Neural mechanisms of oxytocin and serotonin interaction in non-human primates and patients with autism

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N°d'ordre NNT : xxx

THESE de DOCTORAT DE L'UNIVERSITE DE LYON

opérée au sein de

l'Université Claude Bernard Lyon 1

Ecole Doctorale 476

(Neuroscience et Cognition)

Spécialité de doctorat :

Discipline : Neurosciences

Soutenue publiquement le 13 Décembre 2016, par :

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Remerciements

Je tiens en premier lieu à remercier Vendetta, Patch et Jimmy Jazz, les trois macaques ayant participé aux expériences de cette thèse.

Evidemment, je suis très reconnaissant à Angela Sirigu de m'avoir offert l'opportunité de faire ce travail. A son contact, j'ai beaucoup appris sur le monde académique et le fonctionnement de la recherche.

Je souhaite également remercier les personnes sans qui je n'aurais pu réaliser ce travail : Jean-René Duhamel et Manon Dirheimer pour m'avoir appris à manipuler et expérimenter sur des singes ; Nathalie Richard, Jérôme Redouté et Nicolas Costes pour leur aide précieuse dans l'analyse des données TEP.

Beaucoup de collègues ont aussi ma gratitude pour le travail en collaboration que nous avons pu faire, Luc Zimmer et Sylvain Fieux pour l'autoradiographie, Miguel Pedroza pour la SPE, Raphaëlle Mottolese pour l'acquisition des TEP chez l'homme, Pierre-Aurélien Beuriat et Mina Jazayeri pour les TEP chez le singe, Sandra Duperrier pour l'histologie, Jean Luc Charieau et Fidji Francioly pour l'animalerie, Carmine Mottolese et le service de neurochirurgie pour les prélèvements de LCR, Véronique Berthier et le personnel du CERMEP pour leur flexibilité, Jennifer Beneyton pour son efficacité.

De nombreuses personnes m'ont épaulé de manière diverses, merci à Didier Le Bars, Aicha Midouni, May Cha Li, les divers stagiaires de l'équipe, Laurence Chardon, Johan Pacquit et Sylvain Maurin.

J'ai également une pensée pour les patients et leurs familles, qui nous permettent d'avancer, ainsi que l'ensemble des sujets ayant participé aux expériences.

J'ai passé d'excellents moments à l'ISC grâce à la bonne ambiance qui règne entre étudiants, présents ou passés : Martina, Manuela, Song, Laure, Seongmin, David, Rémi, Mathilde, Romain, Sébastien, Augustin, Gustavo et tous les autres.

Pour finir, merci à ma famille, qui m'a toujours soutenu ; à Emma, qui a partagé les hauts et les bas avec moi.

Résumé

La neurohormone ocytocine (OT) est de plus en plus étudiée pour son potentiel thérapeutique dans les troubles du comportement social, comme l'autisme, qui sont associés à une dérégulation de plusieurs systèmes de neurotransmission, notamment l'OT et la sérotonine (5-HT). Dans ce cadre, une étape importante afin de développer des médicaments basés sur des mécanismes biologiques est de caractériser les interactions entre l'OT et les autres neurotransmetteurs. La littérature sur les rongeurs montre que la relation entre OT et 5-HT est fortement impliquée dans plusieurs aspects du comportement social. Par ailleurs, nous avons récemment montré chez le sujet sain que le fonctionnement du récepteur 5-HT 1A (5-HT_{1A}R) est modifié suite à l'administration d'OT.neuro

J'ai donc réalisé une première expérience chez des patients autistes en utilisant le scanner TEP avec le radiotraceur [^{18}F]MPPF (spécifique du 5-HT_{1A}R). Aucune différence n'est apparue, à l'état basal, entre 18 patients autistes et 24 sujets contrôles. Par ailleurs, l'OT n'a pas modifié le système 5-HT_{1A}R. Enfin, alors qu'une corrélation entre la densité de 5-HT_{1A}R et le volume de matière grise du striatum a été observé dans le groupe contrôle, cette relation était absente dans le groupe de patients. Ces résultats suggèrent une altération subtile du 5-HT_{1A}R, ne pouvant être détectée qu'au niveau fonctionnel.

Parce que le scanner TEP ne permet pas de dire si les changements observés sont dus à une libération de sérotonine ou à une modification directe du récepteur, j'ai réalisé une deuxième expérience chez 3 macaques rhésus, avec le [^{18}F]MPPF et le [^{11}C]DASB (marquant le transporteur de la 5-HT). Par rapport au placebo, l'OT injectée dans le ventricule latéral a significativement augmenté la liaison du [^{18}F]MPPF dans l'amygdale et l'insula tandis que la liaison du [^{11}C]DASB diminuait dans ces mêmes régions. Ainsi, nous pouvons dire que l'OT a provoqué la libération de 5-HT ainsi qu'une modification du 5-HT_{1A}R dans ces régions importantes pour les comportements socio-émotionnels. Une étude par autoradiographie a confirmé cette interprétation.

Ces expériences montrent qu'il existe une action régulatrice de l'OT sur la 5-HT chez le primate, mais que ce mécanisme est dérégulé chez les patients avec autisme. Cela ouvre donc la voie à l'investigation de traitements combinés exerçant un effet sur ces deux neurotransmetteurs.

Mots clés : Ocytocine, sérotonine, Troubles du Spectre Autistique, Primate non-humain, scanner TEP

Abstract

The neurohormone oxytocin (OT) is increasingly studied for its therapeutic potential in social disorders, like autism, which are associated with the deregulation of several neurotransmission systems, including OT and serotonin (5-HT). Hence investigating OT's interactions with other neurotransmitters is a relevant step towards mechanism-based treatments. Studies in rodents demonstrated that the interaction between OT and 5-HT, is critical for several aspects of social behaviour. Moreover, using PET-scan in humans we have recently found that 5-HT 1A receptor (5-HT_{1A}R) function is modified after intra-nasal oxytocin intake.

Thus I performed a first experiment in which intra-nasal OT was administered to patients with autism undergoing a [^{18}F]MPPF (a 5-HT_{1A}R radiotracer) PET scanner, in order to study their basal serotonergic system and to look if the oxytocin modulates the 5-HT_{1A}R system. I found no differences of baseline 5-HT_{1A}R concentration between 18 autistic subjects and 24 controls. Critically, in patients, OT did not induce changes on the 5-HT_{1A}R system. Moreover, in controls, there was a correlation between 5-HT_{1A}R and grey matter volume in the striatum, that was not observed in patients. These results suggest a subtle disruption of patients' serotonergic system, that can only be seen at the functional level.

Because PET scan does not tell us if the observed modification is due to a change in 5-HT_{1A}R or 5-HT concentration, I performed a second PET scan experiment on 3 macaque monkeys, using [^{18}F]MPPF and [^{11}C]DASB, that marks the serotonin transporter. Compared to placebo, OT injections in the lateral ventricle significantly reduced [^{11}C]DASB binding potential in right amygdala, insula and hippocampus whereas [^{18}F]MPPF binding potential increased in right amygdala and insula. Thus we reproduced results obtained in healthy humans and extended it by suggesting that OT provokes the release of 5-HT in key limbic regions involved in socio-emotional processing. These results were confirmed with autoradiography.

Taken together, these experiments indicate that OT modulates 5-HT release in primates, but this mechanism is disrupted in patients with autism. This opens ways to investigate combined OT/5-HT treatments, especially since FDA approved drugs targeting the two systems are already available for use in patients with autism.

Key words: Oxytocin, serotonin, autism spectrum disorders, non-human primates, PET scan

Mécanismes neuronaux de l'interaction entre ocytocine et sérotonine chez le primate non humain et les patients avec autisme

Synthèse

L'ocytocine, un nonapeptide prduit par l'hypothalamus, est une molécule fascinante et très importante car elle est impliquée dans la régulation de nombreux processus physiologiques et comportementaux.

La découverte, il y a un siècle, de son rôle dans l'accouchement et plus récemment de ces actions sur le comportement social a ammené les scientifiques à voir en elle « l'hormone de la reproduction », qui synchroniserait le cerveau et le corps dans ce but. Il n'est donc pas surprenant qu'avec une fonction aussi critique, l'ocytocine, ou d'autres nonapeptides d'une structure similaire, aient été trouvés dans l'ensemble des espèces étudiées à ce jour, depuis les nématodes jusqu'aux phocidés, sans oublier bien évidemment les rongeurs et les primates, rendant cette hormone incontournable. Les expériences sur les rongeurs ont montré que l'altération de l'ocytocine ou de son récepteur causaient des déficits du comportement social, plus particulièrement l'accouplement et le maternage. A l'inverse, l'administration d'ocytocine ou d'un agoniste au récepteur à l'ocytocine a fait apparaitre un comportement maternel chez des rongeurs nullipares et a modulé plusieurs aspects du comportement social, comme la mémoire sociale, la préférence pour la grégarité, la formation de couple (pour les espèces monogames), etc…

Ces constatations ont encouragé les chercheurs à administrer de l'ocytocine éxogène à des humains. Cependant, un certain nombre de problèmes, par exemple l'utilisation de spray intra nasal d'ocytocine, la mesure de l'ocytocine dans les fluides périphériques, le manque de connaissance du système ocytocinergique humain ou encore des paradigmes expérimentaux faibles, ont empêché l'obtention d'un résultat clair de ces expériences.

Néanmoins, ces résultats ont largement été relayés par les médias et ont donc attiré l'attention de la communauté scientifique et du grand public, favorisant la recherche sur cette molécule comme jamais auparavant. Ainsi, au cours des 10 dernières années, le nombre de publications scientifiques sur l'ocytocine aaugmenté exponentiellement. D'une manière générale, deux idées majeures ont émergé. Premièrement, il a été demandé si l'ocytocine était impliquée ou même à l'origine des troubles du comportement social, et particulièrement de l'autisme, et deuxièmement, il a été envisagé que l'ocytocine puisse être un traitement à ces troubles. A ce moment là, l'ocytocine était déjà utilisée en routine par les obstétriciens pour induire le travail chez les femmes enceintes et était donc considérée comme étant sans risques.

Dans l'introduction de la présente thèse de doctorat, et suite à une synthèse du système ocytocinergique (Chapitre 1, partie I et II), je présenterai une revue de la littérature se focalisant sur ces deux points clés de la recherche sur l'ocytocine. En premier lieu sera étudié le rationnel pour un rôle causal cette hormone dans les troubles du comportement social, et notamment l'hypothèse que l'ocytocine administrée aux femmes enceintes pour déclencher le travail puisse augmenter le risque d'autisme. Les expériences chez l'animal seront également incluses afin de porter un éclairage sur les mécanismes biologiques sous-jacents.

Le deuxième objectif de cette revue sera de synthétiser les essais cliniques menés sur des patients (autistes de haut niveau). Les études dans lesquelles l'ocytocine était administrée de manière aigüe ont montré des effets similaires à ceux observés chez le sujet sain, bien que ces résultats soient soumis aux mêmes biais. Toutefois, l'utilisation chronique d'ocytocine chez les patients n'a pas aboutie à une amélioration persistente. J'ai donc réalisé une analyse systématique de ces études et les aies mises en parallèle pour conclure quant aux conséquences de l'administration répétée d'ocytocine.

Evidemment, il peut être noté que si le système ocytocinergique est altéré chez les patients avec autisme, l'administration d'ocytocine éxogène ne pourra probablement pas exercer tout son potentiel. Comme mentionné auparavant, cette hormone est impliquée dans un large spectre d'actions. Une question passionnante se pose alors, comment une unique molécule peut-elle avoir des effets aussi variés ? La réponse est très probablement dans la compléxité du système ocytocinergique. Comme il sera détaillé dans le Chapitre 1, ce nonapeptide est libéré *via* de multiples voies (auto- et para- crine, axonale…) par deux types de neurones. De plus, bien qu'il n'existe qu'un seul récepteur à l'ocytocine, on sait qu'il peut être couplé à diverses protéines G, ce qui modifie dramatiquement les voies intra cellulaires qui seront déclenchées. Par ailleurs, ce récepteur forme des homo- et hétéro-dimers qui vont moduler les propriétés de liaison d'autres récepteurs. Cela nous mène au fait que l'ocytocine possède la capacité d'intéragir avec de nombreux autres systèmes de neurotransmission, ce qui a motivé ce travail de thèse.

De grande importance dans le contexte des comportements sociaux, l'ocytocine interagit avec la sérotonine. Ce mono amine est vital pour la régulation de plusieurs composantes du comportement social, comme l'aggressivité, la récompense sociale, l'humeur, l'anxiété ou encore les émotions. On sait que l'interaction ocytocine/sérotonine est impliquée dans le comportement social des rongeurs, et que, comme nous l'avons récemment montré, cette interaction se fait également dans le cerveau des humains. Plus précisément, nous avons trouvé, grâce au scanner TEP avec le [^{18}F]MPPF (marquant les récepteurs 1A de la sérotonine), que l'ocytocine éxogène augmentait le marquage du [^{18}F]MPPF dans l'amygdale, l'insula, et le cortex orbitofrontal, ce qui montre que

l'administration d'ocytocine module ce sous type de récepteur à la sérotonine chez le sujet sain.

En parallèle, nous savons que les patients avec autisme sont affectés d'anormalités du système sérotonergique. Nous avons donc pu formuler l'hypothèse que l'interaction ocytocine/sérotonine était altérée dans la pathologie de l'autisme ce que pourrait contribuer à l'efficacité partielle de l'administration d'ocytocine observée chez les patients. Pour ma première expérience (Chapitre 2 partie II), j'ai ainsi testé si l'administration intra nasale d'ocytocine chez des patients avec autisme produisait des modifications du fonctionnement du récepteur 1A à la sérotonine. Nous n'avons trouvé aucun effet. Nous suggérons donc que ce dysfonctionnement neurobiologique pourrait être lié aux effets incomplets de l'ocytocine observés auparavant chez les patients. Par ailleurs, nous avons également comparé le système sérotonergique à l'état basal entre le groupe contrôle et le groupe de patients. Aucune différence significative n'est apparue, signifiant que la concentration et la distribution des récepteurs 1A à la sérotonine. Cela suggère donc que l'altération de ce système est au niveau fonctionnel.

Un dysfonctionnement du récepteur 1A à la sérotonine a par ailleurs été observé dans plusieurs modèles de souris autistiques. Par conséquent, nous avons voulu tester une autre fonction de ce récepteur (Chapitre 2 partie III). Chez le sujet sain, il a été montré qu'il était positivement associé au volume de matière grise dans plusieurs régions et des expériences chez l'animal ont montré que ce récepteur influençait l'expansion cellulaire. J'ai reproduit ces résultats chez les sujets sains et les patients de mon expérience. Cependant, dans le groupe contrôle, j'ai trouvé une corrélation bilatérale négative entre la densité de récepteur 1A à la sérotonine et le volume de matière grise dans le putamen postérieur, et dans ce même cluster, le marquage du [^{18}F]MPPF était corrélé à la sociabilité des sujets. Aucune de ces associations n'a pu être faite chez les patients avec autisme. Cela indique une fois de plus que le foncitonnement du récepteur 1A à la sérotonine est altéré dans la pathologie de l'autisme.

Cette experience a montré que l'administration d'ocytocine provoque la modulation d'un sous-type de récepteur à la sérotonine, en revanche, le mécanisme sous-jacent à l'augmentation de la liaison du [^{18}F]MPPF demeurait inconnu. De plus, notre propre expérience était soumise au biais de l'administration intra nasale d'ocytocine, étant donné que cela était le seul moyen possible. Pour poursuivre notre investigastion des mécanismes neuronaux de l'interaction entre ocytocine et sérotonine et éviter ce biais, nous avons utilisé des macaques rhésus (Chapitre 3). Ces singes représentent le modèle animal disponible le plus proche de l'homme, vu leur appartenance au même ordre taxonomique. J'ai ainsi pu injecter l'ocytocine directement dans le cerveau, me permettant de contrôler avec précision

la dose délivrée au système nerveux central. En plus du [^{18}F]MPPF, j'ai réalisé des scanner TEP en utilisant le [^{11}C]DASB, un marqueur des transporteurs à la sérotonine, afin de tester si l'ocytocine induisait la libération de sérotonine, comme c'est le cas chez le rongeur. J'ai trouvé que l'ocytocine, par rapport au placebo, augmentait le marquage du [^{18}F]MPPF comme chez l'homme sain mais que celui du [^{11}C]DASB diminuait, suggérant des concentrations plus hautes de sérotonine. Pour aller plus loin, nous avons réalisé une expérience d'autoradiographie pour tester si l'ocytocine était capable d'influencer directement la liaison du [^{18}F]MPPF. Cela n'a pas été le cas, indiquant que la modulation fonctionnelle du récepteur 1A à la sérotonine était probablement liée à la libération de sérotonine.

Finalement, dans le Chapitre 4, je discuterai plus en détail l'ensemble de ces résultats et leurs implications, ainsi que les perspectives et questions clés qui demeurent dans le champ de recherche sur l'ocytocine.

Cette thèse a été réalisée dans l'équipe Neuropsychologie de l'action, au sein du Centre de Neurosciences Cognitives, UMR 5229

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I. Table of contents

Introduction

Oxytocin, a nonapeptide produced in the hypothalamus, is a fascinating and highly important molecule as it is implicated in the regulation of both physiologic and behavioural processes.

The discovery, a century ago, of its role in giving birth and more recently of its actions on social behaviours has led scientists to think of it as the "reproduction hormone", that would synchronize the brain and the body to this aim. Not surprisingly, with such a critical function, oxytocin, or closely related nonapeptides have been found in virtually all species, from nematodes to phocids and of course in rodents and primates, reinforcing the importance of this hormone. Critically, experiments in rodents have consistently shown that disruption of oxytocin or its receptor impairs social behaviour, especially mating and mothering. Conversely, administration of oxytocin or oxytocin receptor agonists were found to provoke maternal behaviours in virgin animals and to modulate social behaviour in various ways, including increase social memory, preference for gregarity, pair bonding, etc…

These ascertainments encouraged researchers to administrate exogenous oxytocin to humans. However, a number of issues prevented to obtain a clear outcome out of those experiments such as the intra nasal administration, problem with oxytocin measure in the periphery, the lack of knowledge about the human oxytocin receptor system or poor experimental designs.

Nevertheless, these results were largely relayed by media and attracted the attention of scientists and general public, favouring research on this molecule as never before. Thus in the last 10 years, the number of scientific publications on oxytocin and social behaviour has grown up exponentially. Critically, two major ideas emerged. First, it was questioned whether oxytocin was involved or even caused social disorders, with a focus on Autism Spectrum Disorders (ASD), and second, it was asked if oxytocin could be a treatment to these disorders. At that time, oxytocin was already routinely used by obstetrician to induce labour in pregnant women and was considered to be safe.

As an introduction to the present PhD, after a general overview of oxytocin physiology and behavioural effects (Chapter part I and II), I will present a review tackling these two key points of oxytocin research. I first looked at the rational for a causal role of oxytocinergic deregulation in social disorders, focusing on the hypothesis that oxytocin gave to pregnant women to induce labour could increase the risk of autism. Experiments from the animal literature will prove to be valuable to conclude.

Regarding the potential benefits of oxytocin in patients with autism, initial experiments using acute intra nasal showed enhanced social behaviour in patients with autism (high functioning) similarly than in healthy subjects, although these results were subject to the same biases as mentioned above. However, chronic use of oxytocin in patients did not led to a chronic amelioration. In the same review, I performed a systematic analysis of clinical trials and put them in parallel with animal experiments which have looked at the consequences of chronic oxytocin administration.

Of course one can note that if oxytocin is impaired in patients with autism, it is likely that exogenous oxytocin administration will not exert its full potential. As previously mentioned, oxytocin is involved in a wide spectrum of actions. A fascinating question therefore arises, how can a single hormone do so much? The answer probably lies in the complexity of the oxytocinergic system. As presented in Chapter 1, this nonapeptide is released in multiple ways (auto- and para- crine, axonal…) in the brain from two different types of neurons. In addition, although there is only one oxytocin receptor, it is known that it can be coupled to various G proteins, dramatically modifying the intra cellular pathway triggered by oxytocin. Furthermore, this receptor forms homo- and hetero- dimers that will modulate the binding properties of other receptors. This leads to the final point, which has motivated this PhD, oxytocin possesses the capacity to regulate many other neurotransmission systems.

Of importance in the context of social behaviours, oxytocin interacts with serotonin. This mono amine is critically involved in several aspects of social behaviour, such as aggressiveness, social reward, mood, anxiety and emotions. We know that the oxytocin/serotonin interaction is involved in social behaviour of rodents, and as we recently shown, it takes place in humans as well. More precisely, we found, using PET scan with the radiotracer [^{18}F]MPPF, binding to serotonin 1A receptor, that exogenous oxytocin increased [^{18}F]MPPF labelling in the amygdala, insula and orbitofrontal cortex, meaning that oxytocin administration modulated this receptor subtype functioning in healthy men.

Interestingly, patients with autism display several abnormalities of the serotonergic system. We thus hypothesized that the oxytocin/serotonin interaction was impaired in autism pathology and that it could contribute to explain the incomplete effects of exogenous oxytocin administration. The first experiment (Chapter 2 part II) therefore tested if intra nasal oxytocin in patients with autism produced some modification of the serotonin 1A receptor functioning. We found no effects. We thus suggest that this neurobiological dysfunction is linked to oxytocin partial efficiency previously observed in patients. Moreover, we also compared the serotonergic system at basal state between patients and the control group. Again we found no differences. This meant that patients had normal

concentration and distribution of serotonin 1A receptor, and thus suggested that the alteration was happening at the functional level.

A dysfunction of the serotonin 1A receptor has indeed been found in several animal models of autism. Therefore, we tested another function of this receptor (Chapter 2 part III). In healthy humans it has been found to be positively associated to the volume of grey matter in several regions and animal experiments have linked this receptor with modulation of cell growth. I reproduced these positive associations in both the control and patients group. However, in healthy men, I found a bilateral negative correlation between serotonin 1A receptor and grey matter volume in the posterior putamen, and in the same cluster, receptor labelling was correlated to sociality. None of these associations were observed in patients. This indicates that the serotonin 1A receptor functioning is altered in patients with autism, which is in line with the absence of oxytocin effects we previously observed.

