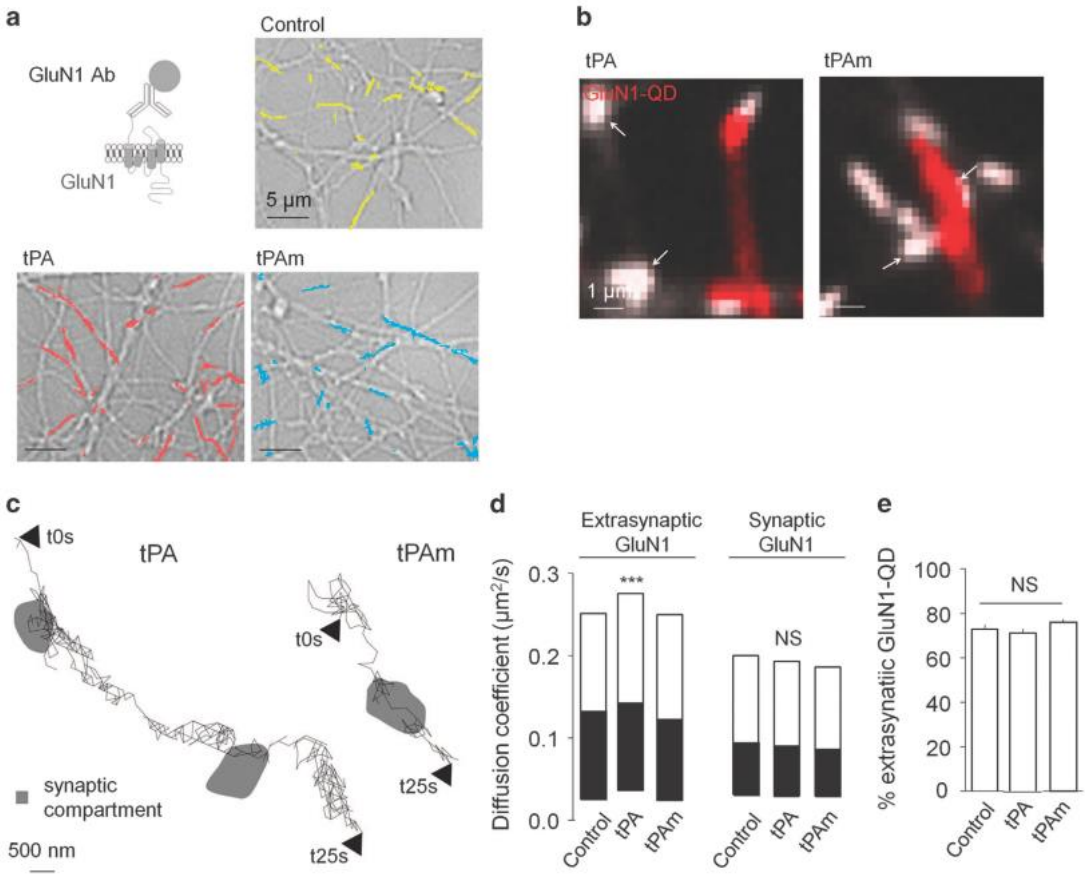


Figure 1 tPA selectively increases extrasynaptic GluN1-NMDAR surface diffusion. (**a**) Schematic representation of the surface labeling of a GluN1 subunit using a single anti-GluN1 antibody QD complex. Representative GluN1-QD (GluN1 QD) trajectories on cultured hippocampal neurons (11–12 DIV) incubated 45 min with buffer (yellow), WT tPA (300 nM, red) or a non-proteolytic tPA (tPAm, 300 nM, cyan). (**b**) Representative trajectories of surface GluN1-QD (red lines, 500 frames, 50-ms acquisition) in the vicinity and within synapses (white arrows). Synaptic trajectories are defined by their colocalization with synaptic labeling (Mitotracker, white), trajectories outside synapses being considered as extrasynaptic. (**c**) Representative trajectories of GluN1-NMDAR tracked with a control GluN1 NTD antibody (control Ab) in the presence of tPA or tPAm (300 nM both). (**d**) Instantaneous diffusion coefficient distributions (median 25–75% IQR,) of extrasynaptic (control $n=3882$ trajectories; tPAm $n=3195$; tPA $n=3701$; $N=3$ independent experiments; ***$P<0.001$, Kruskal–Wallis test followed by Dunn's multiple comparison test) *versus* synaptic GluN1-QD (control $n=4411$ trajectories; tPAm $n=2818$; tPA $n=4283$). Note that GluN1 surface diffusion is specifically increased in the extrasynaptic compartment after tPA incubation. (**e**) Fraction of extrasynaptic GluN1-QD is unchanged between the different incubations (control 73%, $n=18$; tPAm 76%, $n=20$; tPA 71%, $n=17$; NS, not significant, one-way ANOVA; mean ± S.E.M.)

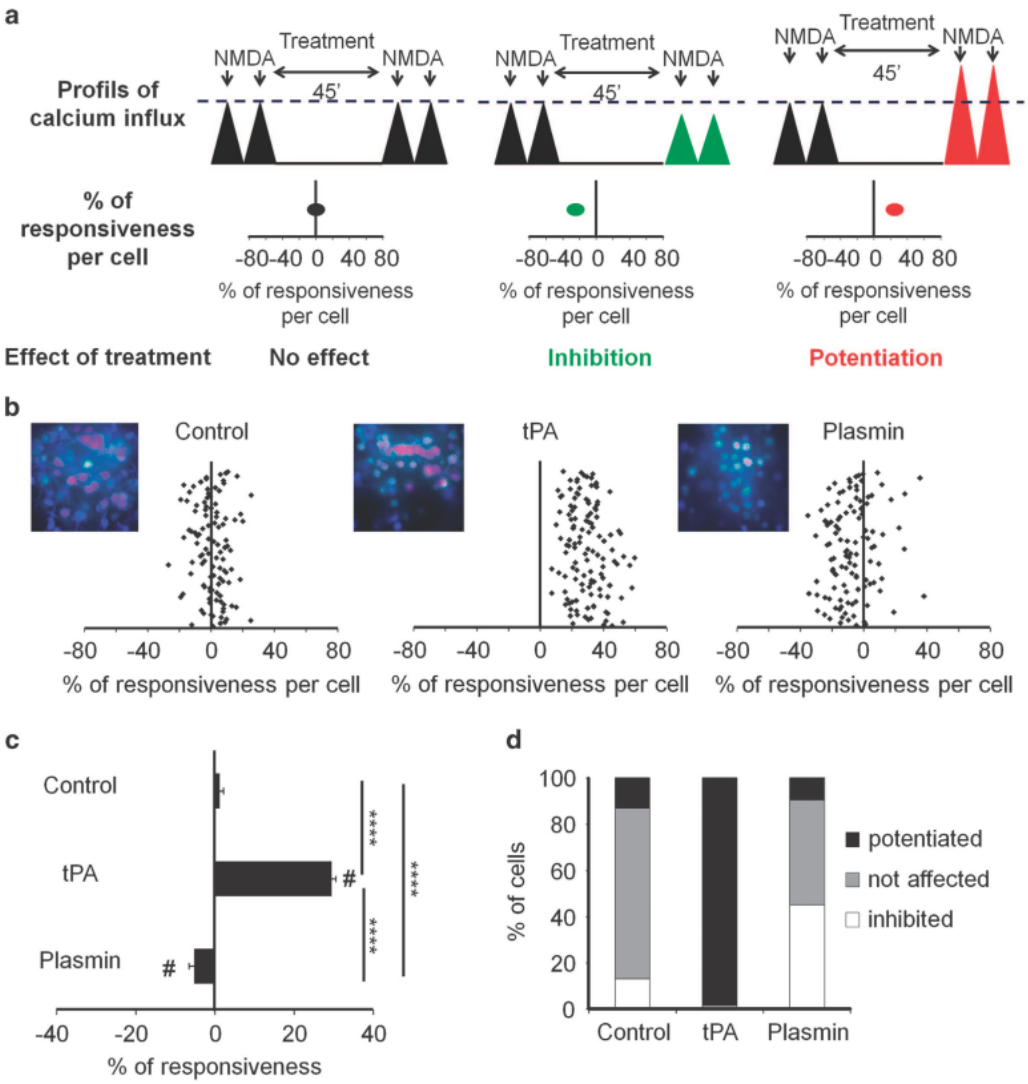
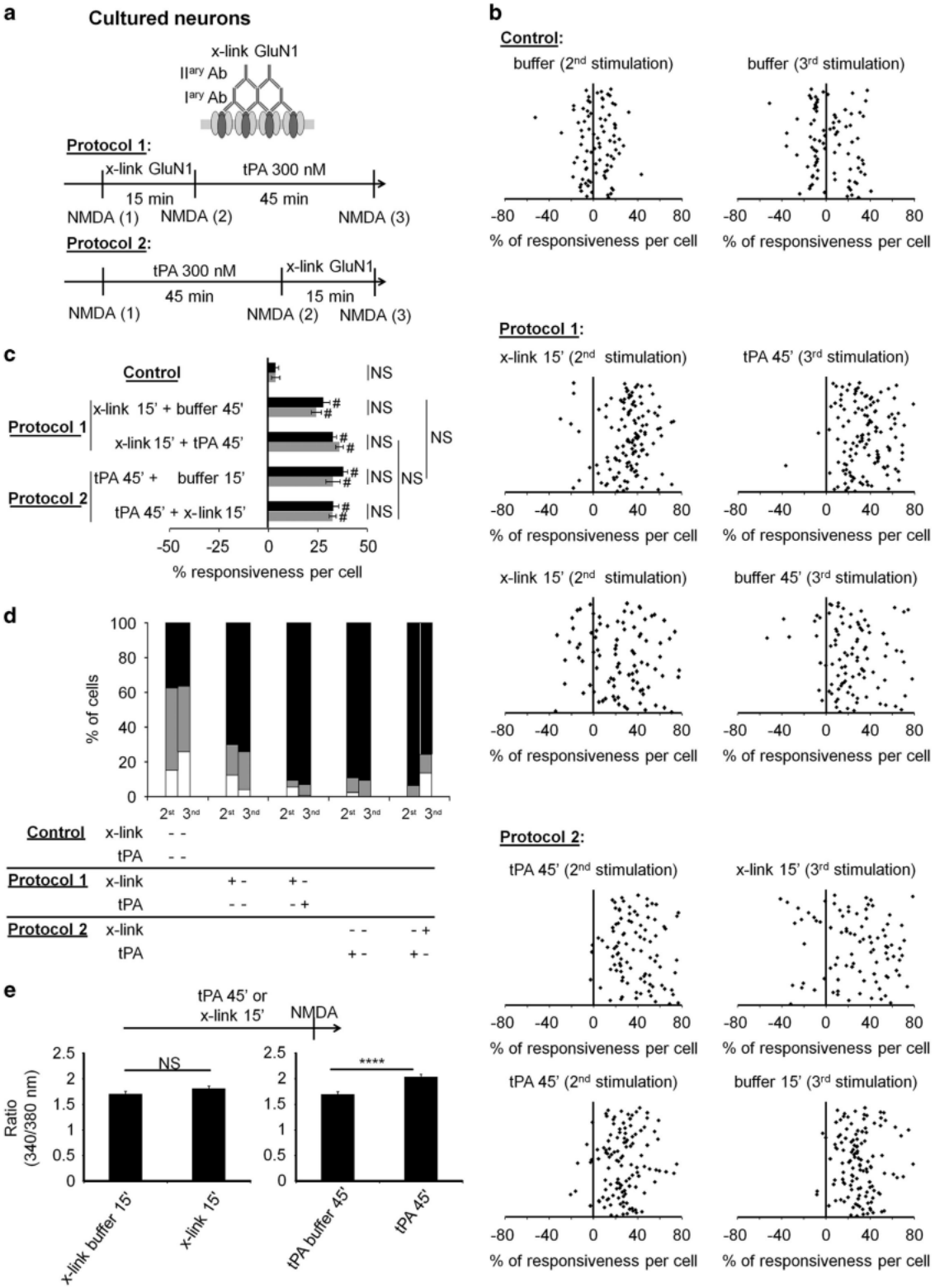