This experiment showed that oxytocin modulated one of the serotonin receptor subtype, however, it was unclear what was the mechanism behind the increase of [^{18}F]MPPF labelling. Moreover, our own experiment was biased by the use of intra nasal oxytocin, as it was the only possible way for us to administer oxytocin to humans. To pursue our investigation of the neural mechanism of oxytocin/serotonin interaction and overcome this bias, we decided to use rhesus macaques (Chapter 3). These monkeys represent the closest animal model available to human, belonging to the same taxonomic order. I was thus able to inject oxytocin directly into the brain, allowing me to precisely control the dose delivered in the central nervous system. In addition to PET scans with [^{18}F]MPPF, I also used [^{11}C]DASB, a marker of the serotonin transporter, in order to test if oxytocin produces the release of serotonin, as it has been demonstrated in rodents. I found that oxytocin increased [^{18}F]MPPF radiolabelling, as in humans, but decreased the one of [^{11}C]DASB, suggesting higher concentration of serotonin compared to placebo. To go further, we performed an autoradiography experiment to test if oxytocin was able to directly act on [^{18}F]MPPF binding, and found that it was not the case, suggesting that the modulation of serotonin 1A receptor functioning might be a consequence of serotonin release.

Finally, in Chapter 4, I will discuss further these results and their implications, as well as the perspectives and key questions that remain to be answered in the oxytocin research field.

I. Chapter 1 - Background: Oxytocin modulates behaviour through various pathways

I.1. An overview of the oxytocinergic system

Oxytocin (OT) is a neuropeptide that was first described in 1906 by sir Henry Dale in experiments in which he injected extracts from posterior pituitary into mammals and found it produced contractions of uterine smooth muscles (Dale, 1906). Subsequently, these extracts where found to provoke milk release, thus indicating that a substance secreted by the brain exerted peripheral actions related to parturition.

Half a century later, the nine amino acids structure of OT (see Figure 1) was described by Vincent Du Vigneaud in 1952, who won a Nobel prize in Chemistry for this discovery (Du Vigneaud et al., 1953). Moreover, this work led to the synthetic production of OT, which was thus the first hormone to be produced in such a way. Following this achievement, OT started to be used to initiate labour in the late stage of pregnancy in women, a procedure that is nowadays happening frequently in every obstetrics department around the globe.

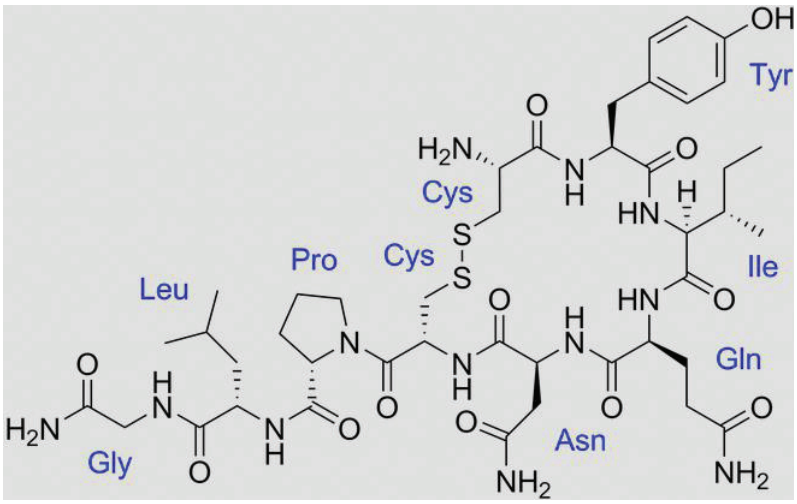

Figure 1. OT structure, showing the cyclic part formed by a disulphide bridge, and the tail formed by the last 3 amino acids (depicted in blue). It is to note that Leucine in position 8 is the most variable part, as many species show dissimilarities at this position.

As one can guess, a hormone involved in such an important aspect of life should be highly conserved across species. Indeed, while some minor variations (generally a substitution of one amino acid) occur in the structure of OT, this peptide is found in almost every animal (Banerjee et al., 2016). Even in simple and distant organisms such as Caenorhabditis elegans, an OT equivalent has been found, and plays a similar role to what is observed in mammals, namely feeding and reproductive behaviours (Beets et al., 2012; Garrison et al., 2012).

Since the initial discovery of OT, an impressive amount of studies have tried to understand its actions. Thus, this nonapeptide has been found to act in the periphery on various organs such as the heart and the gut, but what has made this neurohormone recently even more famous is its effects on behaviour, and more specifically social behaviour.

The aim of this first chapter is to present a general overview of the oxytocinergic system, from the production of the hormone to the behavioural outcomes of its secretion, as well as the literature that will be especially relevant for the subject of this thesis. Across this first part, we will see that despite extensive research on OT, major questions – such as how much OT is in the blood, where are the OT projections and receptors in the human brain, does intra nasal OT produce reliable effects, how can this single molecule be involved in so many functions – still remain to be answered.

I.1.a. Oxytocin Synthetization

OT genes of several mammals, including human, were sequenced in the 80's (Rehbein et al., 1986), it is composed of three exons encoding the precursor of OT, a complex made of Neurophysin 1 and OT (Gimpl and Fahrenholz, 2001). Neurophysin 1 (≈10000 Daltons) is the carrier protein of OT (1007 Datlons), which is separated from OT during the axonal transport of vesicles in which they are stored (Brownstein et al., 1980). While it does not seem to have any biological activity, it is released in the blood at the same time as OT. It should be noted that the OT gene has evolved and flourished, especially in primates (French et al., 2016; Ren et al., 2015).

OT is synthesized in several bilateral nuclei of the hypothalamus: the paraventricular nucleus (PVN), the supraoptic nucleus (SON) and the accessory nuclei (AN) (Sofroniew, 1983) (Figure 2). The PVN is located along the upper part of the third ventricle, the SON is slightly more ventro-lateral just adjacent to the optic chiasm and AN are located around the PVN. In addition, a continuum of OT containing cells have been found outside of the hypothalamus, between the PVN and the bed nucleus of the stria terminalis (Ingram and Moos, 1992). Finally, it seems that OT is synthesized also in the periphery (Chaves et al., 2013; Geenen et al., 1986), but our understanding of this phenomenon is limited and out of the scope of the present work. Moreover, differences in OT expression and synthetization exist between sex and sexual status (see (Scott et al., 2015) for an example), but because all the later presented studies have been performed in adult males, this factor will not be described here.

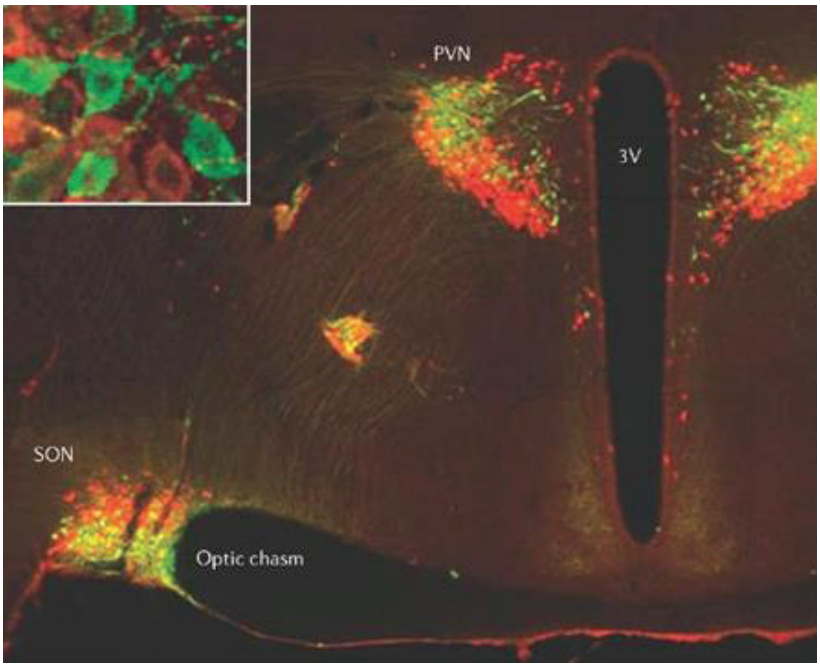

Figure 2. Coronal slice of the rat hypothalamus, in which OT cells have been immunostained in red. The PVN and SON can be seen, as well as what probably are AN in between. The zoom in upper left image shows that neurons express either OT or vasopressin (in green, see part I.2 for details). This image comes from (Ludwig and Leng, 2006).

All these nuclei contain magnocellular neurons, which are synthesizing the majority of OT that the body contains (Ludwig and Leng, 2006). The rate of OT production as well as the total amount contained in the human posterior pituitary (≈21µg) have thus been estimated from the content of one vesicle and the amount of vesicles present in this structure (Leng and Ludwig, 2008; Nordmann and Morris, 1984). While this does not account for the OT contained in the hypothalamus, the real number should be relatively close given that the majority of vesicles are in the posterior pituitary.

I.1.b. Oxytocin peripheral release

Magnocellular neurons are projecting their axons to the posterior pituitary (Figure 3.A), where they release OT in the blood stream *via* exocytosis of large dense core vesicles (Bargmann and Scharrer, 1951). Once released in the blood, OT can virtually reach any organ or peripheral target. However, free OT has a half-life of only a few minutes, as it will be quickly degraded by various enzymes and metabolized mainly by the kidneys (Claybaugh and Uyehara, 1993). Alternatively, it is possible that OT binds to larger blood proteins. This hypothesis has however received poor attention, one preliminary communication - looking at the possibility of complexes regrouping OT and thiol containing proteins such as albumin - that did not led to a subsequent publication, mentions this possibility (Martin L, 2013). Very recently however, a study has proven that it was the case, without however identifying the molecules to which OT is bound (Brandtzaeg et al., 2016). This raises fascinating questions, like which molecule is OT bound to, what

mechanisms regulate this binding, does bound OT has biological significance, and did the EIA performed without extraction measured this bound OT (i.e., is there a correlation between bound OT and OT concentration measured without extraction or filtering?)?

Concomitantly, the fate of Neurophysin 1 is also very unclear, and because it possesses several (three or four) binding sites to OT (Breslow and Abrash, 1966; Rose et al., 1991) it could potentially bind to OT in the blood. Note that since Neurophysin 1 is produced in equimolar quantity to OT, there are at least three times more binding sites than there are OT molecules. Unless it is degraded or bound to a larger protein, OT will diffuse freely between the extravascular fluid and the blood. However, OT does not cross the blood brain barrier, thus OT released from the posterior pituitary does not re-enter the central nervous system (but OT in the brain will reach the blood).

I.1.c. Central release of oxytocin

In addition to their axonal release of OT in the blood, magnocellular neurons have been found to release OT directly from the soma and their dendrites (Ludwig and Leng, 2006) (Figure 3.B). This mode of diffusion has been identified for various molecules and is important for various functions, such as dendro-dendritic modulation, retrograde signalling and synapse morphology (Kennedy and Ehlers, 2011). Interestingly, for OT this involves large dense core vesicles, as opposed to small vesicles releasing "classic" neurotransmitters (Pow and Morris, 1989). Moreover, given the relatively long half-life of OT in the brain (≈20 min) (Jones and Robinson, 1982) OT released in that way is thought to act like a hormone in the brain (with a long temporal action and wide spatial range, contrarily to neurotransmitters that are released in a timely and spatially precise manner) (Ludwig and Leng, 2006). This mode of release is calcium dependant (but electrical activity could prime OT dendritic release) (Ludwig et al., 2002) and therefore semi-independent from axonal release, indeed, magnocellular oxytocinergic neurons can inhibit afferent inputs by releasing endocannabinoids at the presynaptic level (Sabatier et al., 2003). Thus, OT release can happen from both axons and dendrites simultaneously or from one or the other at a time. OT is thought to act both locally inside the hypothalamus *via* extracellular diffusion (Son et al., 2013) and eventually at the whole brain level *via* volume transmission (i.e, diffusion to distant brain sites through cerebrospinal fluid) (Veening et al., 2010).

The volume transmission model was developed to explain the presence of OT receptors in brain regions located far away from the hypothalamus such as the septum, the olfactory bulb or the nucleus accumbens and also because OT receptors are often located extra-synaptically. Moreover, OT neurons are supposed to project dendrites towards the third

ventricle (Landgraf and Neumann, 2004) (Figure 3.C) and eventually to extra hypothalamic regions (Buijs, 1978) (Figure 3.D). It was however suspected that central OT release was more complex than simple volume transmission, notably because local injections of OT in a specific brain region did not seem to diffuse and act on other regions (Landgraf and Neumann, 2004).

Thus, central release of OT must involve long range axonal pathways. Supporting this hypothesis, dense OT fibres were found in the brainstem and spinal cord (Swanson and Kuypers, 1980). These projections came from a second type of OT neurons, named parvocellular cells and located exclusively in the PVN (Figure 3.E). These smaller neurons do not seem to project to the posterior pituitary but to other brain areas, and are supposed not to produce a significant amount of OT compared to magnocellular neurons (van den Pol, 1982). Recently, these neurons have been found to regulate magnocellular neuron activity in addition to their efferent fibres in the spinal cord (Eliava et al., 2016). There is however no direct evidence for parvocellular projections to the forebrain. These parvocellular projections thus could not explain the significant amount of fibres that were found to innervate the nucleus accumbens in several species of rodents (Ross et al., 2009).

This has led to another hypothesis regarding central axonal release of OT. It was proposed that axons in the forebrain would be collaterals originating from magnocellular neurons (Ross and Young, 2009) (Figure 3.F). These projections are forming few synapses in which OT and glutamate are co-released (about 20%), but the majority seems to release OT in a relatively diffuse manner (Ross et al., 2009). More recently, it was demonstrated that a retrograde viral vector injection in the central amygdala and nucleus accumbens was then found in the posterior pituitary, indicating that OT cells projecting to the limbic system were magnocellular neurons (Knobloch et al., 2012). Several studies have now demonstrated the presence of OT fibres in various regions using various viral techniques (Marlin et al., 2015; Mitre et al., 2016; Oettl et al., 2016). The axon collaterals hypothesis is however still not established and was challenged recently, notably because of the morphology of the neurons where these fibres originate, and their location close to the midline, leading some to suggest that such axons would in fact be parvocellular projections (Dölen et al., 2013; Dölen, 2015a) (Figure 3.G). Moreover, some of the observed fibres could be extra hypothalamic dendrites (Buijs, 1978) (Figure 3.D). It is important to note that studies in favour of the axon collaterals hypothesis did not rule out the possibility of parvocellular OT neurons projecting to the forebrain. These questions should be answered within the next few years thanks to the development of virus specific to one or the other type of neurons. We can also wonder to what extent this dichotomous classification of OT

neurons reflects the reality, because the size difference is not that big (15 vs 25 μm) and variations have been reported (Castel and Morris, 1988).

Debates regarding how OT reaches and acts outside of the hypothalamus are still open, as there is no consensus to whether it is through volume transmission, axonal projection targeting synapses, or axons locally diffusing OT in brain regions (Grinevich et al., 2015). These different possibilities are not exclusive and are summed up in figure 3.

Finally, it is important to keep in mind that OT neurons have changed a lot during evolution, one view is that magnocellular neurons have moved away from the ventricle where they initially released the neurohormone, to adopt a more precise axonal release (Kelly and Goodson, 2014; Knobloch and Grinevich, 2014). Interestingly the development of the oxytocinergic system fits the complexification of social behaviour across evolution. Therefore, because primates, especially humans, have the most complex social behaviours, it can be asked how different from rodents is our oxytocinergic system, especially in terms of neuron morphology and projections.

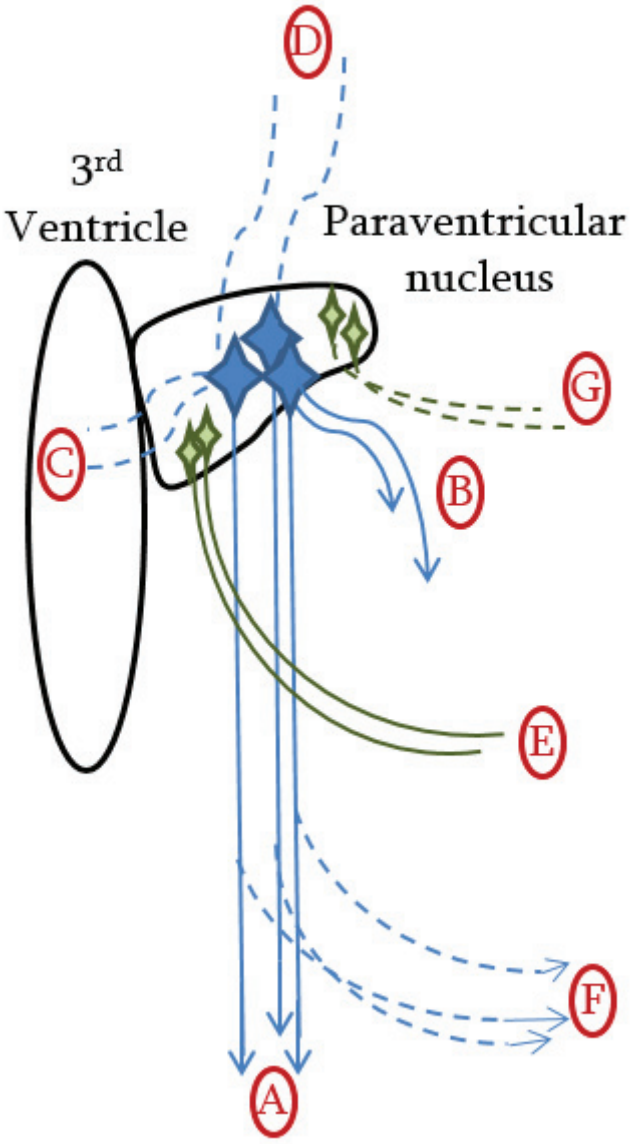

Figure 3. Schematic representations of known (full lines) and theoretical (dotted lines) projections from both parvocellular (in green) and magnocellular (in blue) OT neurons. Only the PVN is shown for clarity reasons and because the SON and AN are not supposed to contain parvocellular neurons.

A – Axonal projection from magnocellular neurons to the posterior pituitary;

B – Somato-dendritic release of OT from magnocellular neurons, which then diffuse within the PVN and to adjacent regions;

C – Magnocellular dendrites ending in the third ventricle, this pathway might be an evolutionary ancient mechanism no longer present in mammals, it is not known if present in primates;

D – Hypothetical dendrites of magnocellular neurons reaching extra hypothalamic areas;

E – Parvocellular neurons are known to send axons towards the brain stem and spinal cord in addition to form connections with magnocellular neurons inside the PVN and with other OT nuclei (SON and AN);

F – Hypothetical axon collaterals emerging from magnocellular neurons' projections to the posterior pituitary. These could account for a large number of fibres found across the forebrain, but this needs further confirmation;

G – Hypothetical parvocellular axonal projections to the forebrain.

I.1.d. Oxytocin receptor

The oxytocin receptor (OTR) gene, discovered over twenty years ago (Kimura et al., 1992), encodes a G protein-coupled receptor with 7 transmembrane domains, and is thus a typical metabotropic receptor. When agonistically stimulated, it is internalised and re-externalised *via* a rapid pathway (≈4 hours) (Conti et al., 2009).

There is only one type of OTR, to which OT binds to with an affinity of 0.79 nanoMolar in humans (Busnelli et al., 2013). As we will see in part I.2, the closely related neuropeptide vasopressin also binds to the OTR, but with a lower affinity. An interesting property of the oxytocin receptor is that it is coupled to various types of G protein, that will have very different effects on the cell. For instance, if coupled to G_q type of protein, stimulation of OTR will trigger phospholipase C, leading in turn to various consequences such as increased intracellular calcium or activation of protein kinase C, a phosphorylating protein. On the contrary, the OTR can be coupled to $G_{i/o}$ protein family, which inhibit cyclic adenosine monophosphate activity and therefore the protein kinase A pathway, involved in various intra cellular functions (Busnelli et al., 2012). Critically, these two pathways can also modify neurons excitability in opposite ways through activation or inhibition of potassium channels (Gravati et al., 2010) (Figure 4 (left)). In addition to this flexibility, OTR activation of G_q or $G_{i/o}$ protein family will vary depending on the OT concentration and the amount of OT receptors coupled to each protein type. This receptor system gives OT the tools to modulate the brain activity in very specific and different ways. Interestingly this also provides great opportunities for drug design (see Chapter 4).

As many G protein-coupled receptors, the OTR forms both receptor homo- and heteromers, which are the couple formed by two OTR or one OTR and another type of receptor (Ferré et al., 2009) (Figure 4 (middle)). Homomerization of two OTR has been reported (Terrillon et al., 2003) as well as heteromerization with vasopressin, dopamine and adrenalin receptors, these interactions will be discussed in section I.2. The role of such phenomenon is still not fully understood but theoretically, heteromerization of OTR could increase the repertoire of actions and the subtlety of its signalling (i.e., OTR effects would then be influenced by the type receptors co-expressed in the same cell) (Agnati et al., 2010; Fuxe et al., 2012). Interestingly, heteromers modulate both signal transduction and receptor trafficking (Bouvier, 2001).

Outside of the hypothalamus, OTR seems to modulate preferentially inhibition mechanisms (Dölen et al., 2013; Marlin et al., 2015; Nakajima et al., 2014; Oettl et al., 2016; Owen et al., 2013), but it has been localized both on excitatory and inhibitory synapses (at the pre- and post- synaptic level) as well as on the soma (Mitre et al., 2016). In addition, OTR are also located on astrocytes which undergo morphological changes following OT

action (Langle et al., 2003) (Figure 4 (right)). Taken together with what we know from OT fibres (that do not always form synapses), it seems that OT acts both as a neurotransmitter at pre and post synaptic level, as well as a more diffused neurohormone. It would be interesting to look if synaptic and somatic OTR are coupled to different G proteins. We could hypothesize for instance that synaptic OTR are coupled to $G_{i/o}$ proteins that need a high OT concentration, something more likely to happen in a synaptic cleft (axonal model of OT); and that somatic OTR are coupled to G_q proteins, needing lower OT concentrations, and therefore activated by simple diffusion of OT (volume transmission model).