Figure 2 tPA-induced potentiation of NMDAR-mediated neuronal calcium influx is independent of plasmin. (**a**) Schematic representation of calcium video imaging on primary cultures of cortical neurons (12–14 DIV). The neurons were stimulated twice with the NMDA, then incubated with the treatment during 45 min prior a second run of NMDA stimulations. Each neuron responsive to NMDA is thus its own control. By comparison between the two runs of NMDA stimulations (% of responsiveness per cell), we visualized the effect of treatment on NMDA-induced calcium influx estimated the % of responsiveness per cell with either no effect of the treatment, an inhibitory effect or a potentiating effect. Each dot represents one individual neuron, with data collected from a minimum of three independent experiments. (**b**) After control NMDA stimulations used as baseline, neurons were incubated for 45 min with either plasmin buffer (control, $n=107$ cells), tPA (300 nM, $n=123$ cells) or plasmin (100 μg/ml; $n=115$ cells). Each dot represents one cell. (**c**) Percentage of stimulation or inhibition after incubation were calculated for each individual cell and reported as the percentages of responsiveness for each group (mean $\pm$ S.E.M.; $N=3$ independent experiments; $^{****}P<0.0001$ Kruskal–Wallis and Mann–Whitney tests for group comparison; $^{\#}P<0.0001$ Wilcoxon signed-rank test for the comparison pre- and post-incubation responses). (**d**) Represents the percentages of potentiated, not affected and inhibited cells

dynamics of NMDARs. It should be noted that these acute applications of tPA did not modify the percentage of detected extrasynaptic GluN1-NMDAR. This indicates a stable pool of membrane NMDAR within this time frame (Figure 1e). Altogether, these data provide the first direct evidence that tPA can increase extrasynaptic,but not synaptic, GluN1-NMDAR surface dynamics in living neurons.

Next, we investigated the influence of tPA on consecutive NMDA-evoked calcium influx (Figure 2). The neurons were stimulated twice with the NMDA, then incubated with the treatment during 45 min prior a second run of NMDA stimulations. Each neuron responsive to NMDA is thus its own control. By comparison between the two runs of NMDA stimulations (% of responsiveness per cell), we visualized the effect of treatment on NMDA-induced calcium influx estimated by the % of responsiveness per cell with either no effect of the treatment, an inhibitory effect or a potentiating effect. Each dot represents one individual neuron, with data collected from a minimum of three independent experiments (Figure 2a). tPA treatment increased NMDA-induced calcium influx (300 nM; 92% of neurons potentiated, +28% stimulation in mean value; $^{****}P<0.0001$ when compared with control neurons; Figures 2b–d). This effect is independent of the plasmin because plasmin did not elicit such a potentiation of

NMDA-evoked calcium influx (Figure 2). To directly address whether this functional effect of tPA on NMDA-induced calcium influx response requires, or could be mimicked by, surface diffusion of NMDAR. We artificially reduced NMDAR surface dynamics and created NMDAR clusters using a cross-link protocol (x-link)[26–28] (Figure 3a). It should be noted that x-link alone, without NMDA application, does not alter the amplitude of calcium influx, as previously reported[28,29] (Figure 3e).



Remarkably, after a first challenge with NMDA, x-link led to an increase of NMDA-induced calcium influx (90% of neurons potentiated, +33% stimulation in mean value; control $n=85$ cells and x-link $n=128$ cells; $^{****}P<0.0001$ when compared with control cells), indicating that NMDA-induced calcium influx response requires dynamic diffusion of surface NMDAR. The same effect was observed following tPA treatment (94% of cells potentiated, +38% stimulation in mean value; protocol 2, $n=95$ cells; $^{****}P<0.0001$ when compared with control cells; Figures 3c and d). Importantly, no additional effect on calcium response was observed when adding tPA after performing x-link (NS, not significant, protocol 1), or when performing x-link after adding tPA (NS; protocol 2; Figures 3b–d). The same results were obtained when x-link was induced by Glunomab (Supplementary Figure 1) instead of polyclonal antibodies against GluN1 NTD subunit (residues 385–399, Figure 3). Altogether, these data demonstrate that NMDA-induced calcium influx requires a fast dynamic diffusion of NMDAR, allowing clustering, as shown by x-link experiments. For extrasynaptic NMDARs, this process is promoted by tPA.

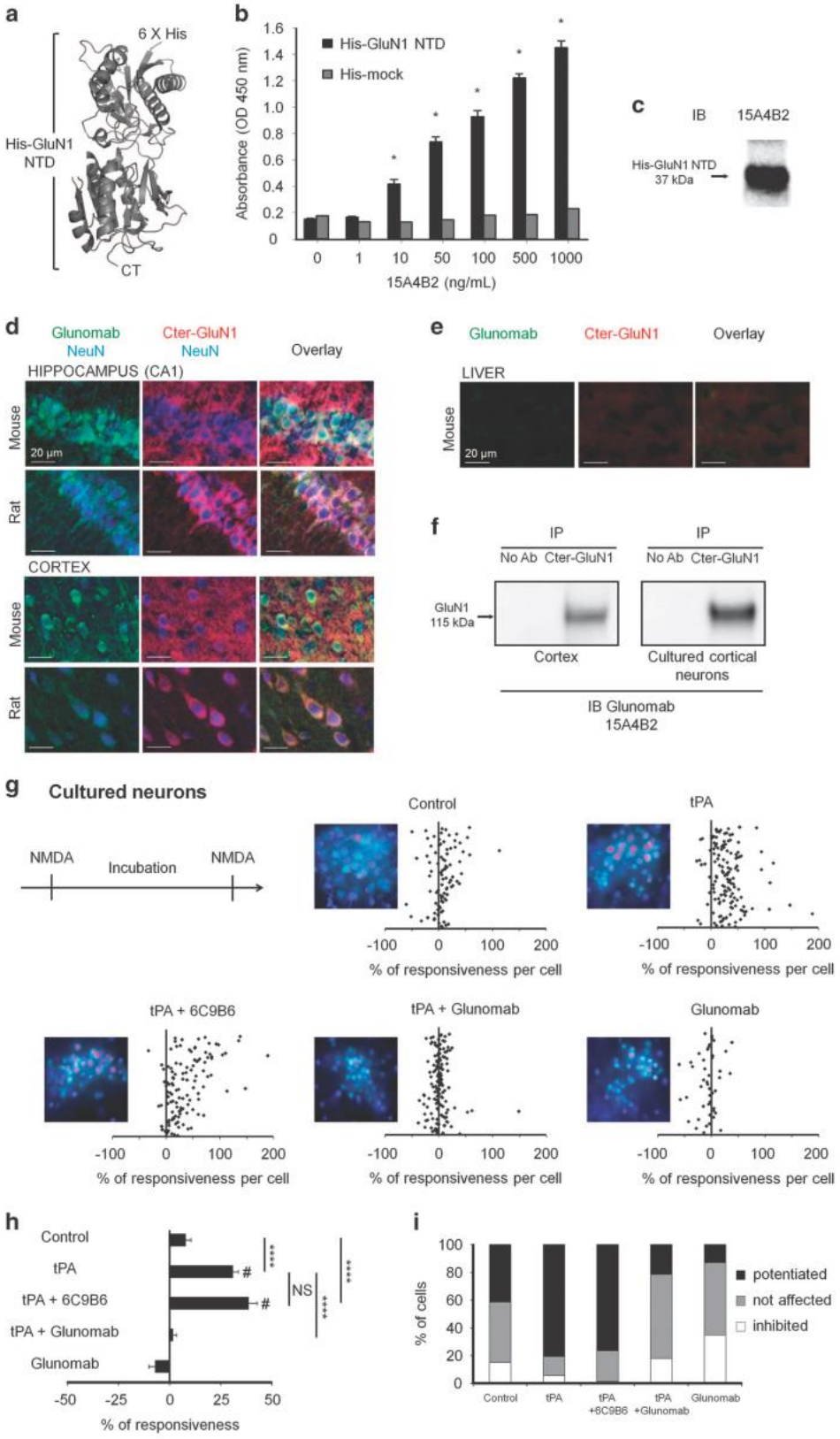
Promotion of NMDARs signaling is caused by tPA interaction with GluN1: critical role of lysine 178 (GluN1 NTD^{Lys178}). To identify the putative binding site of tPA on GluN1 NTD,[22,30–32] we first screened a series of monoclonal antibodies we have generated using recombinant GluN1 NTD as antigen (residues 19–371; Figure 4a). Positive antibodies on ELISA (Figure 4b and Supplementary Figure 2) were further selected by immunoblottings raised against recombinant GluN1 NTD (Figure 4c and Supplementary Figure 2). Then, histochemistry performed with selected antibodies (e.g., clone 15A4B2 also named Glunomab) revealed positive stainings in cortical and hippocampal neurons (mouse and rat). Glunomab-positive staining colocalizes with a parallel immunostaining performed against the C-terminal end of GluN1 (Cter-GluN1) both in cortical and hippocampal but not in non-neuronal tissue sections (Figures 4d and e). As Glunomab is not optimum for direct immunoblotting, we tested its specificity after we have performed a pull down of the GluN1 subunit by using an antibody raised against the Cter-GluN1 (Figures 4e–g. clone 15A4B2, Glunomab). These experiments were performed from whole cortex and primary cultures of cortical neurons protein extracts. We then evaluated the blocking activity of each positive clone on tPA-driven potentiation of NMDAR signaling using calcium video-imaging assays performed on primary cultures of cortical neurons (Figures 4g–i). NMDA exposure led to reproducible waves of calcium influx that were potentiated by 31% in the presence of tPA (300 nM; mean value of potentiation with 81% of cells potentiated; $^{****}P<0.0001$; control, $n=92$ cells and tPA, $n=139$). Antibodies from clone 6C9B6 failed to influence tPA-induced potentiation of NMDA-induced calcium influx (+38% of potentiation with 78% of cells potentiated; $n=119$ cells; $^{\#}P<0.0001$ compared with pre-incubation responses; NS, not significant compared with tPA alone). However, Glunomab completely abolished the tPA-dependent potentiation of NMDA-induced calcium influx ($^{****}P<0.0001$ when compared with tPA alone, $n=139$ cells). Of note, Glunomab did not affect basal NMDAR signaling ($n=46$ cells).

Then, an epitope mapping of Glunomab was performed to refine the binding site of tPA on the GluN1 NTD. Immunoblottings for 141 overlapping pentadecapeptides frame shifted by three residues covering the entire amino-acid sequence of GluN1 NTD (residues 19–371) were revealed with Glunomab (Supplementary Figure 3a). Once reactive peptides were identified, a corresponding alanine scanning (Alascan) was performed to identify key residues contributing to the epitope of Glunomab (Supplementary Figure 3b). These studies revealed that Glunomab binds to the ^{172}EGRAAQKRLETL-LEE^{186} sequence of the rat GluN1 NTD, a sequence 100% conserved between species from mouse to human (Supplementary Figures 3c and d). Alascan also revealed ^{176}AQKRL^{180} as the minimal epitope and both arginine 174 (R174) and glutamic acid 185 (E185) as putative stabilizing amino acids. Positioning of the minimal epitope (^{176}AQKRL^{180}) in the structure of heterotetrameric NMDAR reveals that this epitope is accessible to extracellular molecules (Supplementary Figure 3e).