As for OT fibres, the localization of OTR is still not firmly established. Several aspects need to be taken into account. First, the techniques used to map OTR in the brain have evolved and led to different results. In the eighties, autoradiography was used in rodents and gave relatively good results, allowing the mapping of OTR in various species (Freeman and Young, 2016; Gimpl and Fahrenholz, 2001; Tribollet et al., 1992), and interestingly, the inter- and intra- species variability of OTR distribution patterns was consistent with behavioural differences (Francis et al., 2002; Insel and Shapiro, 1992; King et al., 2015). Importantly, analysis of OTR gene expression gave relatively similar results (the eventual differences can be explained by the logical spatial mismatch between where the receptor is synthetized and where it is located on the plasma membrane), and most recent techniques using gene reporter also confirm the previously observed patterns, with eventually more precision (Dölen et al., 2013; Mitre et al., 2016; Yoshida et al., 2009). It is also to note that other factors influence OTR localization, such as developmental stage (Arsenijevic et al., 1995, 1995), sex, cycle and pregnancy status (Caughey et al., 2011), other hormones (Grozhik et al., 2014; Tribollet et al., 1990), environmental effects (notably socio-emotional events) (Lukas et al., 2010).

From this literature, it is now accepted that oxytocin receptors in rodents are located within the hypothalamus (notably in the PVN and SON), in the brain stem, in the limbic system (amygdala, hippocampus, nucleus accumbens, septum) and in the neocortex (prefrontal, olfactory and auditory) (see (Freeman and Young, 2016; Gimpl and Fahrenholz, 2001; Grinevich et al., 2015) for reviews).

However, when trying to switch to primates, scientists have faced major difficulties. Radio-ligands working in rodents (^{125}I-ornithine vasotocin analogue, or ^{125}I-OTA) did not show the same selectivity in primates (Toloczko et al., 1997). Consequently, radiotracers developed for positron emission tomography (PET) scanning have all failed (Smith et al., 2013a, 2013b, 2016). Recent *in vitro* attempts with new agonist and antagonist molecules have found OTR in very discrete brain regions in several species of primates (Sara M. Freeman et al., 2014; S. M. Freeman et al., 2014; Freeman et al., 2016; Schorscher-Petcu et

al., 2009). While these studies have been carefully controlled (competition assay, RT-PCR), the absence of OTR in the PVN and SON may eventually mean that not all the primate brain OTR have been found. Alternatively, it has been hypothesized that OT could act on vasopressin receptors in primates (Toloczko et al., 1997). For now, what can be concluded is that OTR distribution in the primate brain is not firmly established. Ultimately these long lasting interrogations (Verbalis, 1999) should be answered with the advent of viral targeting techniques, which will hopefully be developed soon (Izpisua Belmonte et al., 2015; Miller et al., 2016).

These studies have nevertheless led to an interesting hypothesis, which would be that cortical OTR are located in areas specific for the main social sensory modality (i.e., olfaction for rodents, audition for birds, vision for primates) (Freeman and Young, 2016; Grinevich et al., 2015). Again, further studies are needed to confirm this pattern of OTR distribution. Finally, it should be noted that OTR are also located outside the brain, in various organs (Gimpl and Fahrenholz, 2001), but again, this is beyond the scope of this work.

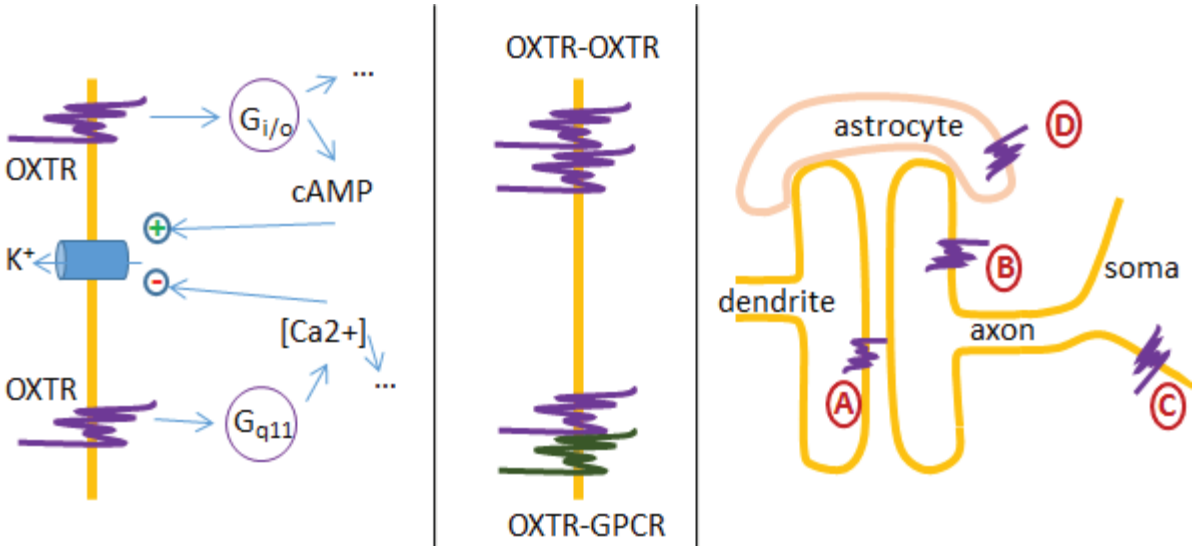

Figure 4. Schematic representation of the OTR (in purple) various ways to modulate information processing. Orange straight lines represent the plasma membrane. (Left) OTR can be coupled to different G proteins that will have opposite effects on the cell. The three dots represent further and more complex intra cellular effects. Some studies but not all have suggested that OTR could also be linked to G_s protein family (Stoop, 2012). (Middle) OTR can form homomers and heteromers which will potentially modify their affinity. (Right) Different possible localizations of OTR: A – post synaptic, B – pre synaptic, C – on the soma and D – on astrocytes and eventually other glial cells. It is not known if OTR coupling to G protein depends on the localization of the receptor on the plasma membrane.

I.1.e. Stimulation of the oxytocinergic system

A less studied but nonetheless important topic is the question of what does trigger OT release and how does this happen?

If OT is involved in so many functions, it is because a lot of stimuli, and molecules provoke its release. First, OT itself enhances the release of OT (while vasopressin, as described in the next part, inhibit it) (Ludwig and Leng, 2006; Moos et al., 1984). This auto regulatory function is important for the pulsatile release of OT, synchronizing magnocellular dendrites and axons projecting to the posterior pituitary, but it is unknown if this phenomenon also modulates OT release from terminals ending in extra hypothalamic regions of the forebrain. The increased extracellular concentration of OT was thought to be linked to somato-dendritic release, but it was recently found that a small population of parvocellular OT neurons were stimulating OT release from magnocellular neurons (Eliava et al., 2016).

Other hypothalamic peptides also trigger OT activity in the context of homeostasis regulation, such as alpha-melanocortin-stimulating-hormone which specifically stimulates magnocellular OT neurons (Sabatier et al., 2003). Histamine administration in the PVN was also found to increase extracellular OT concentrations (suggesting again a somato-dendritic release), but the behavioural outcome of this event is unknown (Bealer and Crowley, 1999). Another peptide, nesfatin-1 (Oh-I et al., 2006) also regulates OT release to produce satiety (Maejima et al., 2009). Given the existence of over a hundred neuropeptides, it is likely that a lot of other hypothalamic molecules are involved in the secretion of OT, and most of them should be related to homeostasis functions. It is to note though that the interaction between Corticotropin releasing hormone and OT is involved in anxiety regulation, which can obviously alter social behaviour in an indirect manner (Dabrowska et al., 2011). However, one them, secretin, seems to be involved in the modulation of social behaviours through its action on OT neurons (magnocellular neurons in the SON) (Takayanagi et al., 2015). Thus neuropeptides other than OT could be involved in the regulation of social behaviours and further research in this direction should lead to interesting findings, notably linking social environment/status to physiologic functioning.

Of course, OT release is also triggered by extra hypothalamic sources. Several afferent fibres to the paraventricular nucleus (PVN, where OT is produced) have already been discovered in the past (Sawchenko and Swanson, 1983). Notably, serotonergic projections from the raphe nuclei (Sawchenko et al., 1983), noradrenergic projections from the locus coeruleus and A1/A2 cell groups (Bealer and Crowley, 1999), dopaminergic projections from the zona incerta (Moos and Richard, 1979) and GABA/Glutamatergic from the amygdala.

In addition, serotonin has also been found to be an important regulator of serotonin release. We know that serotonergic fibres are innervating PVN neurons (Larsen et al., 1996; Sawchenko et al., 1983), and that serotonin released in the PVN increases electrical activity (Ho et al., 2007) of neurons and provokes the release of OT (Osei-Owusu et al., 2005) as well as increased mRNA expression (Jørgensen et al., 2003; Vacher et al., 2002). These effects were found to be dependent on serotonin 1A, 2C and 4 receptors (Jørgensen et al., 2003).

Importantly, all of these extra hypothalamic pathways have the potential to activate OT neurons in a timely, acute and specific manner. This is in line with the recent shift of paradigm indicating that OT is released also with this precise timing in the forebrain. However, these results were obtained more than thirty years ago with classic anterograde/retrograde techniques. New tools, especially viral vector-based tracing, allow us to dissect these networks leading to the release of OT, eventually leading to the identification of sub populations of neurons involved in specific aspects of social behaviours. A recent work has employed such techniques and found a sexually dimorphic dopaminergic pathway stimulating OT involved in maternal care (Scott et al., 2015). This topic is of high importance given that knowing how to stimulate specific sub populations of OT neurons could lead to great therapeutic improvements.

OT is also sensitive to peripheral signals. Several pathways from the gut have been found to release OT (Romano et al., 2013a, 2013b; Ueta et al., 2000). OT neurons also react to various physiologic stimuli involving cardiovascular functions (Gutkowska and Jankowski, 2012) or plasma osmolality (Neumann et al., 1993) and others, as well as external stimuli such as suckling, probably through a serotonergic pathway (Moos and Richard, 1983).

Concerning the inhibition of OT cells, studies have focused on magnocellular neurons. It should first be noted that because of their very low basal firing rates, OT neurons do not need extensive inhibition. We know that opioids are involved through diverse receptors (Brown et al., 2000). Furthermore, OT neurons receive glutamatergic and GABAergic inputs (Pol, 1985). Interestingly, GABA inputs to OT neurons can however be shut down by OT itself (Brussaard et al., 1996). Finally, among the unknown but probable neuropeptides influencing OT neurons, some of them are likely to exert an inhibitory action on OT cells. Note that the view presented in this part (Figure 5) is simplified: there are differences between sub nuclei of the PVN, different OT neurons types, etc…

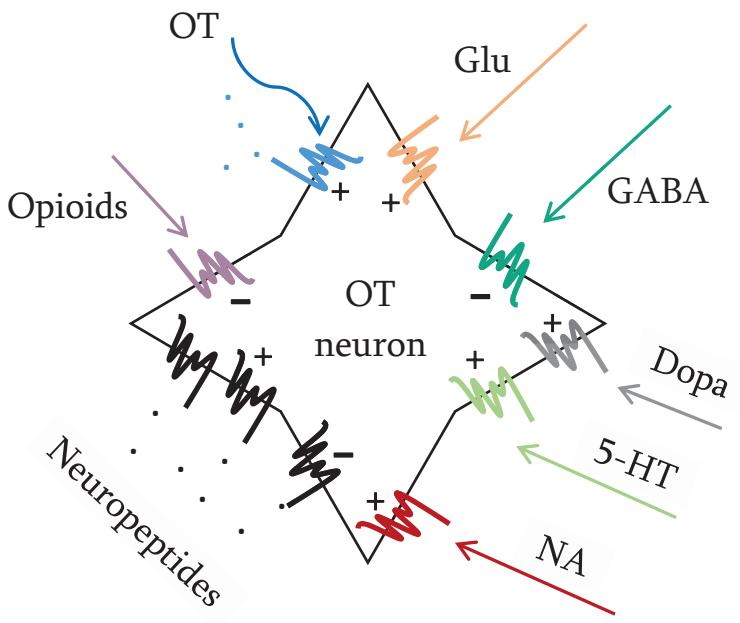

Figure 5. Summary of the neurotransmitters known to excite (+) or inhibit (-) oxytocinergic cells activity. Arrows represent projections making contact with OT cells and dots represent possible hypothalamic factors locally diffused. The schema does not account OT cell sub types differences.

I.1.f. Oxytocin and other neurotransmitters

Apart from the various ways of release and the versatility of its receptor system, OT can modulate brain activity through another way, the control of other neurotransmission system's activity.

First, as described in part I.2., OT often regulates the inhibitory/excitatory balance by acting on inhibitory interneurons (Figure 6.E). This type of action is present in the cortex (Marlin et al., 2015; Nakajima et al., 2014; Oettl et al., 2016), in the amygdala (Huber et al., 2005; Knobloch et al., 2012) and in the hippocampus (Mairesse et al., 2015; Owen et al., 2013). Oxytocin neurons can even co-release OT and glutamate to trigger inhibition instantaneously and on a longer term basis. At the cellular level, OT has been found to induce both excitatory and inhibitory post synaptic currents (Mitre et al., 2016), and this fine tuning of cortical electrical activity ultimately reduces background noise while increasing sensitivity to environmental stimuli (Oettl et al., 2016).

Secondly, OT interacts with many other hypothalamic neuropeptides (Figure 6.B). Briefly, OT is involved in several regulatory loops with other hypothalamic peptides, notably prolactin and gonadotropin, beta-endorphin (Csifáry et al., 1992; Samson, 2016), as well as Corticotropin releasing factor (CRF), with which OT interacts to regulate stress (Engelmann et al., 2004). An inhibition of CRF activity by OT has been found both in rodents (Bosch et al., 2015; Dabrowska et al., 2011, 2013) and in humans (Legros et al.,

1982), leading to the hypothesis that OT has an anxiolytic activity, especially in case of social stress (Neumann and Slattery, 2015). This has led to pre-clinical studies investigating the potential of OT as an anxiolytic molecule, notably in post-traumatic stress disorders (Frijling et al., 2014; Koch et al., 2016; Macdonald and Feifel, 2014; Slattery and Neumann, 2010).

The most well described interaction of OT in the hypothalamus is however with vasopressin (AVP) (Figure 6.A). AVP is a very closely related nonapeptide differing from OT by only two amino acids at least in mammals (note that in other classes of animals, some equivalents have been found, or sometimes only one peptide exists), which was first discovered for its hypertensive and antidiuretic effects. For a review of the vasopressinergic system please report to (Caldwell et al., 2008). AVP also has effects on social behaviours, often in an opposite fashion to OT (Young and Flanagan-Cato, 2012). Thus it is thought that these two neuropeptides down regulate each other to balance their effects (Legros, 2001; Neumann and Landgraf, 2012). Importantly, OT has some affinity (a hundred times less than for OTR) for the AVP 1A receptor and this information should always be kept in mind when using exogenous OT, especially at supra-physiologic levels. For instance, several studies reported OT and OT agonists/antagonists effects *via* an action on this AVP 1A receptor (Hicks et al., 2014; Ramos et al., 2013; Sala et al., 2011; Song et al., 2014).

Outside of the hypothalamus, OT is modulating catecholamines. While there is little or no evidence of OT acting on acetylcholine or noradrenaline (Wrzal et al., 2012), an extensive body of literature has described the interaction between OT and dopamine, the neurotransmitter famous for its role in reward signalling.

Of interest, OTR have been found on neurons in the ventral tegmental area (Figure 6.C), where dopamine is synthetized (Melis et al., 2007; Vaccari et al., 1998), thus indicating that OT stimulates the dopaminergic system, and another study confirmed that OT induced the release of dopamine inside the nucleus accumbens (Shahrokh et al., 2010). Moreover, the OT receptors (OTR) are forming heteromers with the dopamine D2 receptor in the striatum (Romero-Fernandez et al., 2012) and in the amygdala (de la Mora et al., 2016) which creates a facilitatory effect on receptor recognition (Figure 6.D). In this line, it seems that OT is preferentially acting with the dopamine D2 receptor, rather than the D1 receptor (Liu and Wang, 2003). In humans, there has been no direct evidence of an interaction between OT and dopamine, apart from a few fMRI studies (Groppe et al., 2013; Scheele et al., 2013), and some gene polymorphism effects (Love et al., 2012; Sauer et al., 2013). Critically, a Positron Emission Tomography using a radiotracer specific to dopamine D2 receptors did not find a significant effect of intra nasal OT (Striepens et al., 2014). This however does not indicate the absence of the OT-dopamine interaction in humans, since

authors used a specific task in which male subjects had to rate the attractiveness of unknown women, it is possible that in this specific context OT did not trigger dopamine release, because for instance of a ceiling effect. Furthermore, it is unclear if subjects were in a relationship or not (Striepens et al., 2014), as this could lead to differential dopaminergic activity (in voles, pair bonding creates a modification of nucleus accumbens dopaminergic receptors concentration (Aragona et al., 2006)). The behavioural roles of this OT-dopamine interaction will be further discussed in part I.2.e.).

Finally, OT is acting on the serotonergic system. As this is the core subject of the present work, it will be covered by chapters 2 and 3.

Note that most of these interactions are bilateral: OT regulates and is regulated (I.1.e.) by these other molecules.

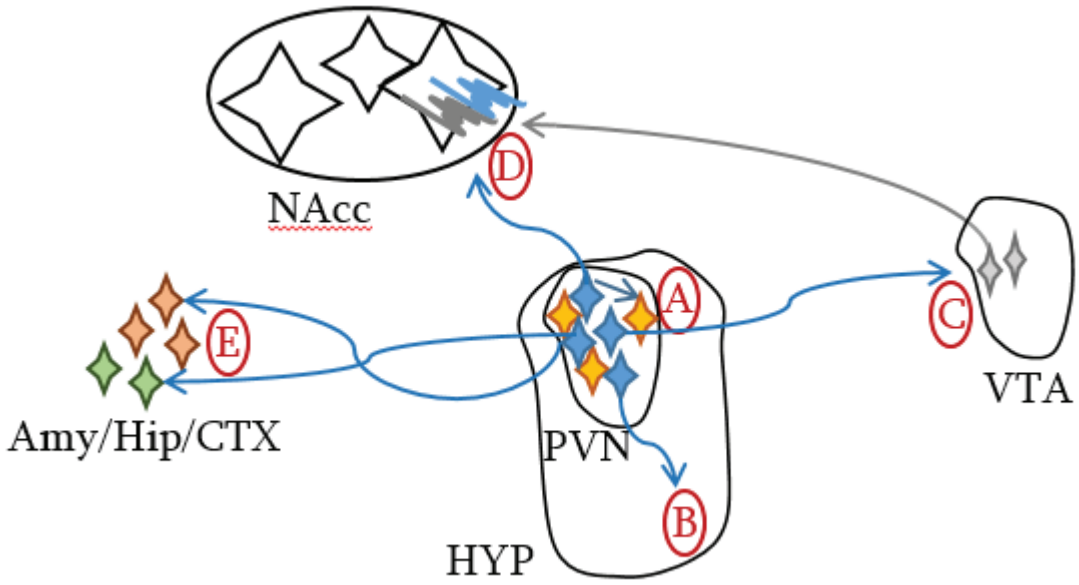

Figure 6. Schematic representation of OT interactions with other neurotransmitters (blue cells = OT neurons, yellow cells = AVP neurons, red cells = inhibitory GABA inter neurons, green cells = glutamatergic neurons). A – OT and AVP regulate each other inside the PVN; B –OT interacts with other hypothalamic (HYP) neuropeptides such as gonadotropin and CRF; C – OT activates dopaminergic neurons in the Ventral Tegmental Area (VTA); D – OT and dopamine D2 receptors form heteromers in the Nucleus Accumbens (NAcc) which potentiate receptors recognition. In addition, it is likely that OT modulates opioids in both the HYP and the NAcc. E – OT regulates the inhibitory/excitatory balance in several regions including the amygdala (Amy), the hippocampus (Hip) and the sensory cortices (CTX). The OT effects on serotonergic neurotransmission are not represented (see part II.1.a.).

I.2. Oxytocin effects in the brain and the body

One of the most fascinating properties of oxytocin is the incredible range of actions it has been found to exert on both physiology and behaviour. After one century of research and the initial discovery of oxytocin role in parturition, we are still discovering new functions for this neuropeptide. In this part, I will review the effects of oxytocin on both the brain and the body.

I.2.a. Oxytocin physiological effects at the periphery

Oxytocin (OT) was first known for its role in reproduction. During labour or suckling, OT is released in a pulsatile manner (synchronized burst of spiking from magnocellular neurons) from the posterior pituitary (Brown, 1997). OT provokes the contraction of uterus and mammary gland muscular cells through its action on intra cellular calcium (Gimpl and Fahrenholz, 2001). This will then induce milk ejection and parturition. It is to note that high OT levels have also been found in the portal vein (Gibbs, 1984), and thus a role for OT at the anterior pituitary has been suggested (Knobloch and Grinevich, 2014).

Since the original discovery of oxytocin effects on giving birth and lactation, other physiological effects has been found.

The most well described one is the role of OT in pain reduction (Martínez-Lorenzana et al., 2008; Rash et al., 2013). Parvocellular neurons of the PVN release OT in the spinal cord to inhibit sensory neurons, producing analgesia. Moreover they trigger magnocellular OT neurons which diffuse OT in the blood that will also reduce pain sensations by modulating ganglion neurons outside of the blood brain barrier (Eliava et al., 2016). It is not clear yet if OT also influences pain processing in the forebrain, although two studies suggest that it would have an action in the striatum (Gu and Yu, 2007; Pan et al., 2016). This role for OT in pain reduction may have appeared during evolution to reduce pain during parturition, although there are yet no evidence for such theory.

This analgesic property of endogenous OT has nevertheless led to the idea of using exogenous OT in humans (Tracy et al., 2015). Two recent studies have shown relatively weak but consistent reduction of pain perception following intra nasal OT administration (Paloyelis et al., 2015; Zunhammer et al., 2015). The utility of such effects is however unclear given the already existing wide range of drugs for pain managing. The advantage of OT probably lies in its apparent complete absence of secondary (negative) effects.

Beyond these well described actions of peripheral OT, this hormone has been linked to a very large spectrum of physiological actions, that will not be detailed here for clarity sake.

These include, the regulation of skeletal homeostasis (Colaianni et al., 2013; Zofkova and Matucha, 2014), muscles maintenance and regeneration (Elabd et al., 2014), energy metabolism (Chaves et al., 2013), body temperature regulation (Kasahara et al., 2013, 2015), cardiovascular regulation (Danalache et al., 2014; Gutkowska and Jankowski, 2012; Kemp et al., 2012), and cancer proliferation (Cassoni et al., 2004; Imanieh et al., 2014; Péqueux et al., 2002) (Figure 7).