We demonstrated previously that the lysine-binding site (LBS) of the kringle 2 domain of tPA was important to promote NMDAR signaling.[33,34] Thus, we postulated that the lysine 178 (K^{178}) in the minimal epitope of Glunomab could be critical for the binding of tPA on GluN1 NTD. To address this question, NMDA-induced calcium influx was recorded in HEK-293 cells transfected with WT GluN2A in combination with either WT GluN1-1b (GluN1-1b WT) or GluN1-1b containing a point mutation of the lysine in position 178 into valine (K178V) or GluN1-1b containing a point mutation of lysine in position 190 into valine as control (K190V) (Figures 5a–c). As observed in cultured cortical neurons, tPA enhanced NMDA-induced calcium influx (+27% of potentiation with 62% of cells potentiated; $^{***}P<0.001$ compared with control; control, $n=34$ cells; tPA, $n=36$) and Glunomab prevented tPA-induced potentiation of NMDAR signaling ($^{**}P<0.01$ when compared with tPA alone; $n=30$ cells). K178V mutated

Figure 3 tPA mimicks x-link-induced NMDARs clustering. (**a**) Schematic representation of the x-link procedure performed on cultured cortical neurons and used to induce an artificial clustering of GluN1-NMDAR,[26–28] as well as the detailed sequence of treatments. (**b**) NMDA-induced calcium influx measured after the following treatments: buffer alone ($n=85$ cells), x-link 15' then tPA 45' (protocol 1, $n=128$ cells) or tPA buffer 45'($n=97$ cells) and tPA 45' then x-link 15' (protocol 2, $n=95$ cells) or x-link buffer 15' ($n=114$ cells). Each dot represents one cell. (**c**) Percentage of stimulation or inhibition after incubation were calculated for each individual cell and reported as percentages of responsiveness for each group. (mean ± S.E.M.; $N=3$ independent experiments; NS, not significant; $^{****}P<0.0001$ Kruskal–Wallis test followed by Mann–Whitney test for group comparison; $^{\#}P<0.0001$ Wilcoxon signed-rank test for the comparison pre- and post-incubation responses). (**d**) Percentages of potentiated, not affected and inhibited cells. (**e**) Comparison of the amplitudes of calcium influx after tPA 45' ($n=87$ cells) or x-link 15' ($n=77$ cells) treatments and their controls (tPA buffer 45', $n=68$ cells; x-link buffer 15', $n=92$ cells) without a pre-treatment with NMDA (as shown on the diagram). Effects of tPA 45' or x-link 15' are not dependent of the pre-incubation NMDA stimulation. In contrast to tPA 45' condition, the x-link does not lead a augmentation of calcium influx amplitude. (mean ± S.E.M.; $N=3$ independent experiments; $^{****}P<0.0001$ Mann–Whitney test for group comparison)

NMDAR were still responsive to NMDA stimulation but failed to respond to tPA ($n=35$ cells). The control point mutation K190V did not modify NMDAR responsiveness to tPA incubation ($n=37$ cells; NS compared with tPA alone), neither the blocking activity of Glunomab (****$P<0.0001$ compared with GluN1-1b K190V in tPA condition; $n=47$ cells). These



mutations do not influence the basal activity of NMDAR in the absence of tPA (Figure 5d). Accordingly, binding of Glunomab was reduced on HEK-293 cells transfected with GluN1-1b K178V compared with cells expressing NMDARs containing either GluN1-1b WT or GluN1-1b K190V (Figures 5e and f). Taken together, these data demonstrate that lysine 178 within the GluN1 NTD is of decisive importance in effecting tPA-induced potentiation of NMDAR signaling and, equally important, these findings revealed Glunomab as a selective antagonist of tPAs action on NMDAR.

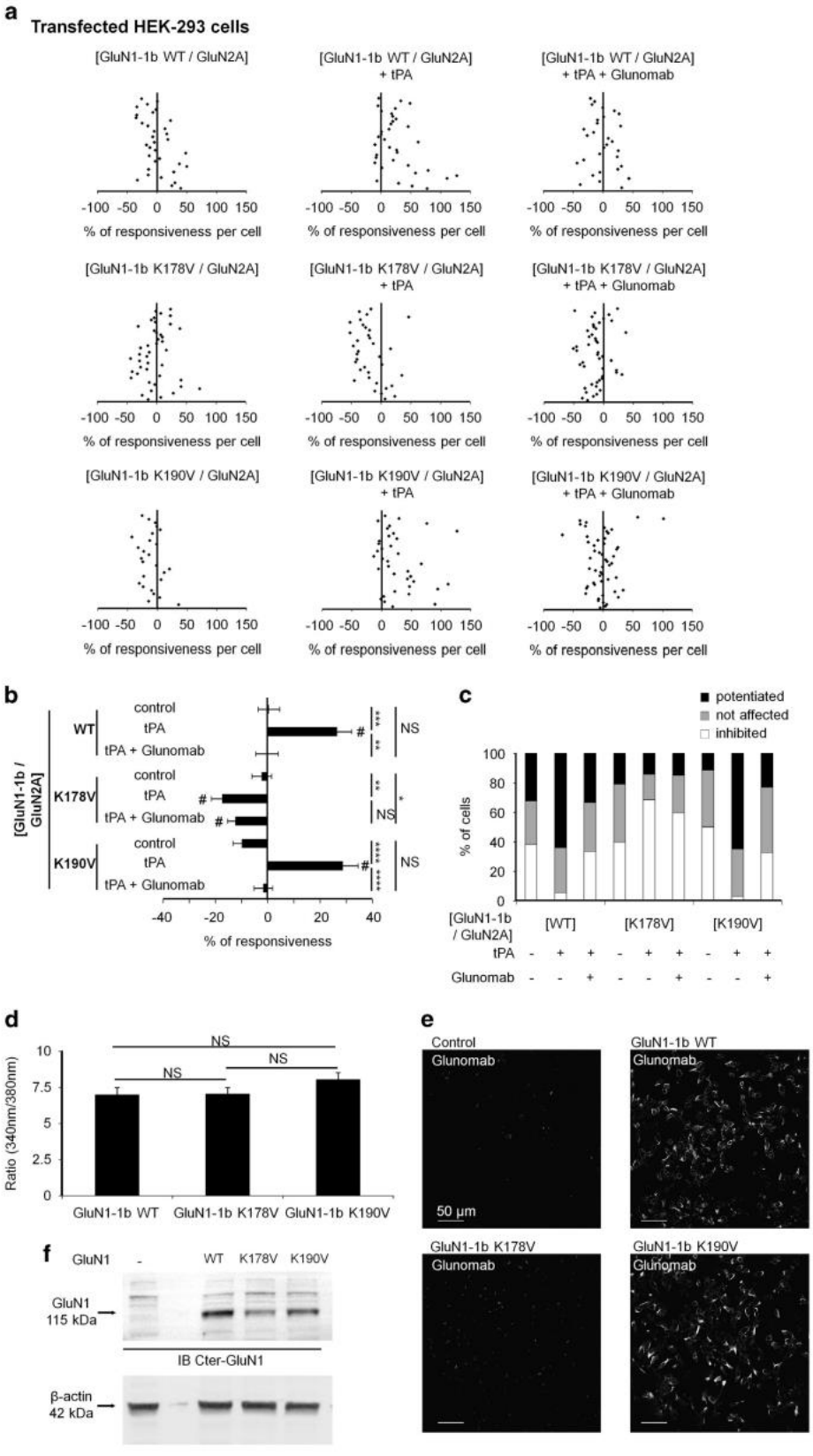
To ascertain whether the LBS contained in the kringle 2 domain of tPA (K2) was indeed the tPA moiety that interacts with the lysine 178 of the GluN1 NTD, we used a previously characterized mutant of tPA containing an inactive LBS (W254R), hereafter termed tPA K2*.[33] NMDA-induced calcium influx was recorded in HEK-293 cells transfected with rat cDNAs encoding both GluN1-1b WT and GluN2A in combination with WT rat tPA (tPA WT) or tPA K2* (Figure 6a). Although Glunomab reduced NMDAR signaling recorded in the presence of tPA WT (–18% of potentiation with 62% of cells inhibited; $^{\#}P<0.01$; $n=29$ cells), it failed to reduce NMDA-induced calcium influx recorded in the presence of tPA K2* ($^{**}P<0.01$ compared with tPA WT; $n=19$ cells; Figures 6b and c). These data show that Glunomab is specific of the tPA/NMDAR interaction directly involving the LBS of tPA and the lysine in position 178 within GluN1 NTD.

Preventing tPA/GluN1 NTD interaction reduces extrasynaptic NMDAR diffusion, tPA-promoted NMDAR signaling and subsequent neurotoxicity. NMDAR surface diffusion was then recorded on cultured hippocampal neurons incubated with single nanoparticle complexes containing a QD associated with either an antibody raised against GluN1 NTD subunit or Glunomab. These experiments were designed to assess the effects of endogenous tPA on the dynamics of NMDAR at the neuronal surface. Figures 7a and b illustrate the distinct diffusion patterns from representative trajectories of surface NMDAR, labeled with control antibodies (control Ab) or Glunomab. The epitopes of the control Ab and of Glunomab are different corresponding, respectively, to the amino-acid residues 385–399 and 176–180 of the GluN1 subunit. However, both control Ab and Glunomab can bind GluN1 NTD. In consequence, the striking reduction in receptor surface diffusion by Glunomab compared with the control antibody can be only explained by the specificity of Glunomab, that is, its capacity to prevent the interaction of tPA with the GluN1 subunit of the NMDAR. Indeed, the fraction of immobile receptors increased more than twofold (Figure 7c). When the curve of the average square displacement (MSD) over time was calculated for the two conditions, the Glunomab curve was shifted to the right, indicating a stronger confined behavior of NMDAR in this condition (Figure 7d). We then recorded and specifically compared the diffusion of synaptic and extrasynaptic NMDAR to discriminate potential compartment-specific effect of tPA binding site. Although the reduced diffusion observed with Glunomab was marked for extrasynaptic receptors ($^{****}P<0.0001$; control Ab, $n=170$ trajectories; Glunomab, $n=146$), only a slight reduction was observed for synaptic receptors ($^{*}P<0.05$; control Ab, $n=148$ trajectories; Glunomab, $n=261$; Figure 7e). Altogether our data demonstrate that endogenous tPA selectively increases the surface dynamics of extrasynaptic NMDAR and subsequent calcium influx.