It is to note that these effects often go along with local production of oxytocin in bones and various organs. They could thus be relatively independent from the role of OT on behaviour, at the central level. But the evolutionary path of OT leading to this diversity of effects is for now unknown. This variety of fundamental actions must however be phylogenetically very ancient because an OT-like peptide was already associated with feeding and reproduction in Caenorhabditis elegans (Beets et al., 2012; Garrison et al., 2012).

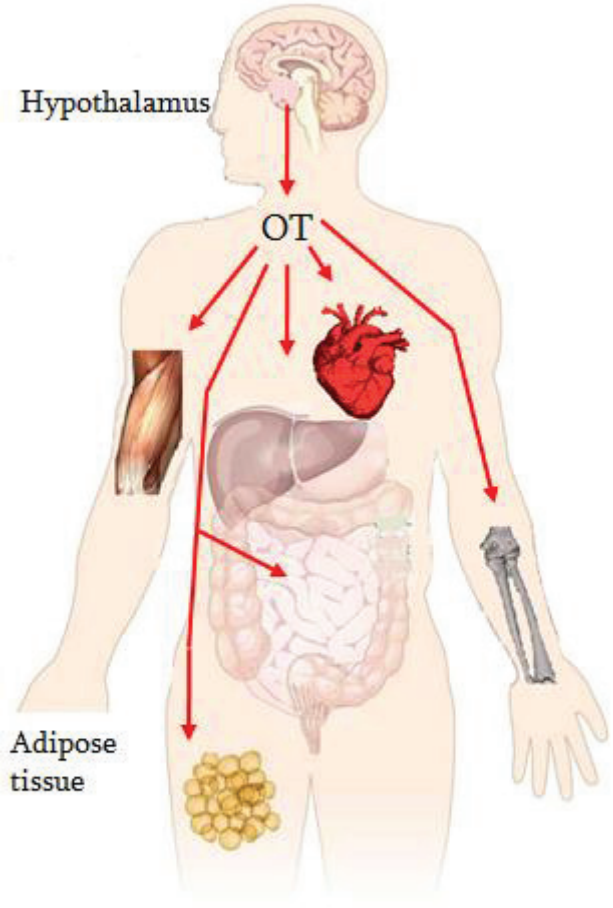

Figure 7. Schematic representation of OT effects on body physiology. It should be noted that this wide range of actions could be mediated by local production and release of the peptide directly from the organs depicted here. In addition, we know that some of those, for instance the gut or the heart, might exert a feedback action on the hypothalamus.

I.2.b. Oxytocin influences on behaviour

The role of oxytocin in social behaviour is now relatively well established in mammals, including humans, although some caveats exist. In this part, I will review studies demonstrating the role played by oxytocin at different cognitive steps of social behaviours, from perception to decision and memory. The aim is to give an overview of the variety of actions involving OT neuromodulation. This part was adapted from a book chapter I wrote recently (see **annexe 1**) (ref) and will focus on humans (and non-human primates to some extent) but will still include some of the rodent literature.

The social brain

The so-called social brain circuit has been divided in two main networks (O'Connell and Hofmann, 2012): the first centred in the amygdala is thought to process the emotional significance of social stimuli (Dalgleish, 2004); the second centred in the nucleus accumbens is known for coding the rewarding nature of objects and events (Ruff and Fehr, 2014). Both networks are present in most vertebrates and their neurochemical properties are very similar (O'Connell and Hofmann, 2012). As previously described, oxytocin (OT) has receptors in most of these regions, and its precise effects will be detailed. However, several factors need to be remember to consider OT actions on social behaviour. We have to take into account the variety of actions OT can exert, through its different modes of release, and the flexibility of the OT receptor (OTR). It is also important to keep in mind that each species has developed expertise in very particular behaviours, such as highly species' specific courtship display (West-Eberhard, 2014), which are presumably underpinned by specialized brain networks (Goodson and Thompson, 2010). Indeed, we already saw that the oxytocinergic system was varying between species. Effects of OT on social behaviour may thus be species dependant.

I.2.c. Oxytocin and perception of social stimuli

Sensory perception

In many species, OT receptors are distributed in many sensory regions (Boccia et al., 2013; Freeman and Young, 2016; Grinevich et al., 2015). Recent studies have revealed the cellular mechanisms mediated by OT during sensory processing. Importantly, OT plays a role very early in brain development. Indeed, in new born mice, OT stimulates sensory plasticity in tactile, auditory and visual areas (Zheng et al., 2014). While this process seems independent of social behaviour, it seems that this restriction of OT action comes later. In

adult mice, OT orients maternal behaviour in response to pup calls by regulating inhibitory/excitatory activity in auditory cortex (Marlin et al., 2015). Another work has dissected the network supporting OT effects on olfactory perception (Oettl et al., 2016). Interestingly, both of these studies showed OT released from fibres in sensory cortices, suggesting a fast axonal modulation. However, electrophysiological recordings displayed activity modification only after a few minutes, indicating a hormonal like action of OT, although direct modulation (*via* co-release of GABA for example) cannot be excluded. It can thus be hypothesized that in mammals, OT fibres have grown to reach and diffuse OT in relevant sensory cortices.

As previously stated, OTR are preferentially distributed according to the species' preferred social modality, i.e., olfaction for rodents, auditory for birds and visual for primates. Data showing improvements in eye contact after OT administration in human and non-human primates (Dal Monte et al., 2014; Ebitz et al., 2013; Guastella et al., 2008) or increasing time of olfactory search in rodents (Witt et al., 1992) support this idea. Of course, it is likely that OT effects are broader and not strictly restricted to a main sensory modality, for instance, auditory related effects were found in humans (Striepens et al., 2012). Nevertheless, OT action on primary sensory regions can be seen as a winning strategy to rapidly process social cues and events (Figure 8). Hence, a first route for OT to influence social behaviour is by modulating incoming information *via* adjusting the inhibition in sensory regions. Importantly, OT does not seem to modulate perception of non-social stimuli.

Emotion perception

A large number of studies have demonstrated that OT is important for emotion perception. OT intranasal administration in humans leads to a better recognition of facial emotional expressions (Shahrestani et al., 2013) and emotional valence (Cardoso et al., 2013). At the neural level this is explained by an important influence OT has on amygdala activity, a limbic region involved in emotional control and processing of social fear. Functional MRI (fMRI) investigations in humans have shown that OT reduces amygdala response to arousing emotional stimuli but that such effect was valence dependant (Gamer et al., 2010; Kirsch et al., 2005; Wigton et al., 2015) (Figure 8). Research in rodents has provided a detailed description of OT neuronal effects for the control of emotional behaviour, especially fear. When OT is liberated in the central amygdala, GABAergic neurons become active in the centro-lateral amygdala where they suppress output neurons signals from amygdala centro-medial region. The result on the animal behaviour of these chemical events is a decreasing fear reaction (freezing) to various social and non-social

stressors (Huber et al., 2005; Knobloch et al., 2012; Peters et al., 2014; Stoop, 2014; Viviani et al., 2011). It is however unclear if the endogenous release of OT happens in every fear context, or if it is specifically released in the case of social fear. Thus, various theories have been trying to model OT effects.

For instance, OT action on socio-emotional behaviour has been embedded in the context of a general framework proposed by Bethlehem and colleagues (Bethlehem et al., 2014) where it is suggested that OT reduction of general anxiety has the purpose to increase the saliency of social stimuli. These independent but complementary effects could be controlled by distinct neural processes. For instance, while anxiety and stress can be regulated by OT fast axonal release in the amygdala, modulation of sensory areas could be achieved *via* OT hormonal-like diffusion, allowing for a longer modulation of social perception. Further studies should investigate the potential correlation between time course of OT action in cortical areas and in limbic regions. It would also be interesting to look if different sub populations of OT neurons project their fibres to sensory and limbic areas. Finally, while OT interacts with other neurotransmitters within the limbic system, it is not known if similar interplay happens in the neo cortex.

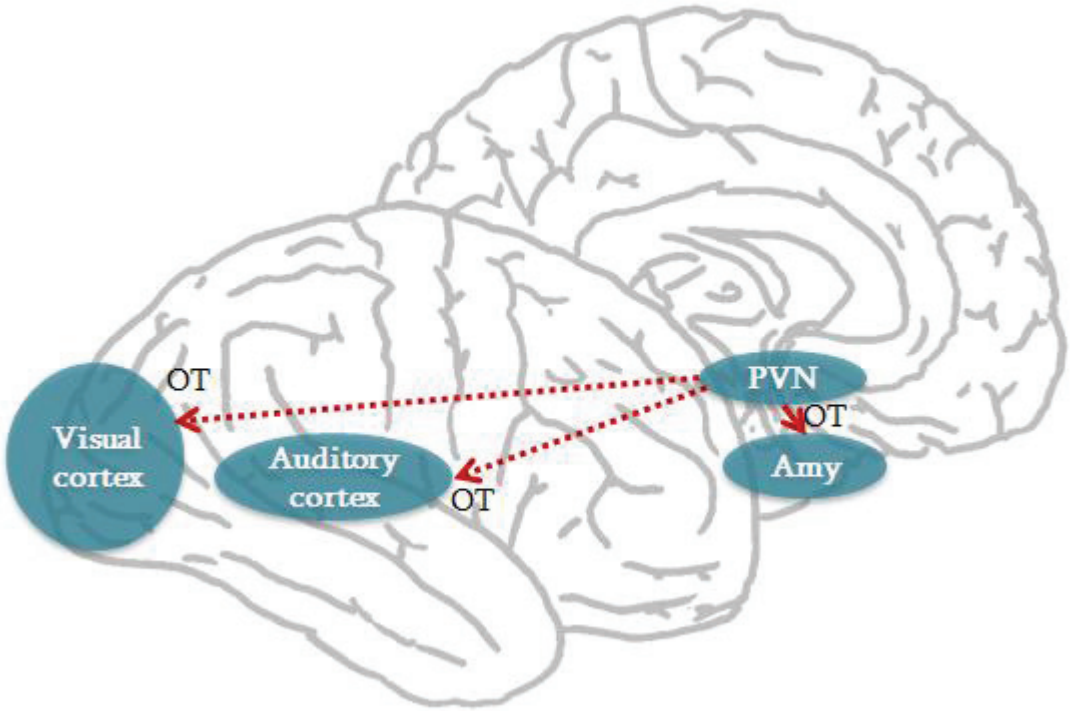

Figure 8. OT acts on the amygdala to influence emotion perception (red line). Moreover, it was shown in animal, but not yet in humans, that OT modulates perception directly in sensory cortices (red dotted lines), and this effect seems specific to social stimuli.

I.2.d. Oxytocin and social decisions

In addition to sensory areas, one of the most consistently region in which OT receptors are expressed the nucleus accumbens (Ross et al., 2009), but also in other reward related regions such as the Ventral Tegmental Area (VTA) and the Subtantia Nigra (Vaccari et al., 1998). Moreover, they are also present in regions important for decision making like the medial Pre-Frontal cortex (mPFC) (Stoop, 2012) or the Anterior Cingulate Cortex (ACC) (Nakajima et al., 2014). The role of OT in these regions is however unclear at the cellular level, but seem similar to what is observed in sensory areas, namely the modulation of cortical inhibition.

Choices and social decisions can be made at three different levels: for self , for others and according to social norms (Ruff and Fehr, 2014). Hence, various experimental approaches have been used to test OT effects in tasks involving (1) trust, (2) empathy and (3) moral judgments.

Trust games and cooperative behaviours

The first type of social decisions encompasses social choices that will affect me directly, such as the decision to marry someone. The finding that OT can bias this kind of social decision in humans has attracted wide media coverage. To understand the relation between OT and decision making, many studies have adapted tasks from the field of economy and added intra nasal OT.

Kosfeld et al.'s study is probably the first one which showed OT effects on trust behaviour (Kosfeld et al., 2005). Since then, a large number of reports have been published. Although some have successfully replicated Kosfeld et al findings, many have failed to show any effect of OT on trust (for a meta-analysis see (Bakermans-Kranenburg and van IJzendoorn, 2013)). A tentative conclusion from this body of research is that OT has none or weak effects on trust which seems context-dependent (Lane et al., 2016). It is to note that in a related topic, OT actions on cooperative behaviours, effects of OT seem to more consistently promote in group prosocial behaviours (see the recent review by (De Dreu and Kret, 2015)). In fact, environmental and personal factors are suspected to largely influence OT effects on trust behaviours (Bartz et al., 2011).

Two fMRI studies using trust games have found that OT has a main action on amygdala, insula, and prefrontal cortex, three regions belonging to the emotional/social brain network (Baumgartner et al., 2008; Rilling et al., 2012) (Figure 9). Enhanced activity in these areas can be linked to increased expectation of a future (social and monetary) reward ("if I trust this person, he will trust me back"), although the meaning of OT action is unknown because

the BOLD signal doesn't allow to distinguish excitatory from inhibitory activity. Hence, like already shown by animal research (see above), whether OT is inhibiting amygdala while facilitating activity in other cortical regions is unclear. Additionally, these two fMRI studies have found also increased activity in the caudate and the putamen, two regions where no anatomical or biological evidences suggest a possible OT action. Indeed, a PET scan investigation have shown that OT does not seem to modulate activity in the caudate (Striepens et al., 2014) (but effects in the nucleus accumbens/striatum ventral are likely, see part I.2.f.). Finally, it should be mentioned that, as in animals, OT effects are greatly modulated by sex (Rilling et al., 2013) (this will not be discussed more since the present work only tested male subjects).

Another way to study OT effect in the brain is by looking at how it modifies functional connectivity between emotional and reward areas. Some studies have reported increased connectivity between amygdala and both mPFC (Sripada et al., 2012), and ACC (Kovács and Kéri, 2015) or between the hypothalamus and the dlPFC (Wang et al., 2013). While once again, the meaning of these results for understanding regional and large scale OT action in the brain remains limited, they still suggest that OT actions are synchronized in the various regions targeted by its modulation. To this end, animal experiments in non-human primates are needed to elucidate the excitatory/inhibitory steps orchestrated by OT across brain regions.

Finally, it must be noted that most of OT experiments in humans have been conducted using intra-nasal administration of this hormone. This method is currently highly debated for various methodological and physiological issues (see (Leng and Ludwig, 2015; Quintana et al., 2014)) regarding whether OT inhalation reaches the brain and if so what are its modes of release (dendritic, axonal, or both). Finally the dose of OT going into the brain of each subject may greatly vary depending on their inhalation strength and the physiognomy of their nose, although some attempts to control this bias have been made (Guastella et al., 2013).

Prosociality and empathy

Another type of social choice an individual can face is when he is deciding for someone else (e.g. choosing a school for your child), which are decisions mainly impacting the other one, with eventually (but not always) a cost to self. We can judge the degree of prosociality of such decisions, and therefore test it under varying conditions (Ruff and Fehr, 2014). Empathy is the capacity to perceive and to feel others' emotions and to act with the aim to improve their well-being. It is thus an important factor influencing prosocial decisions

(Decety et al., 2016). Such cooperative behaviours are highly evolved in primates, and more specifically humans (Burkart et al., 2014; Shultz et al., 2011).

To date, few human experiments have explored the problem of prosocial behaviour and empathy after OT administration. In this kind of tasks, individuals are often prone to give money, or to help others without receiving back any compensation or utility. Human studies have shown increasing prosociality toward in-group but not for out-group members after OT administration (De Dreu and Kret, 2015). This dissociation between in-group and out-group is coherent with animal studies showing that OT is involved in maternal care and maternal aggression (Bosch and Neumann, 2012; Ferris et al., 1992). OT would thus increase positive behaviours towards closely related congeners but increase defiance towards unknown conspecifics (De Dreu and Kret, 2015). Concerning empathy again fMRI experiments have mostly focused on empathy for others' pain (Singer et al., 2008) but no findings are available on the neural correlates of empathic choices in humans after OT. OT prosocial effects have been documented also in non-human primates (Chang et al., 2012; Mustoe et al., 2015), and a recent study, which locally injected OT in the amygdala during a social decision task in macaque monkeys, found that OT increased prosocial decisions (Chang et al., 2015). While these kind of studies are insightful because they will allow us to perform electrophysiology in primates (Chang and Platt, 2013), we should remember that we observe "artificial" social situations between monkeys who generally know each other and have an already established social hierarchy.

Recent prosocial paradigms developed in rodents (Hernandez-Lallement et al., 2014) are promising given the large array of molecular tools like optogenetic or DREADD (Designed Receptor Exclusively Activated by Designed Drug) currently available, and the possibility to record in freely moving animals. Ultimately, this type of technology will eventually transfer to primates, and we will thus be able to study naturally occurring behaviours closer to human ones.

Social norms

Other pro-social choices humans can make are those for the benefit of the society (Fehr and Fischbacher, 2003). These include for instance, giving money to charity, respecting norms and laws or punishing free-riders, etc. Of course this field has been investigated mostly in humans because animals are very limited in this aspect. In males, OT was found to increase the amount of money donated to a charity although it did not increase the number of participants who gave money, suggesting that OT enhances donation behaviour in individuals already keen to donate (Barraza et al., 2011). Using the same paradigm in

females another study found that OT effect was similar to the one found in males but limited to participants who experienced low parental love-withdrawal (van Ijzendoorn et al., 2011). Another study used a social dilemma task where subjects could keep or distribute money to member of their group or to all participants, OT significantly increased decisions to send money to all players (Israel et al., 2012). Hence, this suggests that OT facilitate prosocial choices regardless to group membership. While this is a bit contradictory to what was previously described, it should be noted that the experimental context may lead to diverse outcomes. Interestingly, a recent experiment showed that when people had the choice to distribute a certain amount of money between a pro environmental or a social care charity, OT biased them towards the socially framed one, whereas they equally split the money under placebo (Marsh et al., 2015). The neuronal bases of this facilitation remains however unknown.

In sum, in humans OT has been convincingly associated with various types of social decisions. Nevertheless, this field has suffered of several issues (lack of reproducibility, intra nasal OT, etc…). It should still be retained that OT is involved in human social behaviour but that they may not be that sensitive to exogenous OT. Thus, animal experiments, which allow us to study endogenous OT functioning, will continue to be important in the future.

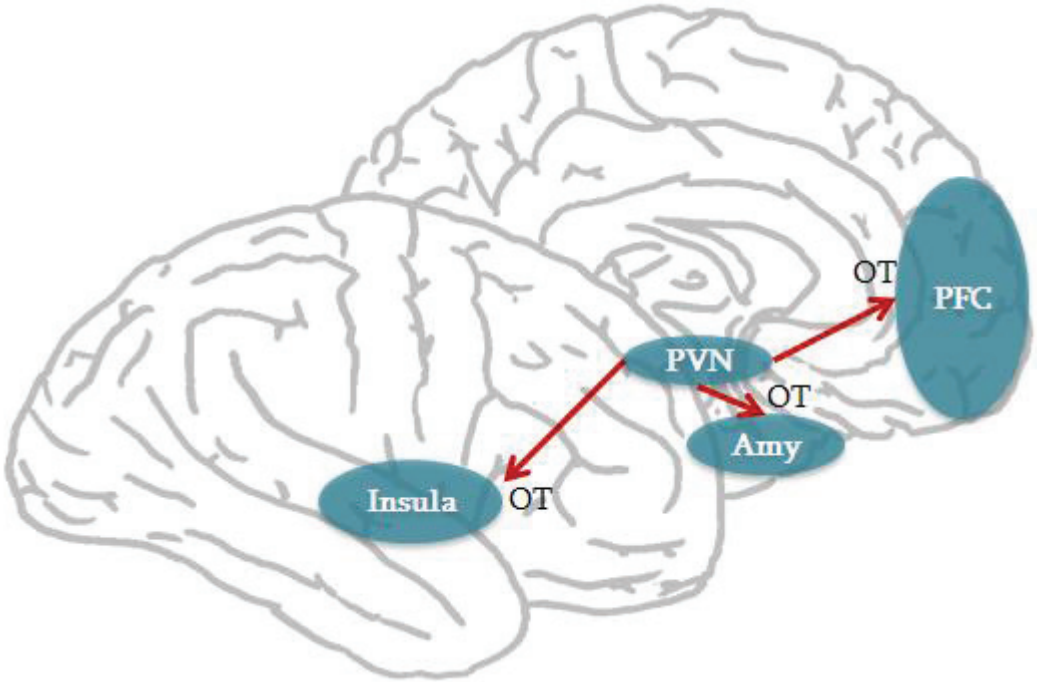

Figure 9. OT influences decisions related to social contexts by modulating the socio-emotional network. Several fMRI studies in humans have found effects of OT in the prefrontal cortex (PFC), the amygdala (Amy) and the insula. Other regions have been occasionally reported but are not depicted here.

I.2.e. Oxytocin and social reward

As stated above, OT receptors are present in the reward system, and OT is known to modulate reward in various ways. A key point is that OT action is expected when reward has a salient social dimension (Shamay-Tsoory and Abu-Akel, 2015). However, we will see that OT is also involved in non-social types of rewards, casting doubts on this social salience hypothesis.

Social reward

Several types of social attachment like maternal, pair-bonding and consociation (friendship) are processed in the brain (Dölen, 2015a). Note that whilst maternal attachment is present in most mammals, only a minority of species (≈5%) display monogamous behaviour (Numan and Young, 2015).

In voles, a monogamous species, OT is required to create pair bonding *via* dopamine modulation in the nucleus accumbens (for a review see (Young and Wang, 2004)). Similar mechanisms seem active in humans as well. OT increases activity in reward areas when subjects see his/her own partner while decreases activity in the same areas when they are seeing others' (opposite sex) unfamiliar faces (Scheele et al., 2013). OT and dopamine interaction also modulate sexual behaviour (control of penile erection in rats) in non-monogamous species without provoking pair bonding (Melis and Argiolas, 2011). Common OT and dopamine mechanisms sub serve attachment/parenting behaviour in both rats (D'Cunha et al., 2011; Shahrokh et al., 2010) and primates (Damiano et al., 2014; Strathearn, 2011) (Figure 10). The nucleus accumbens, the hypothalamus (paraventricular nucleus) and the medial prefrontal cortex (mPFC) constitute the neural circuit where both neuromodulators promote the simplest form of social behaviour (see (Love, 2013) for review). In the nucleus accumbens OT action also interfaces with opioids receptors activity (involved in the "liking" part of reward) (Gu and Yu, 2007), another chemical interaction highly relevant for social rewards (Resendez et al., 2013; Trezza et al., 2011, 2012). The triadic relation formed by OT, dopamine and opioids certainly deserves further investigation (Brown et al., 2000; Csifáry et al., 1992), and it has already been suggested that OT would initiate social reward through the recruitment of the opioids pathway (Wei et al., 2015). The substantial literature on OT effects in the nucleus accumbens and its interaction with dopamine and opioids makes it clear that OT is an important regulator of social reward, generating motivation for socio-sexual behaviours, especially in social contexts linked to reproduction (i.e., involving the partner or the offspring). This fits

perfectly the role of peripheral OT in parturition and lactation, and place OT as the neuromodulator synchronizing the brain and the body for reproduction.