We then tested whether the blockage of the tPA-dependent potentiation of NMDARs surface dynamics could reduce the pro-neurotoxic action of exogenous tPA in a paradigm of NMDA-mediated neuronal death (Figure 8). NMDA alone (12.5 μM), led to approximately 40% neuronal death 24 h later. Whereas, tPA-promoted NMDA-induced neuronal death (60%, $^{*}P<0.05$; $n=3$ independent experiments; $n=9$ dishes; Figure 8a), this potentiating effect was prevented in a dose-dependent manner by Glunomab (0.1, 1, 10 μg/ml; $^{*}P<0.05$; $N=3$ independent experiments; $n=9$ dishes; Figure 8b). *In vivo*, as previously reported,[32] excitotoxic neuronal death, induced by stereotaxic administration of NMDA (10 nmol) in the mouse striatum, was potentiated by an intravenous injection of tPA (10 mg/kg). This potentiation was not observed if Glunomab (160 μg) was co-administered with tPA ($^{*}P<0.05$; $n=7$, 8 or 9 per group; Figures 8c and d). We previously showed that tPA promotes the neuronal ERK(½) activation mediating its pro-neurotoxic effects.[35] Accordingly, we measured the phosphorylated and total ERK(½) levels in primary neuronal cultures subjected to NMDA (50 μM), tPA (300 nM) and Glunomab (10 μg/ml) exposures either alone or in

Figure 4 Production and characterization of Glunomab (clone 15A4B2), a monoclonal antibody raised against GluN1 NTD. (**a**) Structure of the bilobate amino-terminal domain of GluN1 (GluN1 NTD, PDB ID: 3Q41).[50] The GluN1 NTD was used as antigen for immunization assays in mice. (**b**) Example of an indirect ELISA assay performed with conditioned media harvested from Glunomab (clone 15A4B2), using a His-tagged mock protein as a control or the His-GluN1 NTD as the specific antigen (1–1000 ng/ml of antibody, $N=4$ independent experiments; $^{*}P<0.05$ compared between one dose and previous dose; Kruskal–Wallis and Mann–Whitney tests). (**c**) Immunoblotting performed after SDS-PAGE resolution of recombinant His-GluN1 NTD (20 μg loaded per lane) and immunoglobulin Glunomab (clone 15A4B2) as the primary antibody ($N=2$ experiments). (**d**) Immunostaining performed from hippocampal (CA1) and cortical tissue sections (mice and rats) using either Glunomab (clone 15A4B2) (1 : 400, green) or an antibody against the C-terminal GluN1 subunit (Cter-GluN1, 1 : 400, red) or NeuN (1 : 250, blue). (**e**) Control immunostainings performed from liver tissue sections (mice) using either Glunomab (clone 15A4B2) (1 : 400, green) or an antibody against the C-terminal GluN1 subunit (Cter-GluN1, 1 : 400, red). (**f**) Immunoprecipitation (IP) assays from mouse cortices or primary cultures of cortical neurons using Cter-GluN1 subunit antibody followed by immunoblotting (IB) using Glunomab (clone 15A4B2) (representative data of three independent samples). (**g**) Calcium video imaging was performed on primary cultures of cortical neurons (12–14 DIV, see Materials and Methods section). After two successive control NMDA stimulations, neurons were incubated for 45 min with buffer (control, $n=98$ cells), Glunomab alone (10 μg/ml, $n=46$ cells), tPA alone (300 nM, $n=139$ cells) or with immunoglobulins corresponding to Glunomab (clone 15A4B2) (10 μg/ml, $n=139$ cells) or the clone 6C9B6 (10 μg/ml, $n=119$ cells). A second set of NMDA stimulations was then performed. Each dot represents one cell. Each graph is accompanied by an image during NMDA stimulation after treatment. (**h**) Percentage of stimulation or inhibition after incubation were calculated for each individual cell and reported as percentages of responsiveness for each group (mean ± S.E.M.; $N=3$ independent experiments; NS, not significant; $^{****}P<0.0001$; Kruskal–Wallis and Mann–Whitney tests for group comparison; $^{\#}P<0.0001$ Wilcoxon signed-rank test for the comparison pre- and post-incubation responses). (**i**) Percentages of potentiated, not affected and inhibited cells

combination (Supplementary Figure 4). After 5 min of treatment, tPA promotes the NMDAR-ERK(½) activation, an effect prevented by the presence of Glunomab (*$P<0.05$, $N=5$ independent experiments, including a total of $n=20$ individual dishes per condition). These data show that the control of neuronal survival is one of the physio-pathological tPA effects



that involve its ability to influence the surface dynamics of extrasynaptic NMDAR.

Discussion

In this report, we demonstrate that extrasynaptic GluN1-NMDARs surface dynamics and subsequent signaling are increased by the neuronal extracellular serine protease tPA, leading to an enhanced NMDAR signaling and neurotoxicity (Supplementary Figure 5). These effects are the direct consequence of an interaction of tPA with the lysine in position 178 (^{176}AQKRL^{180}) of the GluN1 NTD of NMDAR. Thus, tPA acts as a modulator of NMDARs distribution at the neuronal surface.

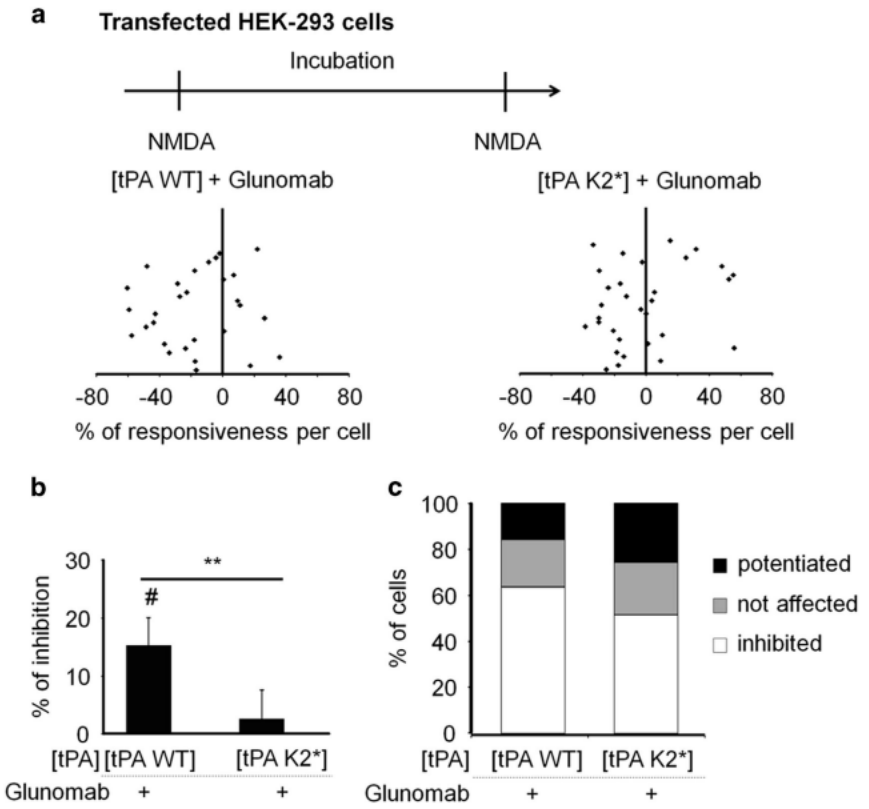


Figure 6 tPA-induced potentiation of NMDAR signaling is dependent of the LBS of the tPA kringle 2 domain (kringle 2-tPA^{LBS}). (**a**) Calcium video imaging performed on HEK-293 cells transiently transfected with GluN1-1b WT and GluN2A in combination with tPA WT or a tPA containing a point mutation within its LBS-containing kringle 2 domain (W254, tPA K2*).[35] After control NMDA stimulations (used as baseline), transfected HEK-293 cells with either GluN1-1b/GluN2A/tPA WT ($n=44$ cells) or GluN1-1b/GluN2A/tPA K2* ($n=31$ cells) were incubated for 20 min with Glunomab (10 μg/ml), before a second set of NMDA stimulations. (**b**) Percentages of inhibition after incubation with Glunomab were calculated for each individual cell and reported as the percentages of inhibition for each group (mean $\pm$ S.E.M.; $N=3$ independent experiments; $^{**}P<0.01$ Kruskal–Wallis and Mann–Whitney tests for group comparison; $^{\#}P<0.01$ Wilcoxon signed-rank test for the comparison pre- and post-incubation responses). (**c**) Percentages of potentiated, not affected and inhibited cells

Figure 5 tPA-induced potentiation of NMDAR signaling involves the lysine 178 of the GluN1 NTD (GluN1 NTD^{Lys178}). (**a**) Calcium video imaging performed on HEK-293 cells transiently transfected with either GluN1-1b WT, K178V mutated GluN1-1b or K190V mutated GluN1-1b in combination with WT GluN2A. After control NMDA stimulations (used as baseline), transfected cells with GluN1-1b WT and GluN2A were incubated for 20 min with either buffer (control, $n=34$ cells), tPA (300 nM, $n=36$ cells) and/or Glunomab (10 μg/ml, $n=30$ cells), before a second set of NMDA stimulations. The same set of NMDA stimulations were applied on cells transfected with mutated GluN1-1b K178V or mutated GluN1-1b K190V incubated with either buffer ($n=43$ cells or $n=26$, respectively) or tPA (300 nM, $n=35$ cells or $n=37$, respectively) or tPA with Glunomab ($n=47$ cells and $n=52$ cells, respectively). Each dot represents one cell. (**b**) Percentage of stimulation or inhibition after incubation were calculated for each individual cell and reported as the percentages of responsiveness for each group (mean $\pm$ S.E.M.; $N=3$ independent experiments; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$ Kruskal–Wallis and Mann–Whitney tests for group comparison; $^{\#}P<0.01$ Wilcoxon signed-rank test for the comparison pre- and post-incubation responses). (**c**) Percentages of potentiated, not affected and inhibited cells. (**d**) Calcium video imaging performed on HEK-293 cells transiently transfected with either GluN1-1b WT, K178V mutated GluN1-1b or K190V mutated GluN1-1b in combination with WT GluN2A. Before an incubation with different treatment, two first NMDA stimulations (100 μM) were performed on transfected HEK-293 cells with GluN1-1b WT (or K178V or K190V) and GluN2A. The K178V and K190V point mutations within GluN1 do not influence the basal activity of NMDAR in the absence of tPA. (mean $\pm$ S.E.M.; $N=3$ independent experiments; NS, not significant; Kruskal–Wallis and Mann–Whitney tests for group comparison). (**e**) Representative images of immunolabelings revealed with Glunomab (as primary antibody, 1 : 800) performed on HEK-293 cells transiently transfected with either GluN1-1b WT, mutated GluN1-1b K178V or mutated GluN1-1b K190V in combination with WT GluN2A ($N=3$ independent experiments). (**f**) Immunoblotting raised against GluN1 (Cter-GluN1, 1 : 250) performed from protein extracts of HEK-293 cells transiently transfected with either GluN1-1b WT, K178V mutated GluN1-1b or K190V mutated GluN1-1b in combination with WT GluN2A and a loading control using β-actin antibody (20 μg loaded per lane, $N=1$ experiment)

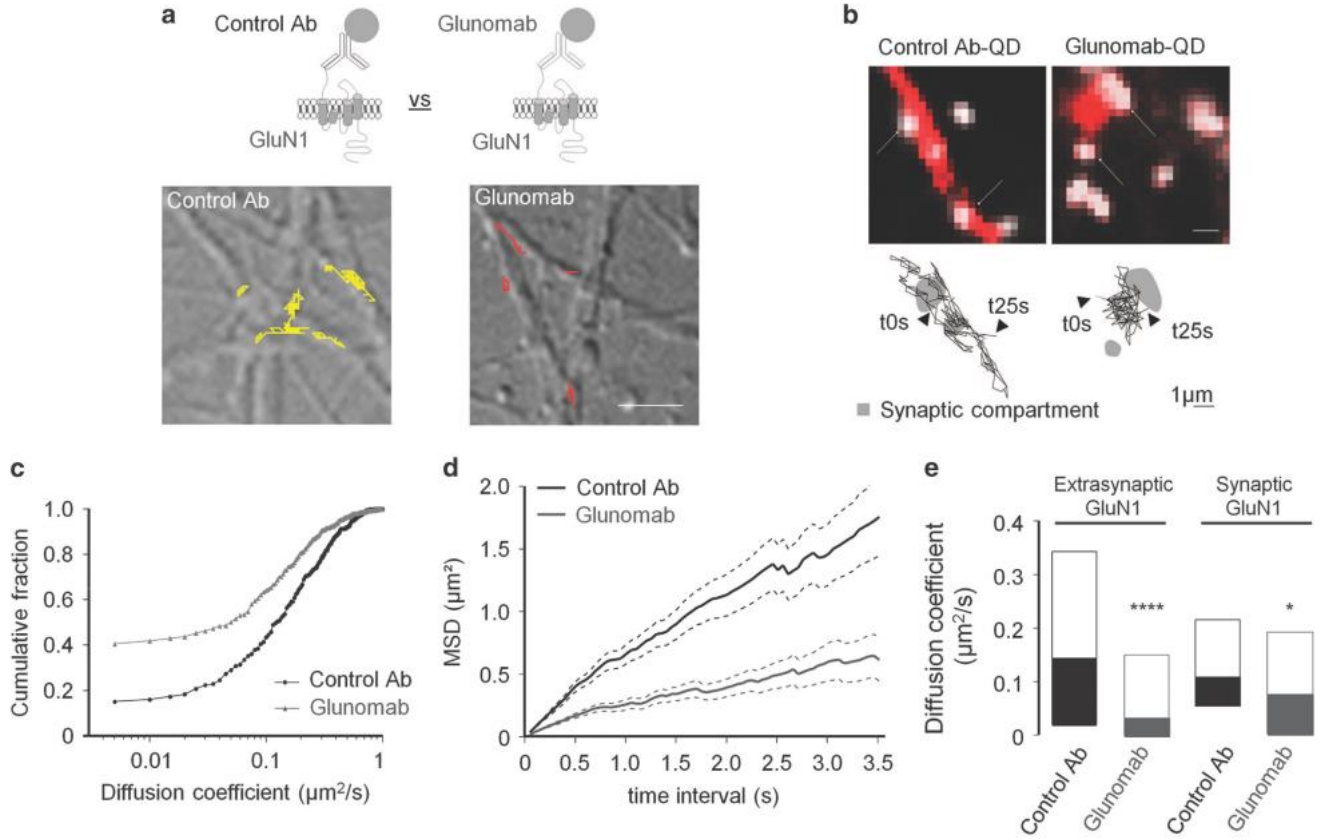


Figure 7 Blockage of the ability of tPA to bind GluN1 NTD alters extrasynaptic GluN1-NMDARs surface diffusion and increases their confined behavior. (**a**) Up: GluN1-NMDAR tracked using a single anti-GluN1 antibody QD complex obtained either by coupling a control GluN1 NTD subunit antibody (control Ab, Alomone Labs, 1 : 200) or the Glunomab antibody (1 : 200) to QDs. Down: representative GluN1-QD (GluN1 QD) trajectories on cultured hippocampal neurons (11–12 DIV) with control Ab (yellow) or Glunomab (red). (**b**) Representative trajectories of surface GluN1-QD (black lines, 500 frames, 50-ms acquisition) in the vicinity and within synapses (white arrows). Synaptic trajectories are defined by their colocalization with synaptic labeling (Mitotracker, white), trajectories outside synapses being considered as extrasynaptic. Note that the diffusion of NMDAR targeted by Glunomab is reduced outside synapses. Scale bar = 1 μm. (**c**) Cumulative distribution of the instantaneous diffusion coefficient of NMDARs targeted by the control Ab or Glunomab. The population of NMDAR targeted by Glunomab shows a reduced diffusion speed and a higher proportion of immobile receptors compared with the one tracked with the control Ab (immobile fraction Control Ab = 15%; Glunomab = 41%). (**d**) Plot of the MSD *versus* time of total GluN1 tracked with control Ab ($n = 273$ trajectories) or Glunomab ($n = 129$). The red curve (Glunomab) tends toward a negative curvature, characteristic of a confined behavior. (**e**) Instantaneous diffusion coefficient distributions (median 25–75% IQR) of extrasynaptic (control Ab = 0.1489 $\mu m^2/s$ IQR = 0.0228–0.3476 $\mu m^2/s$, $n = 148$ trajectories; Glunomab = 0.0318 $\mu m^2/s$ IQR = 0.00008–0.1532 $\mu m^2/s$, $n = 261$) *versus* synaptic GluN1-QD (control Ab = 0.1132 $\mu m^2/s$ IQR = 0.0511–0.2148 $\mu m^2/s$, $n = 170$ trajectories; Glunomab = 0.0764 $\mu m^2/s$ IQR = 0.0014–0.1909 $\mu m^2/s$ $n = 146$; $^*P < 0.05$, $^{****}P < 0.001$; Kruskal–Wallis and Dunn's multiple comparison test)

In the CNS, tPA is a well-known serine protease expressed and released in the extracellular space by many cell types including neurons.[11–14] Among the reported receptors of tPA in the CNS, one is NMDAR with GluN1 as a possible binding site.[22] In this study, we provide molecular evidence that tPA is a ligand of NMDAR. We first demonstrate that tPA directly interacts with the GluN1 NTD of NMDARs and identify the lysine in position 178 in the GluN1 NTD as its binding site. These data are in agreement with previous demonstrations that the tPA-induced potentiation of NMDAR signaling involves the LBS contained in the K2.[33,34] Thus, we can postulate that this interaction of tPA with the lysine 178 of the GluN1 NTD is the first and necessary step of a previously suggested two-step process, which also involves arginine in position 260 of the GluN1 NTD.[36] To date, the cleavage of the amino-terminal domain of the GluN1 subunit by tPA is still debated. However, there is no doubt about the capacity of tPA to enhance the NMDAR signaling.[24] Further investigation is thus needed to determine whether GluN1 cleavage is necessary for the enhancement of NMDAR function by tPA or whether GluN1 cleavage is the result of a bystander effect, inhibits desensitization of NMDA receptors or has other functions. Also interesting, plasmin (which is generated by a tPA-dependent processing of the plasminogen) has also been reported to cleave NMDARs, specifically the GluN2 subunit. This cleavage can occur at two sites: lysine 317 on GluN2A, which relieves Zn^{2+} inhibition and thereby increases NMDAR function,[37] and arginine 67 on GluN2B, which increases sensitivity of the NMDA receptor to glycine.[38]

NMDARs are diverse in their molecular subunit composition, their pharmacological properties and their subcellular localization. The dynamics and pharmacological features of NMDAR NTDs are critical for the control of the functional and pharmacological diversities of NMDARs. Although the regulatory functions of the GluN2 NTDs are well documented,[9] the functions of the GluN1 NTD remain largely unknown. In a

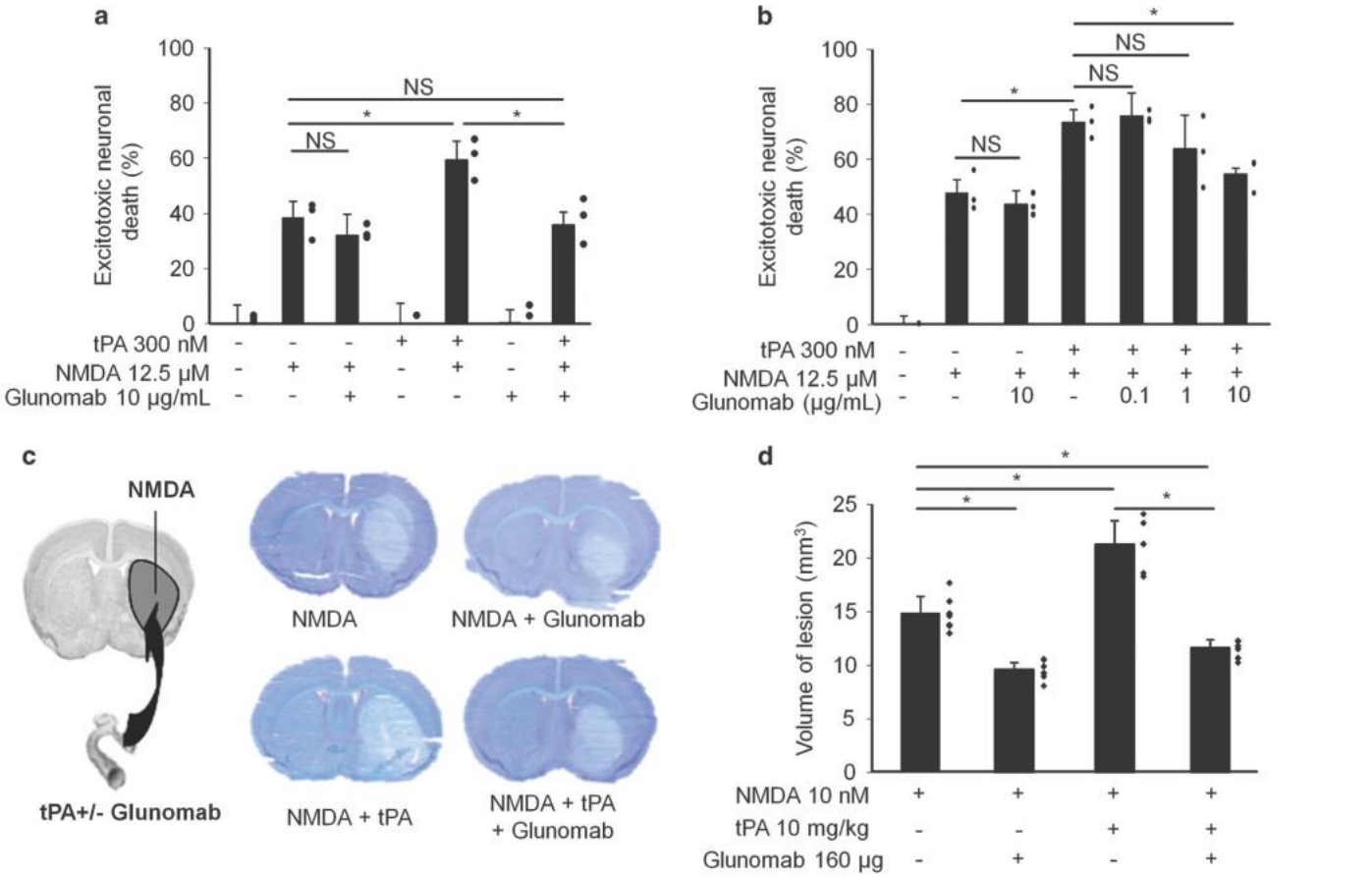


Figure 8 Blockage of the ability of tPA to bind GluN1 NTD interaction prevents the pro-excitotoxic effects of tPA *in vitro* and *in vivo* without alter its anti-apoptotic effect. (**a**) Neuronal death was assessed on primary cultured cortical neurons (12–14 DIV) by measuring LDH release in the bathing media after 24-h exposure to NMDA alone (12.5 μM) or in the presence of tPA (300 nM) and/or Glunomab (10 μg/ml) (mean $\pm$ S.E.M.; $N=3$ independent experiments, including $n=9$ individual dishes per condition; each spot represents an average value for the three independent experiments; NS, not significant, $^*P<0.05$; Kruskal–Wallis and Mann–Whitney tests). (**b**) Same experiments as in (**a**) were performed in the presence of decreasing concentrations of Glunomab (10, 1, 0.1 μg/ml; mean $\pm$ S.E.M.; $N=3$ independent experiments, including $n=9$ individual dishes per condition; $^*P<0.05$; Kruskal–Wallis and Mann–Whitney tests; each spot represents an average value for the three independent experiments). (**c**) Left: schematic representation of unilateral striatal injection of NMDA (10 nmoles, 1 μl). Right: illustrative images of excitotoxic lesions in all conditions. (**d**) Quantification of volume of excitotoxic lesions (mean $\pm$ S.E.M.; $n=7$, 8 or 9 mice per group; $^*P<0.05$ Kruskal–Wallis and Mann–Whitney tests; each spot represents individual lesion volumes).

recent study, it was showed that GluN1 NTD was highly mobile and actively participated in defining the gating and pharmacological profile of NMDAR. As proposed by Paoletti's group, this discovery redefines the possible functional consequences of interactions between GluN1 NTDs of NMDAR and extracellular molecules.[10] Severals studies propose that ifenprodil and polyamines are involved in the modification of NMDAR conformation by shielding negative charges present on GluN1 and GluN2B NTD lower lobes.[39–41] In this study, we propose that tPA is one of these extracellular molecules capable of binding to the GluN1 NTD and, as such, to influence NMDAR signaling. Accordingly, antibodies that target the binding site of such molecules, like Glunomab, are putative modulators of NMDARs. Surface trafficking has recently emerged as a crucial cellular pathway involved in the pathophysiological tuning of excitatory synapse transmission.[42,43] Through the use of single particle imaging in neurons, we showed that, by selectively controlling extrasynaptic NMDARs diffusion, tPA has a key role in synaptic adaptation processes and functions of NMDARs signaling. It is important to note that tPA did not influence the percentages of detected synaptic and extrasynaptic NMDARs, respectively.