A second type of social reward occurs during interaction with unfamiliar conspecifics (neither the partner nor the offspring). Little work has been conducted so far, but the few available data show that OT modulates this type of interaction in a context dependent manner. In women, activity in the ventral tegmental area, a brain region synthesizing dopamine, was found enhanced by OT during the presentation of cues signalling a friendly face (Groppe et al., 2013). OT also seems to strengthen functional connectivity between amygdala and the caudate nucleus during a social learning task (Hu et al., 2015).

Recent data also suggest that serotonin, a neurotransmitter important for approach/avoidance behaviour, also interacts with OT (Dölen et al., 2013). Results in mice show that OT stimulates the release of serotonin from raphe nuclei projections which in turn modulates activity in the nucleus accumbens (Dölen et al., 2013). Interestingly, it has recently been suggested that dopamine would encode reward on the short term time scale and that serotonin would mediate delayed reward (Miyazaki et al., 2014), although this view is probably simplified as contradictory results indicate (Fonseca et al., 2015; Liu et al., 2014). Still, it has been proposed that for social rewards, OT-dopamine interaction would mediate mainly instant rewards from the interaction with the partner (regardless of the persistence of pair bonding) and the offspring, but that for interaction with unknown conspecifics, social reward would be encoded by more long term mechanisms involving the OT-serotonin signalling leading to the formation of trust-like and friendship-like behaviours (Dölen, 2015b).

Finally, it is interesting to note that OT also reinforces social bonds among individuals of different species. For instance, human/animal interaction can produce release of OT. In dogs, administration of intra nasal OT increases affiliative behaviour toward the owner (Kis et al., 2015). Moreover, dog/human interaction triggers peripheral OT (measured in the urine) (Romero et al., 2014). Finally, in lambs OT mediates the stress reaction experienced by the animal after the departure of the human caregiver (Coulon et al., 2013) and in rats, gentle stroking activates OT neurons in the hypothalamus (Okabe et al., 2015). The interpretation of such effects is rather complex and probably lies in between a driftage of OT prosocial effects and an evolutionary advantage from inter species cooperation.

Non-social reward

As previously mentioned, a large body of evidence points to a non-social specific role of OT in reward.

OT has first been linked to feeding behaviour. When centrally released, OT induces satiety *via* the activation of the hypothalamus and the nucleus of the tractus solitarius (Sabatier, 2006; Sabatier et al., 2013). Moreover, apart from this action inside the hypothalamus and brain stem (Maejima et al., 2009; Zhang et al., 2011), OT-induced satiety may be linked to the activation of reward mechanisms in both rodents (Herisson et al., 2016; Mullis et al., 2013) and humans (Klockars et al., 2015; Ott et al., 2013) (Figure 10). It is to note that more specific effects than general satiety have been found, for instance OT modulates sucrose intake (Mullis et al., 2013), and inhibition of appetite for salt (Samson, 2016). This indicates that OT effects on feeding behaviour happens *via* the modulation of nucleus accumbens activity, and very interestingly, dopamine and serotonin seem once again to be involved (Yeo and Heisler, 2012). However, the critical question if OT is modulating social reward and food reward through the same neuronal pathway is yet unknown. At the behavioural level though, it was found that social context could influence the effects of OT on feeding behaviour (Olszewski et al., 2015, 2016). This direction of research could bring new insights to the fascinating question: why OT, the "social neurohormone", is also involved in feeding behaviour? And what kind of evolutionary advantage does it give? For now, it can be guessed at best that linking feeding and social behaviour could potentially enhance cooperative behaviours between conspecifics, thus promoting life in social groups, and in fact, food offering behaviours are part of the sexual parade of many living creatures, from insects to reptiles as well as in birds and mammals (Stevens and Gilby, 2004).

Because of these effects on food intake, OT has been also under investigation as a potential treatment for several feeding related disorders such as obesity and anorexia (Cai and Purkayastha, 2013; Kim et al., 2014; Maguire et al., 2013). Moreover, it has been suggested that autism and anorexia were closely-related diseases (Odent, 2010), which goes in line with the potential interplay between social and feeding behaviours. That being said, there is only a small step to hypothesize that OT sex specific effect are involved (this does not mean OT is a cause or is the only deregulation involved though).

In a slightly different area of research, OT has been found to reduce drug seeking and consumption (Bowen et al., 2011, 2015; Carson et al., 2013; L. Kovács et al., 1998; Sarnyai and Kovács, 2014) especially when it is directly administrated in the nucleus accumbens (Baracz et al., 2014). Conversely, drug consumption has been found to alter the oxytocinergic system (Baracz et al., 2015). This raises the question of an unsuspected role of

exogenous OT on suppression of addiction through the inhibition of the reward pathway. Whether OT modulates reward in general, or social and food related reward more specifically remains debated (McGregor et al., 2009), since all studies looking at OT effects on drug consumption have used exogenous OT (Baracz and Cornish, 2013). Regardless of this interrogation, OT is now a potential target for pharmacological therapies of addiction behaviours (McGregor and Bowen, 2012; Zanos et al., 2013).

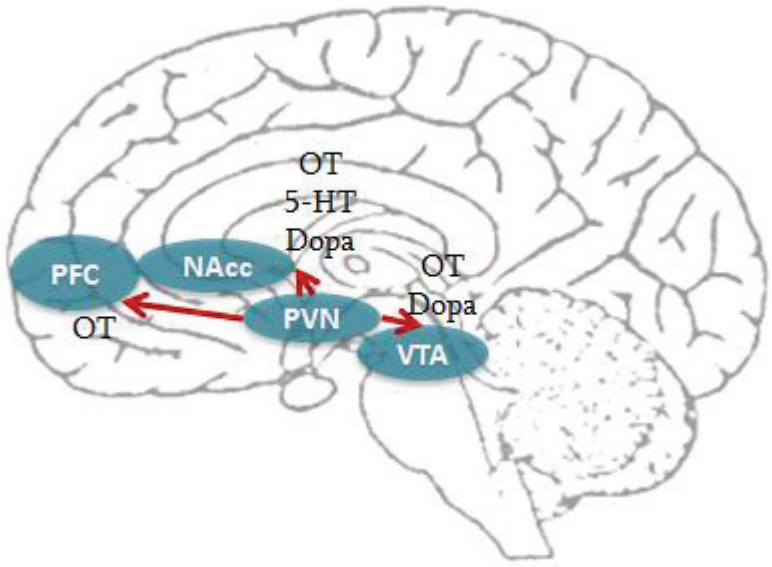

Figure 10. OT is strongly implicated in the processing of reward, by provoking the release of dopamine (Dopa) and serotonin (5-HT) in the nucleus accumbens. These effects seem to be linked to social rewards as well as food rewards.

I.2.f. Oxytocin, learning and memory

One of the first studied effect of OT on behaviour was its influence on memory and learning, two mechanisms important for social behaviours. Since the 60's, studies from DeWied have shown OT role on memory (see (Barbara B. McEwen, 2004) for a complete review). For instance, this hormone has a negative effect on recall of non-social stimuli but a facilitatory one on social or emotional events (Dantzer et al., 1987; Gur et al., 2014; Hurlemann et al., 2010; Savaskan et al., 2008). A recent meta-analysis of human studies confirmed these results (Brambilla et al., 2016). Thus in both rodents and humans, OT is thought to increase social memory by acting in the amygdala and hippocampus.

At the neuronal level, OT can improve the efficiency of hippocampal neurotransmission in a very specific fashion (Harden and Frazier, 2016; Owen et al., 2013). In fact, OT targets a specific type of interneurons, the fast spiking interneurons, by

increasing their firing rate. This in turn lowers the spontaneous activity of hippocampal pyramidal cells and enhances signal-to-noise ratio. This demonstrates that, similarly to its action in the cortex, OT acts on the excitatory-inhibitory balance of hippocampal activity by modulating inter-neurons spiking. It must be noted however that, OT receptors were not found in the hippocampus of human or non-human primates, although this may reflect technologic limitation rather than absence of OT effects in primates' hippocampus.

While a lot of work has been done looking at the effect of OT on social memory, non-social effects has been less studied (Chini et al., 2013). The newly available techniques in rodents should however allow quick progress.

I.3. Oxytocin and Autism Spectrum Disorders (ASD)

The effects of OT on social behaviour have quickly led to studies looking at the links between OT and social disorders, especially autism. The following part will present the characteristics of this disease (of course without being exhaustive to limit the length of this chapter), and how OT could be involved in the expression of autism' symptoms. Finally, the potential of OT as a therapeutic drug in autistic spectrum disorders will be reviewed.

I.3.a. Autism Spectrum Disorders (ASD)

Autism Spectrum Disorders (ASD) are a developmental disease identified relatively recently (Kanner, 1943). It has increasingly attracted attention since its discovery, for instance being declared "Grande Cause Nationale" (Great National Cause) in 2012. The reason for this is the constant raise of patients diagnosed each year: according to a survey conducted every two years in the US, the prevalence of autism has gone up from 1 in 156 in 2002 to 1 in 68 in 2012 (Christensen et al., 2016) (Figure 11). This increase was reproduced when looking at world-wide population (Elsabbagh et al., 2012), although the prevalence was not as high as in the American cohort (6.2/1000 vs 14.7/1000). The discrepancies between regions and countries indicate that the prevalence of autism is very variable and depends on the efficiency of the local diagnosis system. Thus the numbers should be taking carefully. However, the increased number of patients has been repeatedly observed. This can be explained by improved diagnostic criteria (see the evolution between different editions of the Diagnostic and Statistical manual of Mental disorders (DSM) (Kulage et al., 2014)) and awareness of this condition, but a true increase of this pathology's prevalence cannot be ruled out (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators and Centers for Disease Control and Prevention, 2012).

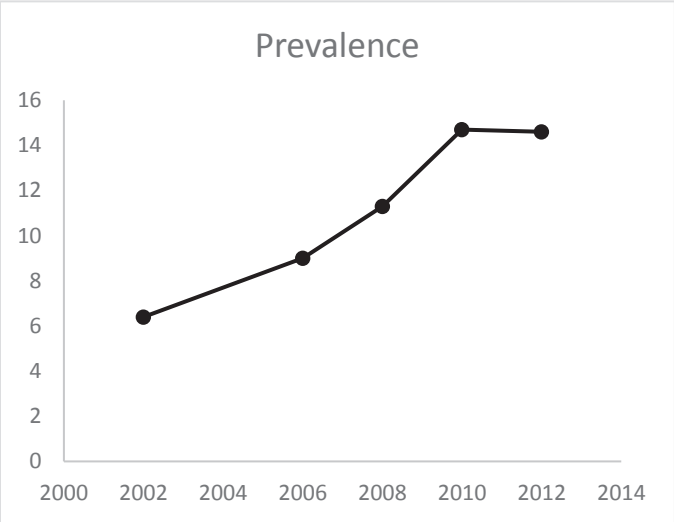

Figure 11. Prevalence of autism spectrum disorders in the United States of America over the last 10 years. The scale represents the number of patients amongst 1000 8 years old Children across 12 different sites. This average does not show the sexual disparity (the prevalence is over 20/1000 in boys and around 5/1000 in girls).

The current definition of ASD, according to the DSM5, is the following "People with ASD tend to have communication deficits, such as responding inappropriately in conversations, misreading nonverbal interactions, or having difficulty building friendships appropriate to their age. In addition, people with ASD may be overly dependent on routines, highly sensitive to changes in their environment, or intensely focused on inappropriate items. The symptoms of people with ASD will fall on a continuum, with some individuals showing mild symptoms and others having much more severe symptoms. This spectrum will allow clinicians to account for the variations in symptoms and behaviours from person to person.".

Thus, patients with ASD present three kinds of trouble: social behaviour, communication and repetitive behaviours; with very various degree of expression.

It is to note that the diagnosis and definition of ASD is complicated by the high comorbidity of this pathology with others, such as depression, anxiety, attention/hyperactivity deficits and epilepsy (Leyfer et al., 2006). The co-existence of these pathologies will also inevitably complicate the development of therapies. Finally, because social or repetitive behaviours are not observable before a certain amount of time in newborns, we do not know if patients are born with autism or if they develop it during the very first moment or their lives.

If the causes of ASD are yet unknown, some factors have however been identified. They can be classified in three categories, depending on when they are acting.

First at the pre-natal level, some genes, or combinations of genes have been associated with increased risks to develop ASD (Bourgeron, 2015). Importantly, the fact that sometimes only one of two monozygotic twin brothers display ASD indicate that this is not a purely genetic disease and explain the rather weak heritability of ASD. It was concluded

from this literature that ASD could be explained mainly by numerous genetic variations, each one acting additively. Regarding the mutations observed, it seems that most of the genes affected are related to synapse formation and brain excitability, suggesting that ASD are linked to hyper or hypo activity of neuronal networks (Kopp et al., 2015). Moreover, these genes are critical for normal foetus brain development.

It thus seems that the expression of these genes are important for normal brain formation, meaning that environmental factors altering their expression could be potential causes as well. Several theories have been emitted in this direction. Indeed, infectious and environmental agents altering normal development and eventually leading to birth defects could explain some cases of ASD. But no agents have been specifically linked to ASD, as they simply alter general development, which result in various conditions.

In the same line, perinatal events, like C-section, labour induction, gestation duration and others have been regularly (but weakly) associated with increased risks to develop ASD (Guinchat et al., 2012). But such associations do not prove any causality and could be consequences of pre-existing genetic factors. One thing to remark is that very few studies have investigated simultaneously genetic factors and environmental factors.

Finally, a number of post-natal factors have been proposed, notably trying to link the often observed gastric disorders seen in patients. Auto immune disease, heavy metals, lack of vitamin D and others potential causes have been suggested but nothing have been scientifically proved so far. One interesting aspect is however the fact that in an autistic mice model, the microbiota is altered, and when researchers corrected it, it improved social behaviour, whereas when they induced this microbiota alteration in healthy animal, they observed social deficits (Hsiao et al., 2013). Again, this phenomenon is probably not restricted to autism. It however has the merit to highlight the importance of non-central nervous system factors.

Given the high heterogeneity of this pathology, it is also likely that several factors, both genetic and environmental, are involved.

Regardless of the cause(s) of autism, researchers have formulated various theories to explain the difference of information processing between healthy subjects and patients. These hypotheses and their potential link to the oxytocinergic system will be discussed in chapter 5.

All these theories are formulated based on experiments highlighting structural and/or functional alteration of patients' brain. It is to note that such differences have now been observed in almost all brain regions (Amaral et al., 2008), depending on the variable observed (cortical thickness, shape and volume of a structure, connectivity, activity at rest

or activity during a task). Critically, a large number of inconsistencies can be found. Interestingly however, a recent study showed, using 26 different mouse models of ASD, that 3 main different patterns of brain abnormalities could identified, notably including hypo and hyper connectivity (Ellegood et al., 2015). In some regions like the amygdala, opposite changes can be found, as in human patients (Greimel et al., 2013). Replicating these results in patients will be critical for our understanding of the heterogeneity of autism, and might allow us to adapt patients' care depending on their "brain profile".

Other abnormalities, regarding molecular pathways, neurotransmitter systems, physiologic regulation, etc. have been observed, but one abnormality particularly relevant for the present work is the serotonergic deregulation found in many patients with ASD. This will be detailed in II.1.a..

I.3.b. Is oxytocin implicated in Autism Spectrum Disorders?

Because of the importance of OT for social behaviour, researchers have looked at its potential involvement in ASD, especially after a study reported low levels of OT in the plasma of children with ASD (Modahl et al., 1998) (but results from peripheral measures must be carefully considered, see Chapter 4).

Several groups have been looking for associations between mutations of the OT or OTR gene and ASD. First it should be mentioned that several Single Nucleotide Polymorphisms (SNP) of OT genes have been linked to social behaviour (perception of faces, reward, sociability) in the general population (Chang et al., 2013; Damiano et al., 2014; Skuse et al., 2013). Several studies have then found some associations between these OTR SNPs and the occurrence of ASD, or the degree of social impairments (Harrison et al., 2015; LoParo and Waldman, 2014; Nyffeler et al., 2014). However, these data are contrasted by other studies which have claimed that OTR SNPs were linked to sociability in both healthy subjects and patients, thus denying a specific association between these SNPs and ASD (Parker et al., 2014). Finally, a meta-analysis (restricted to certain SNPs and a few years old) has failed to find a significant effect of OTR SNPs on several variable, including sociability and ASD (Bakermans-Kranenburg and van Ijzendoorn, 2013). Nevertheless, OTR SNPs remain a good gene candidate to explain social behaviour variability in humans as well as pathology with altered sociability (and studying OT SNPs may be interesting as well), but more studies are needed, especially promising gene x environment experiments might lead to a better understanding of the OT and OTR gene role in social behaviour (LoParo et al., 2015).

Given the suspected importance of perinatal events in ASD, it is relevant to note that OT is a critical molecule for the foetus brain at that particular time. Indeed, OT is required

to trigger the switch in GABA signalling (from excitatory to inhibitory) and this is thought to happen thanks to maternal OT diffusing from the mother to the new-born's brain (Tyzio et al., 2006). OT deregulation at this precise moment has thus been hypothesize to be involved in ASD (Tyzio et al., 2014). These effects of OT are coherent with its action on the excitatory/inhibitory balance in the adult brain (Marlin et al., 2015; Oettl et al., 2016), and give further evidence for a role of OT signalling in social behaviour and ASD.

In the same line, early life events impact on the oxytocinergic system have been thought to determine subsequent social behaviour, and therefore, exogenous oxytocin administration, used to initiate or increase labour, have been investigated in both animals and humans for a potential role in adults' social behaviour. This is the topic of parts 2 and 3 of the review present thereafter (Lefevre and Sirigu, 2016).

I.3.c. Exogenous oxytocin administration in patients with autism

Short term effects of OT

A considerable amount of studies has tried to improve social skills of patients with ASD by administering exogenous OT to them. This started notably after the first experiments using intra nasal OT in subjects, which showed increase gaze to the eye region (Guastella et al., 2008), a hallmark of ASD (patients tend to avoid looking this region of the face (Dalton et al., 2005)). Thus, in 2010, two of the first studies (including one from our team) giving intra nasal OT were published, showing that patients with ASD looked longer at the eye region compared to when they received placebo and were better to perceive and infer emotions (Andari et al., 2010; Guastella et al., 2010). Note that before these two studies, another group has tried to administrate OT *via* an intra venous way showing relatively weak effects (Hollander, 2003; Hollander et al., 2007).

Following these encouraging results, tens of experiments were conducted, often finding weak effects on a very wide spectrum of social behaviours or traits (Guastella and Hickie, 2015; Preti et al., 2014; Yamasue, 2016). However, some studies failed to find an effect of OT, and more importantly, almost all behavioural studies used different outcome variables, making it impossible, or rather hard to compare these clinical trials (Lee et al., 2015). Finally, it is to note that no age-dependant effects have been found so far.

Research on the brain mechanisms which mediate such positive effects have now started. Several studies using social tasks have shown that autistic patients compared to healthy controls, exhibited lower activity in the amygdala (Domes et al., 2013a, 2013b), in median prefrontal cortex (Watanabe et al., 2013) and in orbito-frontal cortex (Gordon et

al., 2013). Remarkably, activity increased in these regions following intra-nasal OT and this went along with improved task performance, suggesting that oxytocin effects on these specific brain regions underlie the behavioural improvement (Bethlehem et al., 2013). However, the mechanisms through which oxytocin increases fMRI signal in these regions are still unclear. According to the animal literature, oxytocin triggers GABAergic activity in the central amygdala, which result in a temporary inhibition of output neurons in this region (Knobloch et al., 2012). Thus, one may speculate that the observed increased fMRI signal is the consequence of an increased inhibitory activity in the amygdala. Nonetheless, where and how intra nasal OT does act in an autistic brain is still blurry. This question is of course being complicated by the heterogeneity of ASD, but no sub group of patients have yet been identified as responding especially well to exogenous OT.

Overall, the lack of result replication, because studies are using different paradigms and different patients, added to the great variability inherent to the use of intra nasal OT makes it difficult to conclude about the potential of this pharmacologic intervention as a potential therapy for patients with ASD. In fact, most of authors now agree that while the oxytocinergic system is the good target, intra nasal OT may not be the best way to reach it (see the discussion in Chapter 5).

Nevertheless, the enthusiasm behind OT, due to its easiness to use and apparent complete lack of negative effects, a now consistent amount of studies has investigated the efficiency of chronic intra nasal treatments in patients with ASD.

Long term effects of OT

This part is covered by part 5 of a review written during the first year of my PhD, in order to address the potential issues with the use of exogenous OT. Note that boxes 1 and 2 were part of the publication but have been already covered previously in this manuscript.

Neuroscience & Biobehavioral Reviews

Volume 63, April 2016, Pages 168–176

Review

The two fold role of oxytocin in social developmental disorders: A cause and a remedy?

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Received 26 June 2015, Revised 5 November 2015, Accepted 27 January 2016, Available online 29 January 2016

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doi:10.1016/j.neubiorev.2016.01.011

Highlights

- Neo-natal oxytocin administration has been causally linked to social deficits in animals.
- Obstetric use of oxytocin and autism have been associated in humans.
- Oxytocin chronic administration in autistic patients has weak effects.

Key words: *Oxytocin, labour induction, early life experience, social behaviour, autism*

Abstract

Oxytocin is widely used by obstetricians to induce or facilitate labour. The long lasting consequences of oxytocin administration remain however unknown. Here, we discuss recent evidence suggesting a link between oxytocin labour induction and developmental social impairments such as autism spectrum disorders (ASD). Because these associations are methodologically questionable, we provide a review of animal studies investigating the long term effects of neonatal injection of oxytocin to shed light on the biological mechanisms that mediate the contribution of early oxytocin supplementation on the development of social impairments. In contrast to this potential negative impact on development, oxytocin has been shown to ameliorate social skills of ASD patients. However, results of chronic oxytocin administration from animal experiments are contradictory, and recent studies looking at chronic oxytocin effects in humans do not allow to conclude. Obstetric and psychiatric uses of exogenous oxytocin both impact on oxytocinergic neurotransmission but the effects may be sharply dissimilar.