Although the relationship between the surface dynamics of NMDAR and their signaling has yet to be proven, our present data support a strong relationship between surface dynamics and subsequent signaling. By increasing the surface dynamics of a fraction of extrasynaptic NMDAR, we can propose that cumulative effects in the whole population of NMDARs are responsible for the potentiating effect of tPA on NMDARs signaling. These observations fit with our previous finding that tPA-promoted neurotoxicity through a mechanism that involved extrasynaptic and possibly GluN2D-containing NMDARs,[35,44] a phenomenon also associated with an activation of the ERK(½) pathway.[35] In this study, we show that Glunomab is also capable to prevent the tPA-dependent increase of neuronal ERK(½) activation. Moreover, the neurotoxicity of tPA can be explained by the conformation (single or two chain tPA; sc-tPA or tc-tPA) and by its concentration.[45] sc-tPA (conformation using in this study) decreased NMDAR-dependent calcium influx at 10 nM causing a neuroprotective

effect, 300 nM tPA led to an increased NMDAR signaling and neurotoxicity. This protective effect of low doses of tPA was also reported by the group of Manuel Yepes.[46] Accordingly, although tPA promotes NMDA-induced excitotoxicity,[22] Glunomab appears to be a promising neuroprotective tool. These data are also in agreement with the proposal that the activation of synaptic NMDARs promotes cell survival, whereas activation of extrasynaptic NMDARs promotes cell death.[2] Interestingly, a non-proteolytic form of tPA failed to promote the surface dynamics of NMDAR, suggesting that at least the catalytic site of tPA is required for this process. Whether the cleavage of GluN1 is needed,[22] remains to be investigated. Although the use of general NMDARs blockers for the treatment of disorders of the CNS is associated with unacceptable effects,[47] the present data suggest that targeting the potentiating effect of tPA on extrasynaptic NMDARs, may offer promising therapeutic avenues.

In conclusion, we identified the extracellular serine protease tPA, produced and released by neurons,[11,12,23] as a ligand of the GluN1 NTD leading to a specific increase of the surface dynamics of extrasynaptic NMDARs, their synaptic diffusion and subsequent neurotoxicity.

Materials and Methods

Ethical statement. Experiments were performed in accordance with French ethical laws (act no. 87–848; Ministère de l'Agriculture et de la Forêt) and European Communities Council Directives of 24 November 1986 (86/609/EEC) guidelines for the care and use of laboratory animals, and were approved by the local and regional ethics committees (authorization code CENOMEXA 0113-03). All efforts were made to limit animal suffering. None of the experimental procedures induced animal mortality except one mouse during surgery (Figure 8 condition NMDA+tPA i.v.). All experiments were performed following the ARRIVE guidelines (www.nc3rs.org.uk), including randomization for the administration of test substances as well as analyses that were performed in a blinded manner. Male mice were housed on a 12-h light (0700 hours)/dark (1900 hours) cycle with *ad libitum* access to water and rodent chow (10 mice per cage).

Chemicals. (5 *R*,10 *S*)-(−)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-iminemaleate (MK801) and *N*-methyl-D-aspartate (NMDA), 2-amino-5-phosphonovaleric acid (AP5) were purchased from Tocris (Bristol, UK). Human tPA (Actilyse) was from Boehringer Ingelheim (Paris, France). tPA buffer was 34.84 mg/ml arginine, 10.72 mg/ml phosphoric acid and 0.1 mg/ml Tween 80. 5-Bromo-4-chloro-3-indolyl phosphate, alkaline-phosphatase-conjugated goat anti-mouse IgG, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, lipofectamine 2000, Dulbecco's modified Eagle's medium (DMEM), poly-D-lysine, laminin, glutamine, cytosine β-D-arabinoside (Ara-C), glycine, HEPES, phosphate-buffered saline (PBS) solution, Freund adjuvants, Trypan blue solution (0.4%), paraformaldehyde, anti-mouse, anti-goat/sheep peroxidase antibody, anti-β-actin (A2066) were purchased from Sigma-Aldrich (L'Isle d'Abeau, France). Goat anti-rabbit IgG and anti-rmouse IgG were purchased from Thermofisher (Villebon-sur-Yvette, France). FuGENE-6 was purchased from Promega (Charbonnières-les-Bains, France). TRITC-, FITC-, Dye Light 649 nm-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Polyclonal antibodies raised against C-terminal end of GluN1 (Cter-GluN1) subunit was purchased from Santa Cruz (sc 1467, Heidelberg, Germany). Anti-NeuN antibody was purchased from Abcam (ab131624, Paris, France). Protein A sepharose was purchased from GE Healthcare (Orsay, France). ECL-Plus detection system was purchased from PerkinElmer Life and Analytical Sciences (Cambridge, UK). Polyclonal antibodies raised against GluN1 NTD subunit was purchased from Alomone Labs (AGC 001, Jerusalem, Israel). QDs 655 labeled goat $F(ab')_2$ anti-mouse (Q11021) and anti-rabbit IgG (Q11421), secondary anti-mouse Alexa 568 antibody (A11004), Green MitoTracker (M7514), RPMI 1640 medium, Fura-2 AM, fetal bovine serum and horse serum, neurobasal medium, B27 supplement (50X) were purchased from Life Technologies (Saint Aubin, France). Lactate deshydrogenase (LDH) detection kit and mouse isotyping test kit were purchased from Roche Diagnostics (Mannheim, Germany). The anti-phosphorylated Erk(½) and anti-Erk(½) antibodies were purchased from Cell Signaling (Saint Quentin Yvelines, France).

Single particle (QD) tracking and immunochemistry. Hippocampal neurons from E18 Sprague–Dawley rats were cultured as previously described.[48] Cells were plated on poly-lysine pre-coated cover slips and maintained in a 3% horse serum containing neurobasal medium. After 3 day *in vitro* (DIV), the original medium was replaced with a serum-free medium. Cultures were maintained at 37 °C in 5% CO_2. To investigate the impact of tPA on NMDAR diffusion, cultures were pre-incubated 45 min with either active tPA (300 nM, 37 °C) or tPAm (non-proteolytic tPA, 300 nM, 37 °C). QD detection and subsequent analysis were performed as previously described.[7] Briefly, hippocampal neurons (11–12 DIV) were incubated for 10 min (37 °C) with polyclonal antibodies against GluN1 NTD subunit (Alomone Labs; 1 : 200). Neurons were then washed and incubated for 10 min (37 °C) with QDs 655 goat $F(ab')_2$ anti-rabbit (1 : 10 000). Green MitoTracker (1 : 1000) was used as a synaptic marker. For the data illustrated in Figure 7, neurons were incubated for 10 min (37 °C) with either the control anti-GluN1 NTD subunit (1 : 200) or the Glunomab antibodies (1 : 200). The same protocol was followed, except that neurons were incubated with QD 655 goat $F(ab')_2$ anti-rabbit or anti-mouse. Signals were detected using a EM-CCD camera (Quantem, Roper Scientific, Evry, France). QDs were followed on randomly selected dendritic regions for up to 20 min. QD recording sessions were processed with the Metamorph software (Universal Imaging Corporation, Chester, PA, USA). Instantaneous diffusion coefficient distributions of GluN1-QD are represented using median and interquartile range. The top white portion, the line between the white and the black portions and the bottom black portion correspond of the first quartile, the median and the third quartile, respectively.

Calcium video imaging and x-link protocol in neurons. Cortical neurons were prepared from E14.5 Swiss mice as previously described.[35] Cortices were dissected and dissociated in DMEM, and plated on 35 mm dishes previously coated with poly-lysine (0.1 mg/ml) and laminin (0.02 mg/ml). Cells were cultured in DMEM supplemented with 5% fetal bovine serum, 5% horse serum and 2 mM glutamine. Cultures were maintained at 37 °C in 5% CO_2. Cultures were transferred into a serum-free HEPES-buffered saline solution (HBBSS: NaCl 116 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 0.8 mM, HEPES 12 mM, NaH_2PO_4 0.34 mM, D-glucose 5.5 mM, $NaHCO_3$ 25 mM, Glycine 10 μM) at DIV 12 and loaded with 10 μM Fura-2 AM for 45 min at 37 °C. The Ca^{2+} bound form of Fura-2 gets excited at 340 nm, the Ca^{2+} unbound form at 380 nm and both recorded at 510 nm. Neurons were washed and NMDA stimulations (2 × 25–50 μM for 30 s) were applied using a peristaltic pump. Neurons were then treated with monoclonal antibodies named Glunomab (10 μg/ml), and/or, its isotypic control 6C9B6 (10 μg/ml), and/or tPA (300 nM) (Figures 2 and 4,Supplementary Figure 1) and/or plasmin (100 μg/ml) (Figure 2) during 45 min. For each cell, the area under curve (AUC) corresponding to the intracellular calcium influx induced by NMDA (ratio 340/380) was calculated before (AUCb) (mean of the two first pre-treatment stimulations) and after treatment (AUCa) (tPA, plasmin, Glunomab, 6C9B6, x-link) (mean of the 2 s post-treatment stimulations). We then compared, for each cell, the amount of NMDA-induced calcium influx (AUC) after treatment with the NMDA-induced calcium influx recorded before treatment. Thus, each cell is its own control with an expression of the modification of the NMDA-induced calcium influx because of the treatment performed between the two rounds of stimulations, expressed as a percentage of response to treatments (% of responsiveness).

$$\% \text{ of responsiveness} = [(\text{AUC})a/(\text{AUC})b] * 100$$

with (AUC)a: sum of area under curve of Fura-2 ratio during both NMDA stimulations after treatment and (AUC)b: sum of area under curve of Fura-2 ratio during NMDA stimulations before treatment.

Experiments were performed at room temperature, on the stage of a Leica DMI6000B inverted microscope equipped with a 150 W Xenon high stability lamp and a Leica 40×, 1.3 numerical aperture epifluorescence oil immersion objective (Wetzlar, Germany). Fura-2 (excitation: 340, 380 nm, emission: 510 nm) ratio images were acquired with a Digital CMOS camera (Hamamatsu, ORCA-Flash2.8 C11440-10C, Massy, France) and digitized (2048 ×2048 pixels) using Metafluor 6.1 software (Universal Imaging Corporation). For x-link procedures, neurons were treated for 15 min with primary anti-GluN1 (1 : 20) and secondary (1 : 30) antibodies (x-link) (Figure 3 and Supplementary Figure 1). The buffer of Glunomab, control 6C9B6 and x-link is PBS solution.

Production and purification of monoclonal antibodies and ELISA. Eight-week-old Balb/C male mice (20–25 g) were immunized by intraperitoneal injection of immunogenic mixtures: complete Freund adjuvant (first injection) and incomplete Freund adjuvant (once a week during 3 weeks) alone (control) or containing the rHis-GluN1 NTD. Sp2/0Ag14 myeloma cell line (ATCC, CRL 1581, Molsheim, France) was fused with splenocytes from selected immunized mouse according to standard protocols and fusion product was plated in culture microplates for 15 days before primary screening.

Clone selection was performed using indirect ELISA along this process: from primary screening, confirmation to sub-cloning screenings. Antigens used were rHis-GluN1 NTD and His-mock as negative control. After primary screening and confirmation, two antibody-producing hybridomas were identified and were sub-cloned twice then frozen in liquid nitrogen. Monoclonal antibodies were produced *in vitro* after collecting concentrated supernatants. Purifications were done by affinity chromatography on protein A sepharose. The mAbs were isotyped with a mouse isotyping test kit according to the manufacturer's recommendations.

6C9B6 is a clone produced in parallel to Glunomab. Like Glunomab, it binds recombinant NTD of GluN1, but it does not block the capacity of tPA to bind to and promote NMDAR signaling, thus targeting a different epitope than GluN1.

Immunoblotting and immunoprecipitation. Proteins (20 μg) were resolved on 7.5% SDS-PAGE blots under denaturing conditions and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with Tris-buffered saline (10 mM Tris and 200 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 0.4% BSA. Blots were incubated overnight with primary antibodies: Glunomab (clone 15A4B2, 0.5 mg/ml, 1 : 1000) or Cter-GluN1 antibody (1 : 250). After incubation with the anti-mouse or anti-goat peroxidase-conjugated secondary antibody, proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system. For the immunoprecipitation, supernatants from TNT buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Triton 100X)-lysed tissues or cultures (500 μg of proteins) were incubated overnight at 4 °C in the presence of the Cter-GluN1 antibody (2 μg). Samples were then coupled to protein G-sepharose beads as described by the manufacturer (GE Healthcare, Orsay, France). Proteins were separated by 7.5% SDS-PAGE, blots were exposed in the presence of Glunomab (1 : 1000) and revealed following the procedure of immunoblotting described above.

Immunohistochemistry. Adults mice (Swiss males; 35–45 g) and rats (Sprague–Dawley males, 350–400 g) were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 150 ml or 400 ml of 0.1M sodium phosphate buffer, respectively. Coronal sections of brain and liver (10 μm) were incubated overnight at room temperature with Glunomab (1 : 500), anti-Cter-GluN1 antibody (1 : 400) and anti-NeuN antibody (1 : 250). Detection was performed using F(ab')2 fragments of donkey anti-goat linked TRITC, anti-mouse linked FITC or anti-chicken linked Dylight 649 nm (1 : 600). All sections were examined with a Leica DM6000 microscope. Images were captured using a CoolSnap camera and visualized with Metavue 5.0 software (Molecular Devices, Sunnyvale, CA, USA).

Epitope mapping and Alascan. Epitope mapping and Alascan are presented in Supplementary Figure 3. A total of 141 overlapping pentadecapeptides, frame-shifted by three or one residue covering the entire amino-acid sequence of GluN1 NTD (residues 19–371), were prepared using the Spot technique according to the protocol previously described by Laune *et al.*[49] Briefly, peptides were assembled using Fmoc chemistry on a cellulose membrane containing an aminopolyethyleneglycol moiety. The C-terminal residue of each peptide was coupled to the moiety. After Fmoc deprotection, the other amino acids were sequentially added as in conventional solid-phase peptide synthesis. Finally, the side-chain protecting groups were removed by trifluoroacetic acid treatment in the presence of appropriate scavengers, whereas the linkage of the peptides to the membrane was maintained. Free cysteines were replaced with non-reactive acetamidomethyl cysteines. Once reactive peptides were identified, alanine scanning of positive sequences was performed to identify key residues contributing to the epitope; signal extinction indicated a key contribution of the aa whereas a decrease in the signal meant partial contribution to the epitope.

Purified mAbs were incubated on the membrane, and then antibody binding was detected by using an alkaline-phosphatase-conjugated goat anti-mouse IgG. For staining, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were used as substrates. A blue precipitate was observed on peptides to which the antibody was bound. To allow the re-use of the membrane, it was sequentially treated with dimethylformamide, then 1% SDS, 0.1% 2-mercaptoethanol in 8 M urea, then ethanol/water/acetic acid (50 : 40 : 10 vol/vol/vol) and, finally, ethanol in order to remove the precipitated dye and molecules bound to the peptides.

Transfection and calcium video imaging in HEK-293 cells. Human embryonic kidney 293 cells (HEK-293 cells) were grown in RPMI 1640 supplemented with 5% fetal bovine serum and with NMDA antagonists (200 μM AP5 and 2 mM $MgCl_2$). Cells were transfected by lipofection (8μl, FuGENE-6), with a mixture containing 2 μg of GluN1-1b WT (or GluN1-1b K178V or GluN1-1b K190V; Figure 5) and 2 μg of GluN2A or with tPA WT or tPA K2* (Figure 6). Transfected HEK-293 cells were loaded with 10 μM fura-2 AM for 30 min at 37 °C in HBSS containing 0.1% pluronic F-127, 20% solution in DMSO, AP5 (200 μM) and $MgCl_2$ (2 mM) and then incubated for an additional 45-min period in HBBSS prior recording. We used the same set up and data analysis as mentioned in the methods section for calcium video imaging in neurons. Concentrations of tPA and Glunomab were also of 300 nM and 10 μg/ml, respectively. NMDA exposures were performed at 100 μM for 30 s and the duration of treatments was of 20 min between the two rounds of NMDA stimulations.

Site-directed mutagenesis. Mutagenesis of rat wt-GluN1-1b (GluN1-1b K178V or GluN1-1b K190V) was performed with the QuikChange XL Kit (VWR International France, Fontenay-sous-Bois, France) and the following primers: 5′-GGCAGCGCAGgtGCGCTTGGAG-3′ and 5′-CGTCCCTCGTGGTCGTCG-3′ (to GluN1-1b K178V), 5′-ACGGGAGTCCgtgAGTAAAAAAAGGAACTATG-3′ and 5′-TC CTCCAGCAACGTCTCC-3′ (to GluN1-1b K190V). Mutations were confirmed by sequence analysis. Site-directed mutagenesis strategy in K2 domain of tPA sequencing procedure have been described previously.[33]

Excitotoxic neuronal death. Cultures of cortical neurons at 12–13 DIV were prepared in same way as that reported above for calcium video imaging. Neurons were rinsed three times with serum-free medium and as previously described,[35] excitotoxicity was induced by exposure to NMDA (12.5 μM) for 24 h. NMDA was applied alone or together with tPA (300 nM) and/or Glunomab (0.1, 1 or 10 μg/ml). Twenty-four hours later, neuronal death was quantified by measurement of LDH released from damaged cells.

***In vivo* excitotoxic lesions.** Twelve-week-old Swiss mice (male) were anesthetized (2% of isoflurane in 1/3 O_2 and 2/3 N_2O) and placed in a stereotaxic frame prior injection. A cortical unilateral injection (coordinates: 0.5 mm posterior, 3.0 mm lateral, –0.8 mm ventral to the bregma; stereotaxic atlas G Paxinos & KBJ Franklin) of NMDA (10 nmoles, 1 μl) was performed. Fifteen minutes after the excitotoxic lesion, a 200 μl intravenous bolus injection of tPA (10 mg/kg) or saline with or without Glunomab (160 μg) was given via a catheter previously inserted into the tail vein. Solutions were injected by the use of a micropipette made with hematologic micropipettes. Twenty-four hours later, mice were killed and the brains removed and frozen in isopentane. Cryostat-cut coronal brain sections (20 μm) were stained with thionine and a volumetric analysis of brain lesions was performed with the Image J software (NIH software, National Institute of Health, Bethesda, MD, USA). Region of interest were determined through the use of a stereotoxic atlas for the mouse and an image analysis system (Scion Image Scion Corporation, Frederick, MD, USA) was used to measure lesion corresponding to the nonstained area ($n=7$, 8 or 9 per group).

P-Erk(½)-dependent NMDAR signaling. Erk(½) activation by phosphorylation was used as an index of NMDAR signaling. NMDARs were activated by exposure of the primary neuronal cultures to NMDA (50 μM), tPA (300 nM) and Glunomab (10 μg/ml) either alone or in combination. After 5 min of treatment, cells were chilled on ice and lysed in buffer containing Tris-NaCl-Triton 1% of protease inhibitor cocktail and 1% of phosphatase inhibitor cocktail. Lysates were clarified by centrifugation at 13 000 *g*, for 10 min at 4 °C. Proteins were quantified and immunoblotted using adequate primary antibodies (anti-phosphorylated Erk(½) and anti-Erk(½), 1 : 1000) followed by incubation with the appropriate peroxydase-conjugated secondary antibody. Erk(½) and p-Erk(½) levels were investigated by running separate gels from the same protein extracts.

Statistical analysis. Normality was tested for all analyses of variance. The instantaneous diffusion coefficients (Figures 1d and 7e) is reported as the median ± 25–75% (interquartile range, IQR). The other data of Figures 1 and 7 are

expressed as mean ± S.E.M. Comparisons between groups for instantaneous diffusion coefficient were performed with Kruskal–Wallis test followed by Dunn's multiple comparison test as post-hoc test. Comparison between extrasynaptic fractions (Figure 1e) was performed with one-way ANOVA. Significance levels were defined as $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and not significant (NS). For calcium video imaging of neurons (Figures 2,3 and 4 and Supplementary Figure 1) and of HEK-293 cells (Figures 5 and 6), the responsiveness was analyzed by Wilcoxon signed-rank test to compare pre-and post-incubation NMDA responses. Significance levels were defined as $^{\#}P<0.0001$ (for neurons experiments) and $^{\#}P<0.01$ (for HEK-293 cells experiments). In addition, for group comparison, Kruskal–Wallis tests were used followed by Mann–Whitney *U*-tests as post-hoc tests, significance levels was defined as $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ and NS. Results are expressed as mean ± S.E.M. For Elisa (Figure 4), the excitotoxic neuronal death (Figures 8a and b), *in vivo* excitotoxic lesions (Figure 8d), P-Erk (½)-dependent NMDAR signaling (supplementary Figure 4), Kruskal–Wallis tests were used followed by Mann–Whitney *U*-test as post-hoc test. Results are expressed as mean ± S.E.M., significance levels was defined as $^{*}P<0.05$ and NS.

Conflict of Interest

DV, CA, K-UP and RM are co-inventors on the following patent 'Treatment of neurological or neurodegenerative disorders' (WO2011023250A1). The original patent W02011023250A1 was completed and now published under the number W02014187879A2 (additional references: EP2805972A1, WO2014187879A3). The present invention relates to the field of antibodies. In particular, it provides an anti-NMDA antibody or fragment or derivative thereof which is effective in inhibiting the deleterious effects of tissue-type plasminogen activator (tPA) mediated by *N*-methyl-D-aspartate (NMDA) receptors being toxic to neurons and inflicting damage to the neurovascular unit/blood–brain barrier (BBB) or regulating it in a way that leads to pathological consequences.