1. Introduction

Best known for the influence it has on delivery and lactation, oxytocin has gained a new interest since the early nineties following the discovery of its effects on social behaviour in rodents (Witt et al., 1992). Several years later, a paper by Modahl and colleagues reporting that plasma oxytocin level is lower in autistic children compared to age-matched healthy controls (Modahl et al., 1998) paved the way for investigating the role of this hormone on social and communicative disturbances of autism spectrum disorders (ASD). Today, a large number of papers are available on the role of oxytocin in social behaviour and social developmental deficits(Young and Flanagan-Cato, 2012), but the comprehension of the exact neural mechanisms of oxytocin's action are still debated. This paper reviews recent contradictory evidences suggesting that oxytocin could be a cause and a remedy for social developmental disorders.

2. Oxytocin shapes social personality during early life

2.1 Behavioural long term effects of oxytocin on animal neonates

Early life experience is crucial for the development of an adapted social behaviour, and several factors like poor maternal contact, social isolation or other stressful events seem to have a major impact on the developing brain (Curley et al., 2011; Lovic et al., 2001; Murgatroyd et al., 2009). Individual responses to these factors are partly modulated by the oxytocinergic system (Veenema, 2012). For instance, in prairie voles, a monogamous social species, animals that have been handled during the first days after birth, express higher alloparenting (care of others' pups) but more anxiety when tested in an elevated plus maze, thereby showing that the same manipulation can induce both positive and negative behavioural effects (Bales et al., 2011). Crucially, early body contacts modulate the expression of oxytocin receptors and oxytocin immunoreactivity in the hypothalamus (Bales and Perkeybile, 2012) (the region of oxytocin synthesis) thus demonstrating that early life events impact on the development of the oxytocinergic system.

These observations raise the question whether oxytocin administration at birth also produces long lasting effects on social behaviour. Bales and colleagues (Bales and Perkeybile, 2012) have examined the influence of exogenous oxytocin administration in prairie voles during the first day of life and reported dosage dependent and sexually dimorphic effects in adulthood. For instance, neonatal administration of oxytocin increased alloparental behaviour in adult females in a dose-dependent manner: the more oxytocin the animals received, the more parental care they expressed (up to 4 times) for the pups of others animals (see table 1 for details). Higher doses of oxytocin (8mg/kg) also changed

partner preference: females prairie voles spent more time with a stranger rather than interacting with a familiar partner as was the case in control animals (Bales et al., 2007a). According to the authors, the shift in partner preference can be considered as an oxytocin-induced social impairment.

By contrast, in males, using the same procedure, early neonatal oxytocin was found to reinforce partner preference formation (Karen L Bales and Carter, 2003). In mandarin voles, a closely related species it facilitated pair bonding in females but this behaviour decreased over time (Rui Jia et al., 2008). These findings suggest that early oxytocin administration unbalances the basal oxytocinergic system yielding conflicting gender effects on social behaviour (see Table 1 for details).

The consequences of neonatal oxytocin administration are not limited to pair bonding and parenting behaviour. For instance, in both prairie and mandarin voles, early oxytocin exposure also increased aggressive behaviour and again this response is modulated by social context and it has sexually dimorphic consequences (Karen L. Bales and Carter, 2003; R. Jia et al., 2008). Specifically, adult female mandarin voles that received oxytocin at birth show increased aggressive behaviour toward other females but only after being exposed for the first time during one hour next to a male. This effect is not observed in females without a male experience nor in males (R. Jia et al., 2008).

Similar findings have been found in other species like pigs where neonatal oxytocin administration seems to alter the development of social behaviour by acting on the Hypothalamic/Pituitary/Adrenal (HPA) axis (Rault et al., 2013), a system important for regulating stress response. Pigs receiving oxytocin showed increased aggression and stronger reaction to aggression. Thus, increased neonatal oxytocin through exogenous administration modifies partner attachment and also affects specific dimensions of social behaviour like the approach/aggression balance (see Table 1).

Table 1. Summary of studies investigating behavioural and biological effects of neonatal oxytocin administration in animals. In this survey we omitted 4 studies reporting no effect of neonatal oxytocin on social behaviour (Bales et al., 2004; Kramer et al., 2003, 2006; Young et al., 2005) nor those looking at oxytocin antagonist or vasopressin administration. i.p = intra peritoneal, s.c = sub cutaneous, IN= intranasal, PND = post-natal day.

Species	Dose	Effects of neonatal oxytocin	Reference
prairie vole female	1 mg/kg i.p PND 1	Decreased social behaviour and increased aggression	(Karen L. Bales and Carter, 2003)
prairie vole male	1 mg/kg i.p PND 1	Facilitated pair bonding	(Karen L Bales and Carter, 2003)
prairie vole female	1 mg/kg i.p PND 1	Alteration of mating behaviour (reduced latency, decreased bout frequency)	(Cushing et al., 2005)
prairie vole female	1, 2, 4 or 8 mg/kg i.p PND 1	Non linear dosage effects (ameliorations and deteriorations) on alloparental care and pair bonding	(Bales et al., 2007a)
mandarin vole	1 mg/kg s.c PND 1	Females: facilitation of partner preference formation but decreased maintenance. Decreased C Fos in limbic brain regions. Males: Altered C Fos response. Males and females: Decreased aggression toward strangers.	(Rui Jia et al., 2008)
mandarin vole	1 mg/kg s.c PND 1	Females : increased context specific aggression Males and females: increased social behaviour and modified C Fos activity in limbic brain regions.	(R. Jia et al., 2008)
prairie vole male	1 mg/kg i.p PND 1	Increased C Fos in the supraoptic nucleus	(Cushing et al., 2003)
prairie vole female	1 mg/kg i.p PND 1	Increased oxytocin immunoreactivity cells in the paraventricular nucleus	(Yamamoto et al., 2004)
prairie vole female	1 mg/kg i.p PND 1	Increased estrogen receptor alpha immunoreactivity in ventromedial hypothalamus	(Yamamoto et al., 2006)
prairie vole female	1 mg/kg i.p PND 1	Decreased vasopressin receptor 1A concentration in several limbic zone	(K L Bales et al., 2007)
prairie vole male	1 mg/kg i.p PND 1	Increased number of serotonin fibers in hypothalamus and amygdala	(Eaton et al., 2012)

Pig	0.035 mg/kg IN PND 1, 2 & 3	Altered aggressive behaviour and HPA axis.	(Rault et al., 2013)
Rat	5 mg/kg s.c PND 1	Altered brain and serum concentrations of serotonin, dopamine and their metabolites.	(Hashemi et al., 2013)

2.2 Biological consequences of neonatal oxytocin administration

Neonatal oxytocin administration moreover induces molecular modifications. Sexually dimorphic alterations of oxytocin and vasopressin (a neuromodulator originating like oxytocin in the supraoptic and paraventricular nuclei (Ludwig and Leng, 2006)) immunoreactivity have been observed in the hypothalamus, the bed nucleus of the stria terminalis and the lateral septum at post natal day (PND) 21 (see Table 1 for details) (K L Bales et al., 2007; Yamamoto et al., 2004). Within these regions, the C Fos protein, a marker of neural activity, was also found altered after birth (Cushing et al., 2003) and later adulthood (R. Jia et al., 2008; Rui Jia et al., 2008). Interestingly as observed for behaviour, oxytocin interference with C Fos activity is dependent on gender, species and social context (Cushing et al., 2003; R. Jia et al., 2008; Rui Jia et al., 2008), suggesting that a supplement of oxytocin at birth has multidimensional influences on brain and behaviour.

The long term impact of oxytocin administration at birth is likely to occur within a critical time window where an excessive amount of this hormone may permanently alter the brain structure and functioning. Several biological mechanisms can be responsible for these changes. First, recent preliminary data in prairie voles have shown that pitocin (synthetic oxytocin commonly used to induce birth in women) alters DNA methylation of the oxytocin receptor gene (OTR), a candidate pathway whereby neonatal oxytocin can influence the neonate's brain (Connelly, Jessica J et al., 2013). Congruent with this idea is the finding showing in humans abnormal high levels of DNA methylation of OTR (i.e., lower expression of OTR) in the temporal cortex of autistic patients (Gregory et al., 2009). Moreover, DNA methylation of OTR assessed in blood cells correlates with higher BOLD activity in the anterior cingulate cortex and superior temporal gyrus, two brain regions important for social perception (Jack et al., 2012). Altogether these results suggest that neonatal oxytocin administration may induce long term changes in social behaviour by altering epigenetic OTR mechanisms. Second, it is likely that oxytocin effects need to be examined within the interactive network of other neurotransmitters' action (see Box 1). For instance, a study in prairie voles has shown that neonatal oxytocin administration increases the number of serotonin fibers in the hypothalamus and in the amygdala at PND 21 (Eaton

et al., 2012). Another study has found modifications of dopaminergic and serotoninergic pathways at 4 months following oxytocin injection at birth (Hashemi et al., 2013). Hyper-serotonemia is a clinical feature of autism which has been linked to oxytocin's dysfunction in this psychiatric condition (Hammock et al., 2012). In agreement with these findings Yang and colleagues (Yang et al., 2014) have proposed that neonatal oxytocin administration has long lasting effects on the serotonin system. This hypothesis is supported by anatomical evidences showing in mice the presence of oxytocin receptors in raphe nuclei (the locus of serotonin synthesis) from the embryonic stage to adulthood (Yoshida et al., 2009). Moreover, evidence of oxytocin action on serotonin neurotransmission has been recently provided. Dölen and colleagues, (Dölen et al., 2013) have demonstrated that the joint action of oxytocin and serotonin on raphe nuclei terminals is critical for modulating glutamatergic signals ending on medium spiny neurons in the nucleus accumbens. This cascade of neural events seems mandatory for promoting the animals' preferences for social contexts. Recently we have also provided results showing serotonin and oxytocin interaction in human subjects. We demonstrated that after intra-nasal oxytocin intake, serotonin 1a receptor function is modified in the amygdala, hippocampus and insula, brain regions which are highly relevant for socio-emotional processing (Mottolese et al., 2014). Hence, there are corroborating findings suggesting a possible dysfunction of the basal oxytocinergic activity after oxytocin neonatal administration. Importantly, the consequences of such dysfunction are likely to rebound on a chain of serotonin and dopaminergic neurotransmission events, also important for the developing social brain.

Finally, Ben Ari and co-workers have highlighted the role of early oxytocin exposure by showing that this hormone plays a crucial role in fetal development. In rodents, oxytocin switches GABA neurons (the major inhibitory system in the adult brain) from excitatory to inhibitory (Tyzio et al., 2006). Critically, oxytocin perturbation during this period of parturition produces autistic like behaviour at the adult age (i.e., altered vocalizations pattern) (Tyzio et al., 2014). The study also shows that the oxytocin system affecting the offspring's brain originated from the mother, thus confirming that the placental barrier is permeable to oxytocin. This adds to the idea that oxytocin given to the mother during labour enters the child's brain and potentially perturb the infant oxytocinergic system. To sum up, during parturition and at birth, oxytocin triggers major changes in the neonate's brain by modulating the action of different neurotransmission systems like serotonin, dopamine, vasopressin and GABA.

Altogether these findings suggest that giving exogenous oxytocin during the neonatal period has long term consequences at the behavioural and molecular level on the developing social brain. It must be stressed however, that most of these studies were

performed in animals, mostly in rodents, thus leaving open the question of how neonatal oxytocin administration affects the human brain and how this can be linked to autism.

2.3 Oxytocin and early life events in humans

Recent studies suggest that oxytocin and early life events are also linked in humans. In adult women, for instance, cerebrospinal fluid level of oxytocin (which represents the global activity of this hormone in the brain) is lower in individuals who have been abused during childhood compared to controls (Heim et al., 2009) thus implying that early traumatic events influence the human oxytocinergic system. Another study has revealed that cerebrospinal fluid level of oxytocin at birth predicts children's sociability at six months. Babies with high level of postnatal oxytocin cried more at six months to attract adults' attention and to obtain body contact. They also behaved more socially compared with babies showing low neonatal oxytocin levels (Clark et al., 2013). These data suggest a link between the default state of the oxytocinergic system at birth and future social skills.

Studying the mother's oxytocinergic system is also relevant. First, because this modulates the neonate's oxytocinergic system at birth (Tyzio et al., 2006) and second because it may play a role on the quality of maternal behaviour. Indeed, in a original study, plasma levels of oxytocin were reported to be lower in mothers of autistic children (Xu et al., 2013). One explanation could be that low oxytocin levels lead to lower maternal care, which in turn affects the child's oxytocin system. In the end the outcome of this process is a disturbed social behaviour. In line with this idea, Apter-Levy and colleagues (Apter-Levy et al., 2013) have reported that depressed mothers showing low salivary oxytocin levels, were more likely to have children expressing Axis 1 disorders (anxiety and ADHD) and diminished social skills. These disturbances have been associated with a specific allele (GG) of a single nucleotide polymorphism (SNP) (rs2254298) in the oxytocin receptor (OTR) found predominantly in depressed mothers. While the exact molecular mechanisms triggered by this SNP are not known, it may be suggested that it alters the efficiency of the oxytocinergic system. In other words, the mother's oxytocinergic system affects the infant's brain, first by genes' heritability, and second upon the quality of the maternal relationship the baby receives early after birth. However, a causal link between maternal oxytocinergic system and infant social skills still needs to be firmly established.

Box 1. Oxytocin interactions with other neurotransmitters.

Anatomical and functional evidence indicate that oxytocin influences vasopressin, dopamine, serotonin, GABA and opioids.

- Vasopressin: Vasopressin is similar to oxytocin regarding its structure, localization, mode of release and functioning. It is believed that the balanced action of these two neuropeptides is important for the control of social behaviour (Neumann and Landgraf, 2012). Moreover, oxytocin can bind to and activate vasopressin receptors (Busnelli et al., 2013; Song et al., 2014).

- Dopamine: OTR are present in the ventral tegmental area (Vaccari et al., 1998), a core region for dopamine synthesis. Magnocellular neurons of the paraventricular and supraoptic nuclei receive dopaminergic inputs (Buijs et al., 1984). In prairie voles oxytocin modulates dopamine neurotransmission in basal ganglia leading to important behavioural effects on pair bonding (Young and Wang, 2004). In humans, oxytocin modulates activity in ventral tegmental area depending on social cues (Groppe et al., 2013). For a more complete review see (Baskerville and Douglas, 2010; Love, 2013).

- Serotonin: Serotonin transporter is located in oxytocin cells of the paraventricular nucleus (Emiliano et al., 2007) and OTR have been found on serotoninergic neurons of raphe dorsalis nucleus where they trigger behavioural effects linked to social preferences (Dölen et al., 2013; Pagani et al., 2015).

- GABA: OTR are present on inhibitory neurons in several brain regions such as amygdala, hippocampus or auditory cortex where they modulate various behavioural functions (Huber et al., 2005; Marlin et al., 2015; Owen et al., 2013).

- Opioids: oxytocinergic synapses have been found on beta-endorphin neurons of the hypothalamus (Csifáry et al., 1992) and opioids regulate oxytocin secretion (Brown et al., 2000).

3. Is neonatal oxytocin involved in developmental social disorders?

As described in the previous section, exogenous oxytocin given at birth may lead to unexpected (adverse) outcomes. This point is critical given that artificial oxytocin (pitocin or syntocinon) is now routinely used in many hospitals to induce labour. For instance, in United States, about 25% of births reported in 2006 were induced or augmented (i.e., helped) with artificial oxytocin against less than 10% in 1990 (Joyce A. Martin et al., 2009). This pattern is also observed in Europe (Oscarsson et al., 2006). An important question is whether oxytocin given to induce or augment labour has a later an impact on children's social behaviour. According to Wahl this procedure can alter the infant oxytocinergic system, and be partially responsible for the later appearance of autism spectrum disorders

(ASD) (Wahl, 2004). This hypothesis is supported by two biological facts: first, oxytocin administered to the mother reaches the fetal brain through the blood stream; second, exogenous oxytocin on human culture cells provokes the internalisation of oxytocin receptors, which do not re-externalise later thus causing a reduction of oxytocin efficiency (Wahl, 2004).

As reviewed by Guinchat and colleagues (Guinchat et al., 2012) contradictory findings have been reported on the association between oxytocin labour induction administration and autism. Using a large cohort (n = 625 042), Gregory et al (Gregory et al., 2013) revealed for instance that ASD children are more likely to have a mother that received artificial oxytocin than control children. Nevertheless, these results have been challenged since no differences were made between oxytocin induction and others labour induction procedures like prostaglandins, intracervical balloon catheters or amniotomy, the latters being probably not less harmful for the development of autistic disorders (Vintzileos and Ananth, 2013)).

Furthermore, some of the diagnostic criteria for atypical autism have been incorrectly reported in the 1994 DSM-IV edition. As quoted at page 77-78, the sentence *"impairment in social interaction **and** in verbal **or** nonverbal communication skills*" has been removed and replaced by "*impairment in reciprocal social interaction **or** in verbal **and** nonverbal communication skills*". According to Vintzileos and Ananth (Vintzileos and Ananth, 2013) this editorial mistake may be the cause of an overdiagnosis of autism between 1994 and 2000. Thus, children may have been diagnosed as autistic while only suffering from mild social impairments. As a consequence, the finding of Gregory et al. (Gregory et al., 2013) might rather be taken as meaning that early oxytocin supplementation is linked with mild social deficits *only*.

As a cautionary note, altogether these results are not the proof of a straight link between oxytocin administration and ASD but can be considered as indirect evidence in favor of such hypothesis. A recent study has examined a large population of children (n = 547 040) born between 2000 and 2009 (thus avoiding the overdiagnosis bias) sorted as a function of birth procedure, i.e., induction or augmentation (Weisman et al., 2015). The authors reported a modest but significant association between oxytocin induction and / or augmentation with increased risk of autism in males but not in females. In the authors' view, receiving oxytocin at birth can increase the risk of developing autism in a vulnerable population, probably through epigenetic modulation (i.e., methylation of OTR). Let's us stress again however, that this study cannot be considered as totally conclusive and that we tremendously need further human research on the link between early oxytocin use and the emergence of ASD.

Hence here we would like to suggest that three important factors should be monitored in order to establish a link between oxytocin given through labour induction and ASD: (1) Oxytocin Dosage. If oxytocin induction in humans is involved in developmental disorders, one should expect a dose dependent response, as it has been reported in animals studies where social behavioural modifications in adult animals are linked to the amount of oxytocin they received at birth (Bales et al., 2007a); (2) Oxytocinergic basal state in the mother and the newborn. Because only a small fraction of infants receiving exogenous oxytocin at birth are prone to develop autism, looking at oxytocin concentration, OTR gene variants and epigenetic factors (e.g., DNA methylation) might be relevant to identify individual cases in which oxytocin's use may be harmful; (3) Severity of social disorders and developmental comorbidity. Because neonatal oxytocin, as demonstrated by the animal literature impacts on social abilities in various ways (see Table 1), the severity of patients' social disorders should be evaluated carefully in term of degree and type of disturbance. More generally, it would be informative to look at the link between neonatal oxytocin and social skills. In addition, one should also investigate potential relations between oxytocin induction or augmentation and other developmental disorders, since a pilot investigation has suggested that pitocin (artificial oxytocin) could also be linked to ADHD (Kurth and Haussmann, 2011).

Recently, the Committee of Obstetric Practice recommended not changing the current use of oxytocin (&Na;, 2014), because there are no firm evidence so far for a causal role of peri-natal oxytocin and increased risk of autism in humans. Nonetheless, in the light of the animal results reviewed here demonstrating a causal role of oxytocin in (1) the long term social and physiological perturbations and (2) the growing incidence of autism and peri-natal oxytocin use in humans (around 15% increased chances to develop ASD in children whose birth was induced or augmented with oxytocin (Gregory et al., 2013; Weisman et al., 2015)) we believe this question urgently deserves further clinical and experimental investigation. A very recent study reported contrasted results, with the association of labour induction and ASD on overall population, but the disappearance of this relation when comparing siblings who were or were not inducted (Oberg et al., 2016). While this suggests perinatal OT administration might not be a cause by itself, this nonetheless argues in favour of a role of perinatal OT in long term social behaviour, as a potential moderator of other risk factors. This latest study raises additional questions, such as the differentiation between induction and augmentation, which is linked to the reasons of exogenous OT administration (i.e., why was OT given?). Ultimately, this problematic would deserve research in animal models, which could be very relevant to assess the role of genes and environment that are easily controllable in rodents.

4. Oxytocin and social behaviour in humans

Because it is suspected that dysfunction of the oxytocin system is a potential cause of social disorders, it is not surprising that oxytocin administration has attracted attention as a potential remedy for such psychiatric conditions. Positive effects of oxytocin are now classically described as improved emotion recognition, enhanced memory of faces and increase of trust, eye contact or social motivation (Bartz et al., 2011).

Oxytocin has been used to improve the impairments of adults and adolescents suffering from developmental and psychiatric disorders such as Prader-Willi syndrome, Williams syndrome, fragile X syndrome, schizophrenia and depression (Bakermans-Kranenburg and van IJzendoorn, 2013). A recent meta-analysis study suggests, however, that intra-nasal oxytocin may have a specific effect on ASD compared to other psychiatric conditions (Bakermans-Kranenburg and van IJzendoorn, 2013).

It is worth noting that all of these experiments have focused on short term effects of acute oxytocin administration, given its short half-life (few minutes in the blood and around 90mn in the brain). The reader should be aware that the use of intra-nasal spray to dispense oxytocin is not free of concerns (see Box 2). Future studies using longer follow-up designs need to be conducted to investigate the behavioural and biological modifications following chronic intake of oxytocin. The promising beneficial effect of oxytocin in ASD patients raises indeed important questions concerning the safety of this hormone after daily administration. This issue will be addressed in the next section.

Box 2. Intra nasal oxytocin administration: facts and pitfalls

Typically, oxytocin is administrated to humans with a nasal spray. This method supposedly allows oxytocin to reach the cerebrospinal fluid, bypassing the blood brain barrier thanks to the specificity of the nasal cavity. While evidence indicates that brain oxytocin concentration raises after intra nasal administration (Born et al., 2002; Modi et al., 2014; Neumann et al., 2013), the specific neural pathways of oxytocin spreading remain unclear. Because OTR are present outside the brain (Gimpl and Fahrenholz, 2001), an alternative hypothesis would be that oxytocin stimulates the autonomic system, such as the vagus nerve, which would in turn provoke a central release of oxytocin (for recent reviews on this issue see (Leng and Ludwig, 2015; Quintana et al., 2014). Additionally, the amount of oxytocin administered to humans is about 24 IU (approximately 50µg) which is above the total volume of oxytocin in the pituitary gland (see (Leng and Ludwig, 2015). This amount is also enormous compared to the cerebrospinal fluid concentration of 10pg/mL, and the fact that this concentration raises to 50pg/mL after intra nasal administration meaning that most of the oxytocin given is not reaching the brain, if any does. Such considerations are highly important when we are considering chronic administration. The risk of desensitization of the endogenous oxytocinergic system cannot be overlooked. This is why dose dependant studies in humans, trying to lower the amount of oxytocin administered to subjects are highly desired and welcome. To date, only one recent attempt has tried to tackle these issues, showing that lower doses can achieve similar effects (Quintana et al., 2015). Finally, given the complexity of the oxytocinergic system (wide spectrum of behavioural and physiological actions, the OTR can be linked to various G protein, etc), an alternative research strategy would be to design partial agonists, to selectively stimulates a subpopulation of OTR. Ideally, these would be small molecules that can cross the blood brain barrier to allow oral administration.

5. Long term effects of chronic oxytocin administration

5.1 Animal studies

The effects of intra-nasal administration of oxytocin raise the question of the biological consequences (e.g., hormonal deregulation) following its chronic exposure. Moreover, it also important to establish the right dosage should be given to humans (e.g., what is the minimal efficient dose, what is the optimal frequency of administration, etc.) to facilitate social behaviour. Bales and colleagues investigated these questions using an animal model. They daily administered different doses of oxytocin (intra-nasal) to adolescent prairie voles. Tested as adults, male voles receiving the low dose of oxytocin exhibited, according to the authors, “disturbed” bonding behaviour, meaning that they spent more time with an unknown female as much as they did with their preferred partner (Bales et al., 2013). This

is in contrast with control animals' behaviour showing a clear-cut preference only for the familiar partner. Interestingly, this effect was neither found for medium and high doses nor in females. Although the authors interpreted partner preference change as a disturbance of attachment (Bales et al., 2013), an alternative hypothesis might be that chronic administration of oxytocin during adolescence does not impair social behaviour *per se* but rather increase novelty seeking activities thus pulling attention toward new partners (Love, 2013).

Two recent experiments in rats have compared chronic effects of oxytocin using intracerebroventricular (i.c.v) or intranasal administration (Calcagnoli et al., 2014, 2015). After 7 days of treatment, both reduced aggressive behaviour toward an unknown conspecific (using the resident – intruder paradigm) and increasing explorative social behaviour were observed. However, only after i.c.v treatment, the effects lasted for 7 extra days after cessation of chronic oxytocin administration. It must be stressed that the intra nasal daily dose was 20µg compared to 240ng for i.c.v (delivered at 10ng/h using an osmotic minipump). This shows that central and peripheral administration of oxytocin has a similar influence but the duration and the dose required to reach the effects are different.

Another study has looked at the biological impact of chronic oxytocin (Bowen et al., 2011). After daily oxytocin administration in adolescent rats (intra-peritoneal) authors have reported decreased anxiety and increased social contact with strangers compared to a placebo group. Moreover, they also found an up-regulation of oxytocin receptor mRNA from hypothalamic extracts. This means that chronic oxytocin during adolescence may modify long term expression of oxytocin receptor *via* epigenetic modulation and this process in turn affects social behaviour.

If chronic oxytocin induces long lasting neural modifications, one may wonder whether it has distinct effects in typically developing individuals compared to those socially impaired. This is a legitimate question since the action of exogenous oxytocin depends on the status of the default oxytocinergic system. This hypothesis was recently tested by giving chronic intra-nasal oxytocin to adult healthy mice (Huang et al., 2013). The results demonstrated that additional oxytocin impairs social behaviour and reduces the expression of oxytocin receptors. Hence, and even if it may seem contradictory, chronic oxytocin in pathologic subjects could be beneficial while adding daily oxytocin to a functionally optimal oxytocinergic system could result in a long term deregulation. Therefore, oxytocin should not be recommended for the healthy population. Finally, as discussed above, the time window of oxytocin administration is critical and chronic oxytocin probably does not have similar effect during neonatal period, childhood, adolescence or adulthood.

Ultimately, only two studies have employed mice models of autism to investigate the effect of repeated oxytocin administration. Teng and colleagues used two models to show that 4 administrations of oxytocin over a week improved social behaviour when animals were tested 2 weeks after the end of treatment (Teng et al., 2013). This demonstrates that recurrent administration of this hormone can be helpful to treat social disorders. Please note, however, that Bales et al., (Bales et al., 2014) just reported a contrasting finding in a single mouse model: neither positive nor negative effects of oxytocin were obtained following 30 days of intranasal administration.

Aside from autism models, chronic oxytocin has been studied for its potential effects on anxiety. First, in rats selectively bred for high anxiety-related behaviour, it was found that i.c.v oxytocin given in a continuous manner *via* a minipump (10ng/h), decreased anxiety in females only. This effect was not found in the low anxiety rats, although a trend appeared in males (Slattery and Neumann, 2010). Secondly, chronic i.c.v oxytocin at high dose (10ng/h) increased anxiety in healthy single housed mice while decreasing OTR binding in the amygdala and lateral septum, both important for the control of this disturbance (Peters et al., 2014). Thus, a desensitization of OTR due to an overstimulation has antagonistic effects. On the other hand, the low dose of chronic i.c.v oxytocin (1ng/h) prevented the manifestation of a hyper anxious behaviour in animals exposed to social stress. Hence, chronic oxytocin is beneficial only if subjects already display anxiogenic traits or if already exposed to a stressful situation. Furthermore, the right dosage need to be determined and sex differences must be taken into account. Since no clear rules are available, a possible procedure is to scale the amount of exogenous oxytocin on behavioural personalities scores (e.g., anxiety or social isolation) or physiological measures (the level of basal oxytocin).

To sum up, animal findings suggest that chronic oxytocin may be detrimental in normal subjects. Unfortunately, no clear conclusion can be reached from animal models of autism.

Table 2. Summary of studies investigating mid and long term effects of chronic oxytocin administration in animals and human patients. Doses are converted as follows: 1 IU is equivalent to 2 µg of oxytocin. i.p = intra peritoneal, IN= intranasal, PND = Post Natal Day.

Species and sex	Oxytocin treatment	Effects	Reference
human (autistic patients)	50 µg IN twice a day for 8 weeks 25 children (3 to 17 years old)	Small improvements of social cognition (pilot study to be confirmed). Reduced irritability. No adverse effect reported.	(Sikich L. et al., 2013)
Human (autistic patients)	36 or 50 µg IN twice per day for 8 weeks 50 adolescents (12 to 18 years old)	No effects. Positive reports from parents who believed their child received oxytocin.	(Guastella et al., 2014)
Human (autistic patients)	100 µg IN per day for 6 weeks 18 adults (24 to 43 years old)	Improved clinical score of reciprocity (ADOS). Increased cingulate and dorsomedial prefrontal cortex response in a social task.	(Watanabe et al., 2015)
Human (autistic patients)	25 µg IN twice per day for 5 weeks 31 children (3 to 8 years old)	Improved social responsiveness, assessed by caregivers.	(Yatawara et al., 2015)
prairie vole	0.04, 0.4 or 4 µg/kg IN PND 21-42	Males spent more time with a stranger compared to the time devoted to the partner. No effect in females.	(Bales et al., 2013)
rat male	1000 µg/kg i.p PND 33-42	Decreased anxiety, increased social contact with unfamiliar rat, increased oxytocin receptor expression and plasma oxytocin concentration.	(Bowen et al., 2011)
mouse	10 or 20 µg/kg twice a day for 7-21 days IN adult	Reduced social interaction, decreased oxytocin receptor binding in several limbic areas, increased vasopressin receptor 1A in lateral septum.	(Huang et al., 2013)
mouse male (autistic model)	4 x 1000 µg/kg within 8 days i.p PND 21-40	Increased social behaviour with a stranger.	(Teng et al., 2013)

Mouse (autistic model)	1.8µg/kg IN 30 days PND 21-50	No effects	(Bales et al., 2014)
Rat (high and low anxiety)	10ng/h i.c.v 6 days adults	Decreased anxiety in highly anxious females but not in males; no effects in animals showing low anxiety.	(Slattery and Neumann, 2010)
Mice	1 or 10ng/h i.c.v 15-19days single housed or social stressed adults	High dose increased anxiety in single housed animals and decreased OTR binding in various brain regions. Low dose decreased anxiety in socially stressed mice.	(Peters et al., 2014)
Rat	10ng/h i.c.v 6 days adults	Decreased aggressive behaviour, increased social exploration of an unknown congener. Effects lasted after treatment ended.	(Calcagnoli et al., 2014)
Rat	20µg per day 6 days intra nasal adults	Decreased aggressive behaviour, increased social exploration of an unknown congener. Effects did not last after treatment cessation.	(Calcagnoli et al., 2015)

5.2 Human studies

Several preclinical trials have investigated how long term oxytocin administration permanently improves patients' behaviour. It is to note that all the humans studies reported here have been conducted with intra-nasal oxytocin, which brings issues that need to be considered carefully (see Box 2). In a preliminary study, 19 adults patients with a diagnosis of high-functioning autism or Asperger syndrome (Anagnostou et al., 2012) self-administered 24 IU (international unit) of oxytocin or placebo twice per day during six weeks. The outcomes measures showed mild improvements on motor repetitive behaviour, increased performance at recognizing emotions and a self-reported benefit on quality of life measures. No side effects were observed. Although encouraging these findings should be taken with caution since patients' self-evaluation of their own improvements were not fitted against parents' rating, classically considered good evaluators of patients' behaviour in the clinical setting. Another pilot study recruited 8 children with severe to high functioning autism (Tachibana et al., 2013). Oxytocin was administered twice daily. The dose was increased every two months (8, 16 and 24 IU) and interleaved (before each increase) by a placebo period during one or two weeks for a total of seven months. The results showed small improvements in communication and social interaction on the

Adolescent diagnostic observation scale. Five parents reported improvements on social behaviour and on the quality of interaction with family members. Notably, in these cases, children were moderately impaired with bad language expression but communication skills still possible. The selectivity of this effect deserves further investigation because it could help targeting ASD patients that can benefit from oxytocin therapy. Yet, the reader should be aware that both studies do not allow drawing firm conclusions on the effects of chronic oxytocin in ASD because of their low statistical power and methodological shortcomings (absence of placebo group or condition, differences at baseline level etc..). Hence, we recommend caution when considering these results given that much rigorous clinical trials are still needed. This note of caution is warranted by a recent report demonstrating no oxytocin effect in 50 adolescents with severe autistic or Asperger's disorders. The study also reported a paradoxical effect: caregivers believing their children received oxytocin reported greater improvements compared to a group believing they received placebo (Guastella et al., 2014). This highlights the importance of using a controlled placebo condition on this type of studies. Other important factors seem to modulate the effect of chronic IN OT. For instance, a recent study found dose and gene dependant effects (Kosaka et al., 2016). Finally, a large scale clinical trial conducted by Sikich and colleagues is currently in progress. In this trial oxytocin or placebo will be administered twice a day for six months to 300 children with severe to mild ASD. Behavioural outcomes will be evaluated as well as DNA methylation levels of OT genes (Sikich L. et al., 2013) (http://projectreporter.nih.gov).

In summary, contradictory results have been reported in the current oxytocin literature. This may be due to differences in the age of administration (children, adolescent or adults) or the variety of measures used to assess social functioning. Future studies looking at the long term effects of oxytocin should focus on the use of tasks known as optimal paradigms (eye contact, mind reading in the eyes test, cyberball game (Andari et al., 2010; Guastella et al., 2010)) for testing oxytocin effects on social behaviour. Ultimately, future trials should include both patients and parents' evaluation on patients' improvements given that contradictory assessments have been found between patients and parents (Lefevre, A et al., n.d.). It is also important to highlight that none of these studies have found serious side effects after chronic oxytocin (see Table 2 for a summary).

6. Conclusion and perspectives

In this review we have raised issues regarding the short and long term use of oxytocin and highlighted the consequences of its administration during labour induction. Many questions related to the long term effects of this hormone remain unanswered. We suggest that decisions of oxytocin's use for obstetric purposes should be carefully weighted in the

light of animal evidence clearly showing behavioural and physiological alterations following peri-natal use of oxytocin.

A significant effort needs to be undertaken in order to understand the biological impact of this hormone, specifically in terms of epigenetic changes. The other side of oxytocin action reveals that sporadic use of this hormone in the autistic condition seems full of promises to tentatively alleviate patients' social disturbances. Yet, its long term efficiency remains unclear. Finally, further research should look at the functional links between oxytocin and others major neuromodulators like serotonin and dopamine also involved in the regulation of socio-emotional health and in the expression of several neuropsychiatry disorders.

Acknowledgments

We are grateful to Cortex Labex, ANR and Fondation Fondamental.

II. Chapter 2 – Experiment 1: Oxytocin and serotonin interaction in patients with ASD

II.1. Aim and methods

II.1.a. The serotonergic system

The corner stone of this PhD is the interaction of OT with serotonin, a monoamine neurotransmitter synthesized in the raphe nuclei. Serotonin (5-hydroxytriptamine, 5-HT) is an important molecule for the brain which is released in almost all forebrain regions by projections mainly originating from the dorsal raphe nucleus, and a little bit from the median raphe nucleus (Charnay and Leger, 2010; Parent et al., 2011; Wallman et al., 2011) (Figure 12). This neurotransmitter has been linked to many functions at both the central nervous system (sleep, reward, impulsivity, aggressiveness, mood…) and the periphery (gut, cardiovascular function, pain, thermoregulation…) (Charnay and Leger, 2010), but most relevant for the present work, it has an important role in social behaviours.

As oxytocin, this social function of 5-HT has been well conserved across evolution. It was indeed found to be responsible for switching desert locusts from solitary behaviour to swarm formation (Anstey et al., 2009). In mammals, experiments on rodents and primates have shown that 5-HT levels influenced social play, reactive aggression, sensitivity to social cues and other aspects of social behaviours at different stages of life (Kiser et al., 2012). These levels of 5-HT are also highly important in humans, where they are linked to depressive state and mood disorders. The amount of 5-HT depends on the release from fibres and the 5-HT transporter (5-HTT), which also has been found to be an important modulator of social behaviours (Canli and Lesch, 2007), however a lot of studies looking at the impact of 5-HTT allelic variations on behaviours have been conducted with small samples size and subsequently questioned (McGuffin et al., 2011).

Serotonin actions are exerted through a complex receptor system with 14 subtypes (Barnes and Sharp, 1999). As a review of all their roles would be too long, I will therefore focus on the serotonin 1A receptor (5-HT_{1A}R). This receptor was one of the first to be discovered and thus most well-described, at the end of the eighties (Hamon et al., 1990). It is also widely distributed across the brain, with notably high concentrations in the limbic system (amygdala and hippocampus, but not in basal ganglia), in the prefrontal and temporal cortex and in the dorsal raphe nucleus itself (Pazos et al., 1987) (Figure 12). Their role at the cellular level is region dependent since their coupling to G protein varies accordingly (Mannoury la Cour et al., 2006). For instance, in the dorsal raphe nuclei, 5-

$HT_{1A}R$ are inhibitory auto-receptors (located on the soma and dendrites of serotonergic neurons) reducing serotonin release, while in the hippocampus, they seem to be post synaptic receptors with more divers effects (regulation of adenylate cyclase and potassium channels) (Albert et al., 2014; Kennett et al., 1987; Palchaudhuri and Flügge, 2005; Raymond et al., 2001). In line with this, the trafficking of this receptor was also found to be region dependant. Indeed, the 5-$HT_{1A}R$ is internalised quickly (from a few minutes to a few hours) after agonistic stimulation in the Dorsal Raphe Nucleus (DRN) but not in the hippocampus (Riad et al., 2001). Regulation of post synaptic 5-$HT_{1A}R$ (i.e., outside of raphe nuclei) has not been described so far, apart from constitutive internalisation (Bouaziz et al., 2014), and the characteristics of 5-$HT_{1A}R$ externalisation are yet not known, especially in terms of temporal pattern.

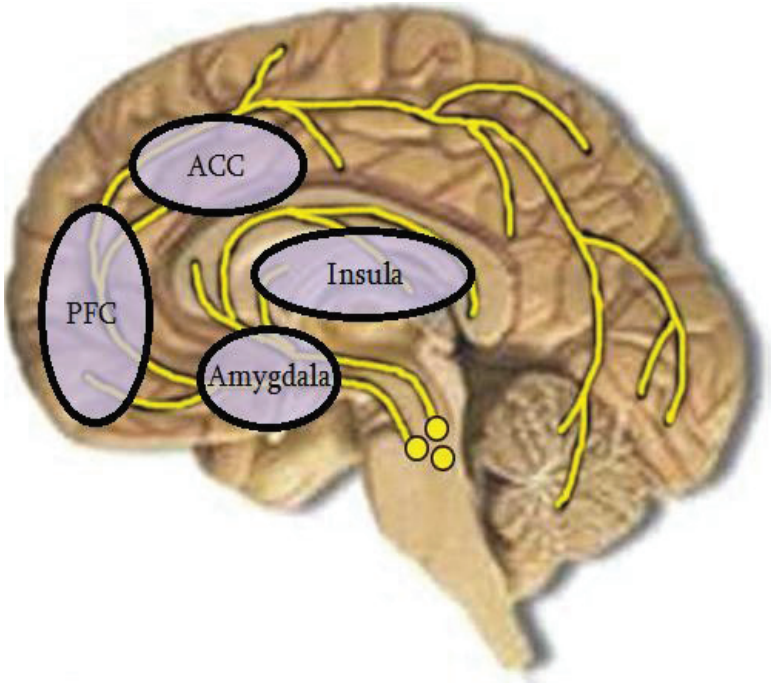


Figure 12. The serotonergic system in the human brain. Yellow dots represent raphe nuclei, yellow lines represent serotonergic fibres projecting across the brain, purple areas indicate the main regions containing serotonin 1A receptors. The hippocampus and raphe nuclei also contains a lot of 5-$HT_{1A}R$ but this is not depicted here for clarity.

At the behavioural level, it has been shown that 5-$HT_{1A}R$ stimulation increases adjacent lying in rats (Thompson et al., 2007) and reduces aggression (Bell and Hobson, 1994; Joppa et al., 1997). Moreover, the 5-$HT_{1A}R$ plays an important role in depression, as all almost therapies are provoking an increase of 5-$HT_{1A}R$ concentration (Savitz et al., 2009) and low concentrations of 5-$HT_{1A}R$ have been associated with suicide (Underwood et al., 2012). Finally, there is a large literature on the importance of 5-$HT_{1A}R$ for anxiety regulation. Animals lacking this receptors were thus found more anxious in various behavioural tests (Gross et al., 2002; Ramboz et al., 1998).

It is to note that several molecules targeting the 5-HT_{1A}R are currently used in various pathologies such as depression, anxiety and, interestingly, some symptoms of autism spectrum disorders (Bandelow et al., 2002; Celada et al., 2013; Vasa et al., 2014).

II.1.b. Positron Emission Tomography of the serotonergic system

Positron Emission Tomography (PET) is the only way to investigate the activity of a specific neurotransmission system *in vivo* in an almost non-invasive manner (along with SPECT). The only clinical manipulation required is the installation of a venous catheter through which the radiotracer is going to be injected. Thus, PET scan has been the key tool of the experiments presented thereafter. The versatility of the PET scan comes from the fact that virtually, a lot of molecules can be tagged by an isotope emitting positrons (this depends on several chemical conditions regarding the possibility to attach an isotope to the molecule). Thus for the serotonergic system, several radiotracers have already been created, allowing scientists to study different receptors sub types (5-HT receptors 1A, 1B, 2A, 4 and 6) (Zimmer and Le Bars, 2013), as well as the serotonin transporter (Stehouwer and Goodman, 2013). Recent research is now trying to develop partial agonists radiotracers that would give the opportunity to study only a precise pathway (represented by a G protein subtype) of a receptor (Becker et al., 2016). Note that tracers are designed to be used at a dose that does not exert any significant biological effects.

PET scan is based on the radioactive tagging of molecules, typically with unstable isotopes such as ^{18}Fluor and ^{11}Carbon. These isotopes will emit positron which will travel for about 1 millimetre before annihilating with an electron. This will generate two photons moving in opposite directions (Figure 13). PET scan consists in a detection unit, made of several circles of scintillators which create bursts of light when gamma photons reach them. Light is then "transformed" in electricity by photomultiplier tubes. When two photons are simultaneously detected from opposite scintillators, they will be attributed to the same annihilation event and the localization of this event will be estimated from the time-of-flight differences of the two photons. This is the information produced and stored by the scanner. A reconstruction algorithm then retro-projects the events and creates an image of voxels containing the number of annihilations over time. This represents the raw images that will be manipulated by researchers.