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Tissue-type plasminogen activator controls neuronal death by raising surface dynamics of extrasynaptic NMDA receptors.

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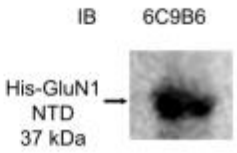
Summary of supplementary data:

This supplementary information includes 5 figures supporting the main text, detailed data from each supplementary figure has been mentioned in the main text.

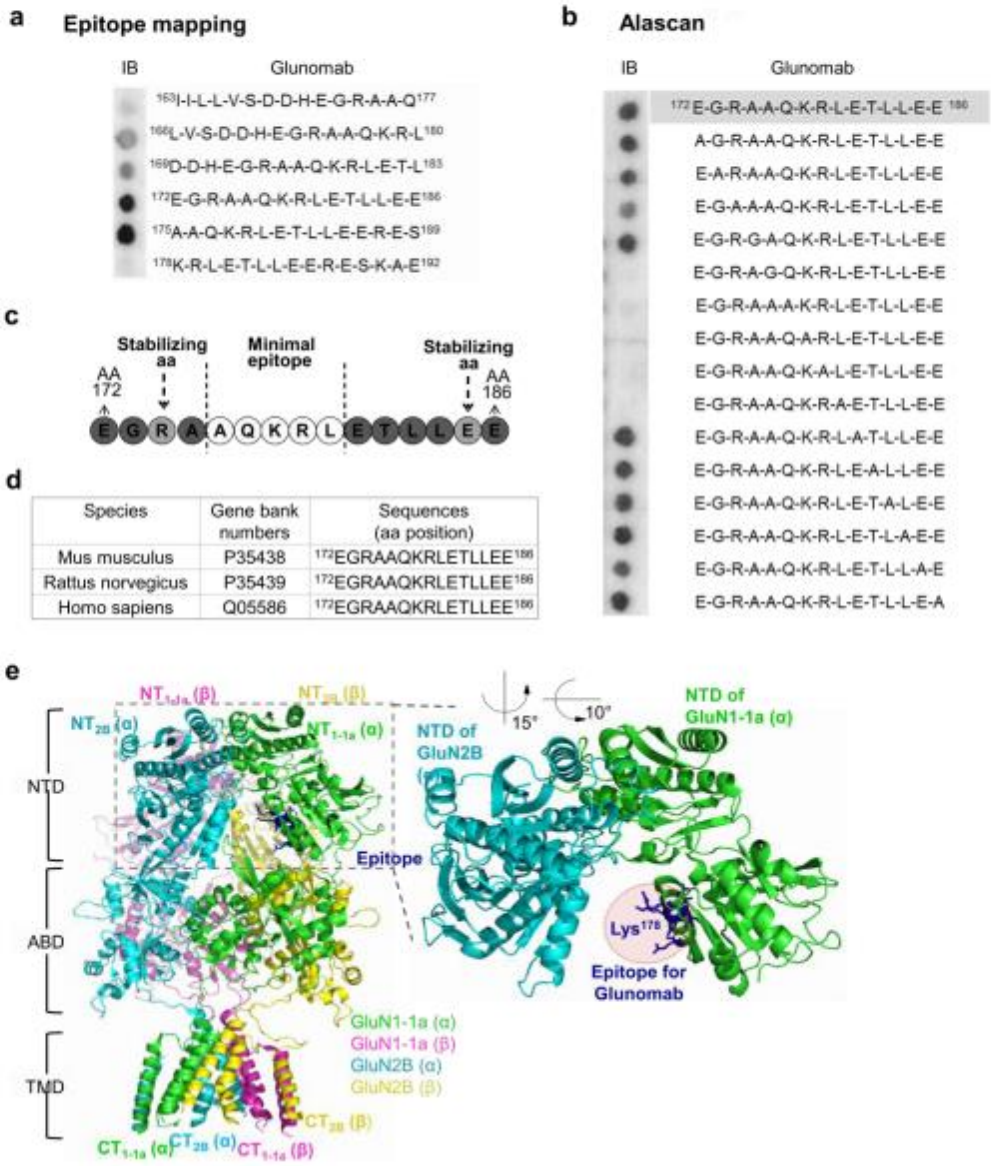
a

Clone Name	Supernatant dilution	His-GluN1 NTD	His-mock	ratio
15A4B2	1/2	2.8	1.6	1.7
	1/10	2.1	1.3	1.6
	1/100	0.6	0.4	1.3
	1/1000	0.2	0.1	1.4
6C9B6	1/2	2.5	1.6	1.4
	1/10	2.3	1.6	1.5
	1/100	1.9	1.3	1.5
	1/1000	1.0	0.6	1.7

b

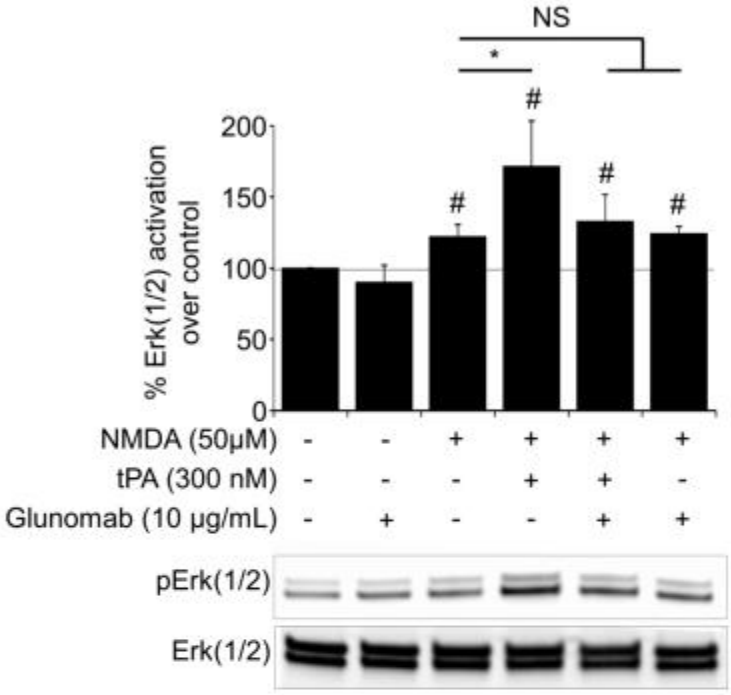


Supplementary Figure 2. Characterization and selection of positive clones. (**a**) Example of indirect ELISA assays performed with conditioned media harvested from Glunomab (clone 15A4B2) and clone 6C9B6, using a His-tagged mock protein as a control or the His-GluN1 NTD as the specific antigen. (**b**) Immunoblotting performed after SDS-PAGE resolution of recombinant His-GluN1 NTD (20 µg loaded per lane) by using the clone 6C9B6 as primary antibody. The immunoblot performed by using the clone 15A4B2 as primary antibody can be visualized figure 4c.

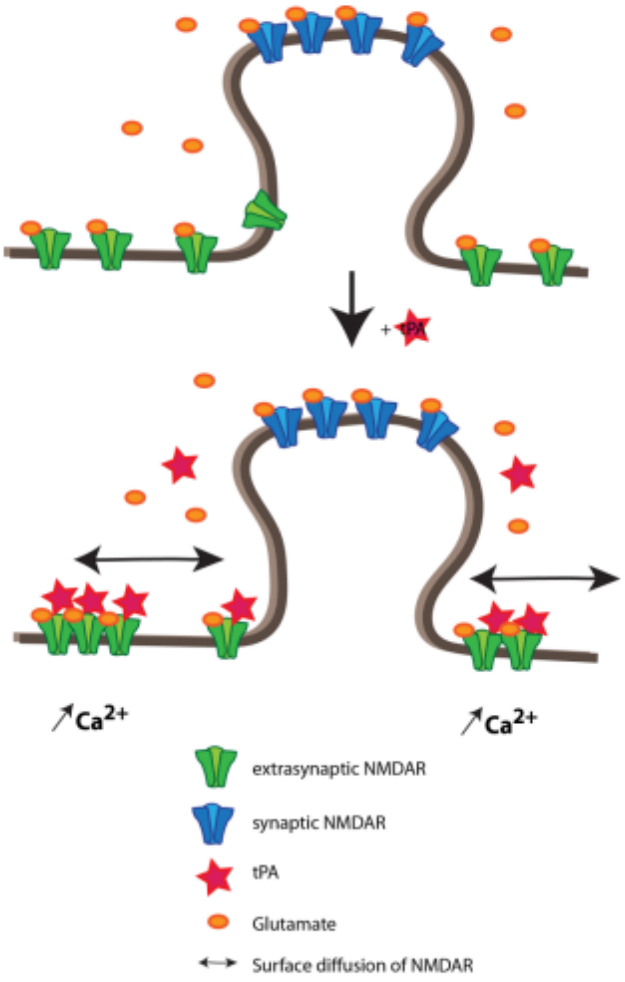


Supplementary Figure 3. Glunomab prevents tPA-mediated potentiation of NMDAR signaling. (**a**). Immunoblotting of a total of 141 overlapping pentadecapeptides frame-shifted by three residues covering a part of the amino acid sequence of GluN1 NTD (19-371aa) was revealed with Glunomab. The figure represents 6 (163-192aa) out of 141 peptides analyzed. Epitope mapping identified the putative epitope for Glunomab as being the following amino acid sequence in GluN1 NTD "^{172}EGRAAQKRLETLLEE^{186}". (**b**) Alanine scanning (Alascan) of the amino acid sequence of the GluN1 NTD "^{172}EGRAAQKRLETLLEE^{186}". (**c**) The epitope identified revealed that the key amino acids within this minimal epitope were A^{176}, Q^{177}, K^{178}, R^{179}, L^{180} and that amino acids R^{174}, E^{185} may stabilize binding of Glunomab

to GluN1 NTD.(**d**) This epitope is conserved at 100% between mice, rats and humans. (**e**) Overall structure of GluN1a-GluN2B NMDAR (PDB ID: 4PE5; ref 50). GluN1a and GluN2B subunits, labelled as GluN1-1a (α), GluN1-1a (β), GluN2B (α), GluN2B (β) are coloured in green, pink, cyan and yellow, respectively. The epitope of Glunomab is coloured in dark bleu and showed in GluN1a (α). This epitope may have possibly access to extracellular molecules which are represented by a red circle.



Supplementary Figure 4. Glunomab prevents the tPA-dependent increase of NMDA-mediated neuronal Erk(½) activation. Immunodetection of activated and total Erk(1/2) performed from primary cultures of cortical neurons (12–14 DIV) subjected to a 5 min exposure of NMDA either alone (50 µM) or with tPA (300nM) and/or Glunomab (10µg/mL). The graph shows the quantification of Erk(½) activation relative to control (mean ± SEM; N= 5 independent experiments, including n= 4 individual dishes per condition NS: not significant; *: $p< 0.05$; Kruskal-Wallis and Mann-Whitney tests for group comparison; #: $p< 0.05$; Kruskal-Wallis and Mann-Whitney tests for comparison with control conditions).



Supplementary Figure 5. tPA promotes the surface diffusion of extrasynaptic NMDAR and subsequent signaling. Interaction of tPA with extrasynaptic NMDAR promotes their capacity to diffuse at the neuronal surface, likely favouring clusters formation and subsequent NMDA-dependent calcium influx and excitotoxicity.

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