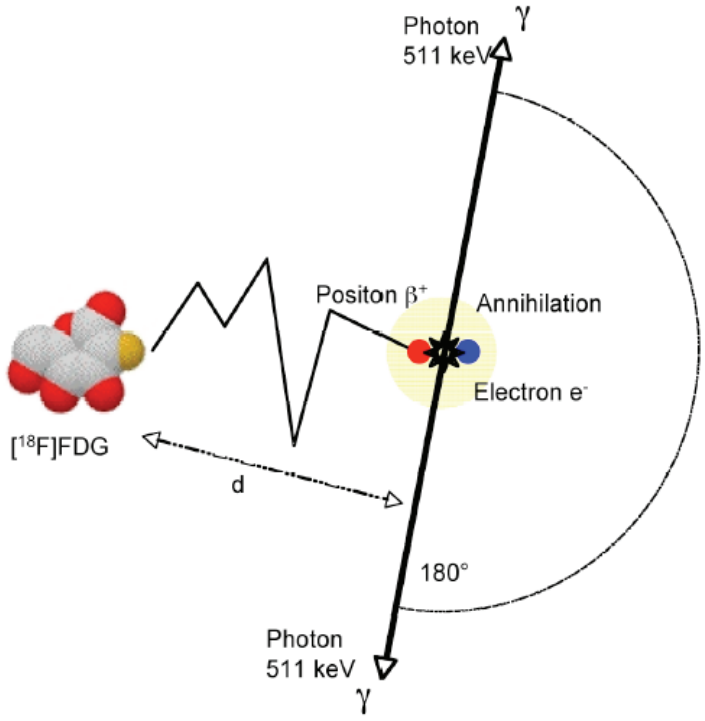
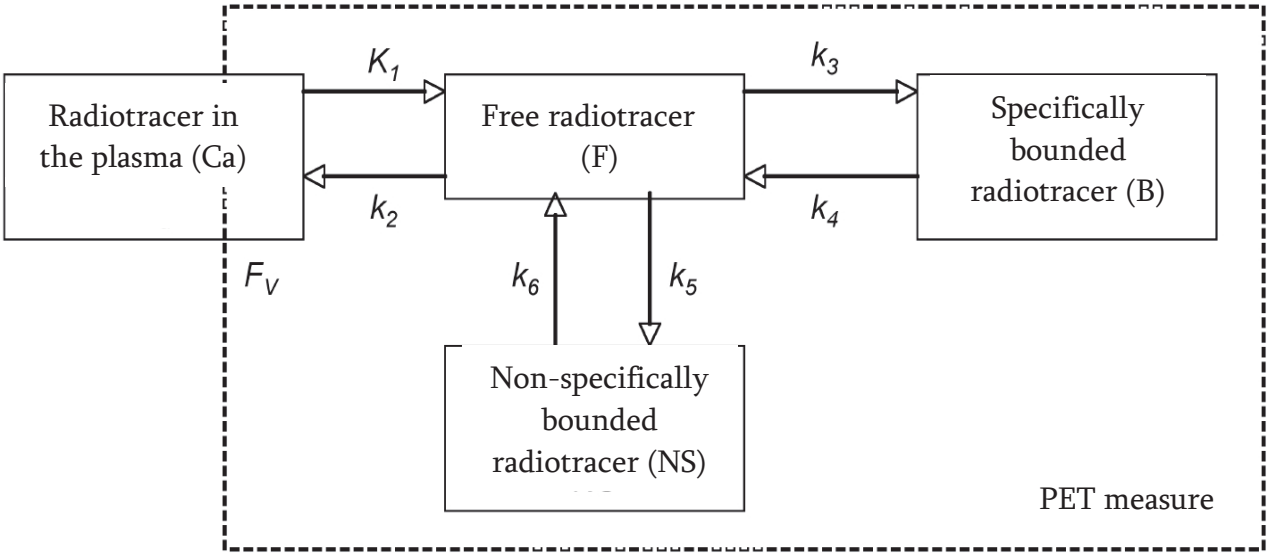


Figure 13. A molecule (Fluorodesoxyglucose, FDG) has been tagged with an isotope (^{18}F) which emits positrons travelling for a distance d (depending on their energy) until they annihilate with an electron. This will provoke gamma radioactivity, and two photons will be generated in opposite directions. The energy and timing of photons allow the scanner to attribute them to the same annihilation event or not, and this will permit the localization of the radiotracer.

(Image from N. Costes)

From these images, it becomes possible to calculate the non-displaceable Binding Potential (BP_{ND}) of a radiotracer to its targeted receptor (i.e., the amount of radioactive molecules bound to their specific target). It is important to note that this value is no longer a raw biochemical information but an estimation made from a model, called the tracer kinetic model, which assumes a compartmental system (Ichise et al., 2001) (Figure 14). In this model, the signal measured by the PET scanner is considered to be the sum of the unbound free radiotracer, the radiotracer specifically bounded to its target and the non-specifically bounded radiotracer, plus the radiotracer contained in the blood that is flowing in the brain (Figure 14). By estimating the parameters (k) it becomes possible to calculate the BP_{ND}. This measure will depend on various factors, such as the affinity of the radiotracer for its target and for other possible sites of binding, the degradation rate, the amount of targets, etc…



$$PET = (1 - Fv)(Cf + Cs + Cns) + FvCa$$

Figure 14. Schematic representation of the different compartments of the model, and equation of the model. The PET scan measures the radioactivity emitted from the radiotracer that is bounded or free and from the blood present in the brain. The model's parameters are quantified through multi-injections, saturation, arterial blood sampling experiments. (Image adapted from N. Costes).

In order to avoid additional experiments to estimate the model's parameters, a simplified model has been developed: the Simple Reference Tissue Model (SRTM) (that has been used in all experiments presented thereafter). The idea is to compare the signal from a region rich in receptors to a reference region known to contain no receptors (these *a priori* can be investigated with *post mortem* autoradiography for instance). This model is based on the assumption that all parameters are equals between the two region, apart from the receptor density. Notably, this assumes that the perfusion rates (radiotracer from the blood to the tissue and *vice-versa*) and the metabolism are identical. In the facts, this method has been estimated to lead to error inferior to 10% compared to experiments including multi injections and arterial blood sampling for full model calculation (Lammertsma and Hume, 1996). It has therefore become the norm to use the SRTM to evaluate receptor systems in the living brain (Heiss and Herholz, 2006).

The Binding Potential (BP_{ND}) is susceptible to several biological factors (Figure 15). First, the number (density) of receptors will determine how much sites of binding are available for the radiotracer. Therefore, phenomenon such as internalisation and externalisation can influence the BP_{ND}. In the case of the 5-HT_{1A}R, we know for instance that internalisation is different between regions and governed by different mechanisms

(Riad et al., 2001). The time scale of internalisation and externalisation is also important, because the PET scan exam lasts for typically 1 hour, this allows 5-HT_{1A}R to undergo such trafficking. For our experiments, we have used 2'-Methoxyphenyl-(N-2'-pyridinyl)-p-18F-fluoro-benzamidoethylpiperazine ([^{18}F]MPPF), a 5-HT_{1A}R antagonist that possibly only binds to receptors that are on the plasma membrane (on the contrary to [^{11}C]WAY100635, another widely used radiotracer for 5-HT_{1A}R, that might bind to all receptors regardless if they are inside the cell or on the plasma membrane).

Secondly, another factor regulating 5-HT_{1A}R radiotracers BP_{ND} is its state of affinity. Indeed, 5-HT_{1A}R exists in a high affinity state, when the receptor is coupled to a G protein, or a low affinity state, when the receptor is not bound to a G protein (Sundaram et al., 1993). Interestingly, this affinity state will strongly modulate the binding of agonist and antagonist molecules, so that agonists will bind more specifically to the high affinity state 5-HT_{1A}R, and antagonist will bind equally to both states (Gozlan et al., 1995). This has led to the idea that using agonists radiotracers gave a more "functional" picture (agonists would be more sensitive to endogenous serotonin) while antagonists radiotracers would be more general, sensitive to overall receptor density. There are approximately equivalent proportions of high and low affinity state 5-HT_{1A}R in the primate brain under normal conditions (Kumar et al., 2012). The transition from one affinity state to another depends on intra cellular mechanisms, and therefore susceptible to many factors.

Finally, the radiotracer injected will be in competition with the endogenous ligand to bind to its target (e.g., MPPF and endogenous serotonin will compete for 5-HT_{1A}R) (Figure 15). It is however important to note that this competition impacts more or less the BP_{ND}, depending on several factors. As previously said, agonists radiotracers that preferentially bind to high affinity state receptors will compete more for these available receptors with endogenous serotonin, while antagonists radiotracers are thought to be more independent of endogenous serotonin because they will always bind to low affinity state receptors, meaning that about 50% of their signal will not be affected by endogenous serotonin (Zimmer and Le Bars, 2013). Moreover, the competition will depend on the affinity of the radiotracer for its target, compared to the endogenous ligand, e.g., MPPF has a higher affinity (2.8nM) for the 5-HT_{1A}R than endogenous serotonin (3.17nM) and is therefore less sensitive to competition (Aznavour and Zimmer, 2007). A critical review of the literature has indicated that MPPF BP_{ND} does not seem sensitive to decreases of endogenous serotonin concentrations, but could be impacted by large increases of serotonin (supra-physiologic modulations induced by pharmacologic manipulations) although a full agreement on this question has not been reached yet (Paterson et al., 2010).

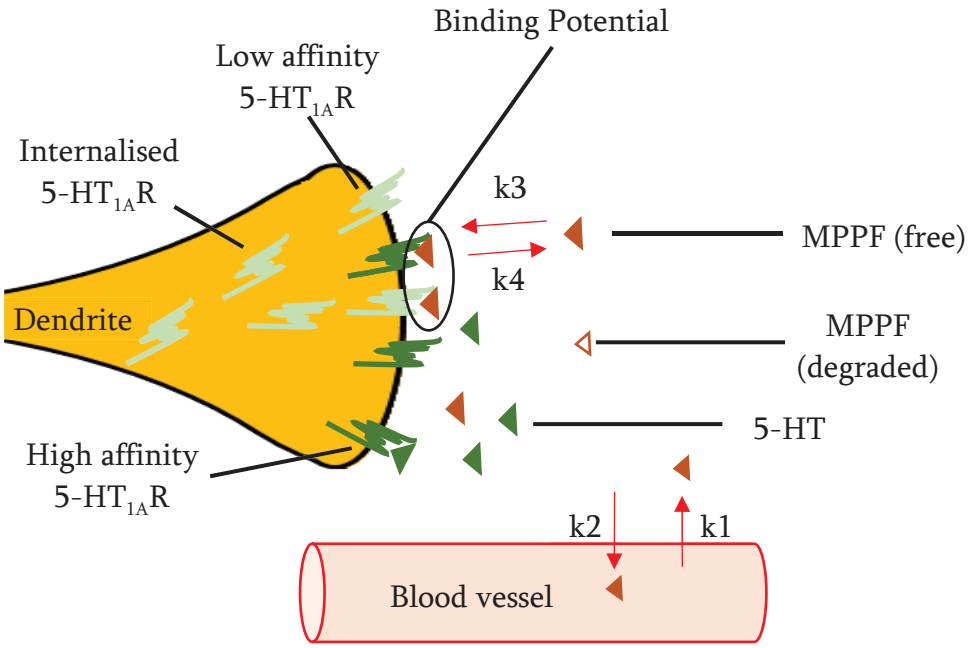


Figure 15. Schema of the various factor influencing the PET scan measure. In the SRTM, parameters k1, and k2 are estimated to be identical between a reference region and regions of interest. Moreover, degradation rate and k4 (dissociation constant) of MPPF are really low compared to the duration of a PET scan exam. This means that since we know the affinity of MPPF (k3), we can calculate the non-displaceable Binding Potential of MPPF. *In vivo* variations of BP_{ND} will mainly occur after changes of externalised 5-HT_{1A}R concentrations. Competition with endogenous 5-HT will occur only for high affinity 5-HT_{1A}R and MPPF will be advantaged by its higher affinity for the receptor.

II.2. Oxytocin fails to recruit serotonergic neurotransmission in patients with ASD

This first experiment of my PhD was done in the continuation of a previous PET scan study performed by our team in healthy subjects (Mottolese et al., 2014). In this work, it was shown that oxytocin (OT) modulated the Binding Potential of MPPF in 24 healthy men (Figure 16). Subsequently, we decided to run the same paradigm in patients with Autism Spectrum Disorders (ASD) (see next section for detailed hypotheses), so when I started this PhD, my first aim was to finish the testing of these patients (started by R. Mottolese) and to analyse the data.

The following section is an article that is currently in review in the "*journal*".

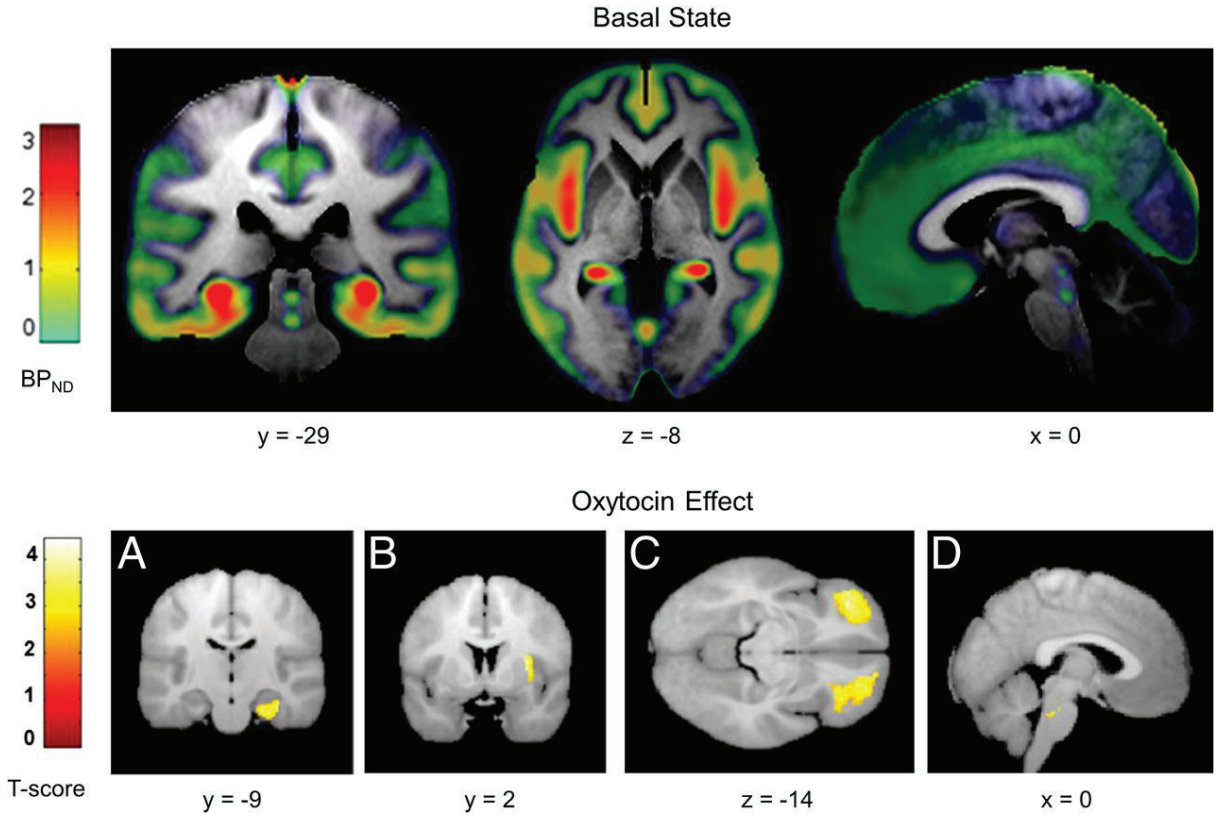


Figure 16. (Upper) Brain mapping of the MPPF binding potential (BP_{ND}) of healthy men at basal state (n = 24). 5-HT_{1A} binding is localized in amygdala, hippocampus and para-hippocampus, insula, DRN, orbitofrontal cortex, and anterior cingulate cortex. The PET functional image is projected on the normalized average brain of the group.

(Lower) T-map SPM analysis (P < 0.01 uncorrected) showing the effect of OT administration on MPPF BP_{ND} in the OT group (n = 12) compared with the basal state: (*A*)

right amygdala/hippocampus/para-hippocampus complex, (*B*) right anterior insula, (*C*), right and left orbitofrontal cortex, and (*D*) DRN. No significant effect in the placebo group ($n = 12$) was found.

This figure is taken from (Mottolese et al., 2014).

Oxytocin fails to recruit serotonergic neurotransmission in patients with ASD

Lefevre A., Mottolese R., Redouté J., Costes N., Le Bars D, Geoffray M.M, Leboyer M., Sirigu A.

Abstract

Oxytocin, a neuropeptide involved in affiliation, has been shown to improve social skills in patients with autism. Oxytocin improvements on patients' sociability are however ephemeral. Animal research has shown that oxytocin reinforces sociability by preferentially acting on the serotonin pathway where stimulation of raphe nuclei terminals causes dopamine release in the nucleus accumbens. We previously showed oxytocin/serotonin functional coupling in the healthy brain. Whether such molecular interaction also occurs in the brain of autistic patients is unknown. We studied oxytocin/serotonin neurotransmission using the radiotracer [18F]MPPF, a selective serotonin 1A receptor (5-HT_{1A}R) antagonist in 18 autistic subjects assigned to placebo or oxytocin group. We investigated the effect of oxytocin, placebo administration and baseline on the [18F]MPPF binding potential (BP) and after. Previous published data from 24 healthy volunteers enrolled with an identical protocol served as control comparison. Blood samples were also collected to evaluate the impact of oxytocin on peripheral free serotonin.

Comparisons between controls and patients did not led to any significant differences on baseline [18F]MPPF BP. Moreover, there were no differences between controls and patients under placebo condition. Neither oxytocin nor placebo spray exerted any effects in patients when compared to baseline, contrary to the oxytocin effect we previously found in controls. Finally, there were no differences between oxytocin and placebo [18F]MPPF BP in patients. Moreover, free peripheral serotonin level in patients did not increase after oxytocin while it did in controls. Our findings suggest a functional disturbance of serotonin through oxytocin stimulation in autism. This may limit the potential benefits of oxytocin in these patients and open the ways to investigate combined oxytocin-serotonin treatment.

II.2.a. Introduction

Recent psychiatric drugs (e.g., atypical antipsychotics, non-benzodiazepine anxiolytic) are agonizing or antagonizing several neurotransmission systems. Yet, little is known on how mutual regulatory actions occur between neurotransmitters in the normal and in the pathological human brain (Baskerville and Douglas, 2010). For instance, take the case of the oxytocinergic system and its relation to Autism Spectrum Disorders (ASD). Oxytocin (OT), a neurohormone produced in the hypothalamus (Ludwig and Leng, 2006) and critical for social behaviour (McCall and Singer, 2012), has gain lot of attention since the first evidence of OT-induced improvements of ASD patients' social skills (Andari et al. 2010; Guastella et al. 2010;Watanabe et al. 2015; Yatawara et al. 2015; Lefevre and Sirigu 2016). In spite of such promising beginning, designing an effective OT therapy is challenging because OT effects although significant, remain modest. The reason may lie on the fact that patients' oxytocinergic system is dysfunctioning (LoParo and Waldman, 2014) and as a consequence other neurotransmission paths may not be stimulated.

In accordance with this hypothesis recent animal results showed that OT action is at the root of a series of neurochemical events which represent important mechanisms for the reinforcement of social behaviours. For instance, OT, that can slowly diffuse as a hormone or be liberated in a timely manner from axons (Knobloch and Grinevich, 2014), will activate GABAergic neurons (Knobloch et al., 2012; Marlin et al., 2015), dopaminergic nuclei (Young and Wang, 2004), Corticotropin-Releasing Factor (CRF) neurons (Bosch et al., 2015; Dabrowska et al., 2011) and serotonin terminals (Dölen et al., 2013). From a fundamental and from a clinical perspective, it is thus relevant to investigate OT neurochemical interactions, in order to evaluate what is preserved and what is dysfunctioning in ASD patients. Amongst these various pathways, the OT-serotonin interaction is of interest because both are involved in the control of social behaviour (Crockett, 2009; Harmer et al., 2003) and because of the well described hyperserotonaemia in ASD patients (Chugani et al., 1999). The tight link between OT and serotonin (5-HT) is now widely acknowledged. Oxytocin receptors are located on serotoninergic cells (Pagani et al., 2015; Yoshida et al., 2009) and as shown in mice, social reward is supported by an oxytocin-induced release of 5-HT in the nucleus accumbens (Dölen et al., 2013). In line with these results, we recently showed for the first time in humans that oxytocin modulates 5-HT neurotransmission (Mottolese et al., 2014). We found, after intranasal oxytocin administration, an increase of [^{18}F]MPPF non displaceable binding potential (BP_{ND}), suggesting the upregulation of serotonin 1A receptor (5-HT_{1A}R) in several key brain areas such as the amygdala, the insula

and orbitofrontal cortex, important for the control of social behaviour. Whether OT can guide 5-HT signalling in the brain of autistic patients is however currently unknown.

According to the existing literature, we can hypothesize that this interaction is likely to be altered. 5-HT system has been suspected to be disrupted in ASD patients (Chugani, 2002). For instance, decreased level of serotonin transporter and serotonin 2 A receptor have been consistently observed in PET scan studies (Zürcher et al., 2015). There are no *in vivo* data yet on 5-HT_{1A}R in human patients. Only two post mortem studies suggested either no differences on the amount of 5-HT_{1A}R in the hippocampus (Blatt et al., 2001) or decreases of 5-HT_{1A}R concentrations in cingulated cortex and in the fusiform gyrus (Oblak et al., 2013). Importantly, we know from autistic mice models (BTBR and SERT ala56) that 5-HT_{1A} receptors, despite showing normal distribution and quantity, display a functional oversensitivity, indicating an altered serotonergic state (Gould et al., 2011; Veenstra-VanderWeele et al., 2012). Finally, it has recently been proposed that the broken interplay between OXT and 5-HT might be partially responsible for the autistic symptomatology (Dölen, 2015b; Yang et al., 2014). Therefore, we asked if *in vivo* 5-HT_{1A}R distribution is normal in ASD patients and if oxytocin could modulate 5-HT_{1A}R activity in the same way that we already observed in healthy subjects.

To answer these questions, we used our previous protocol, which successfully showed an oxytocin-serotonin interaction in healthy subjects (Mottolese et al., 2014). We performed a randomized double blind experiment in which 18 male patients with high functioning autism or Asperger syndrome received either intra nasal oxytocin or placebo. To assess the effects of oxytocin on the serotoninergic system, we used PET scan to measure the binding potential of the 2′-methoxyphenyl-(N-2′-pyridinyl)-p-[^{18}F]fluoro-benzamidoethylpiperazine ([^{18}F]MPPF) radioligand, a selective antagonist of 5-HT_{1A}R. The 5-HT 1A receptor is one of the most widespread serotonin receptor across the brain and therefore gives a good picture of serotoninergic system status (Hamon et al., 1990). Each patient underwent two scans, one under baseline and another one under spray condition. For each condition we computed a map of the [^{18}F]MPPF BP_{ND}. Two analyses were performed, first, we looked for the basal differences in localization or quantity between healthy subjects and patients' serotonin 1A receptors, and second, we evaluated the effects of OXT spray administration on 5-HT in autistic brains. In addition, using High Performance Liquid Chromatography (HPLC), we measured blood serum free serotonin concentration change following administration of spray, and correlated this peripheral

measure with levels of [18F]MPPF BP_{ND} in the Dorsal Raphe Nucleus as a proxy of brain serotonin level.

II.2.b. Methods

Participants

Healthy participants (referred to as the "HC group") of this experiment were the same as in our previous publication (Mottolese 2014).

Nineteen autistic patients (referred to as the "ASD group") were tested identically to the HC group (mean age: 34.3 ±7.6 y). They all had a clinical diagnosis of Asperger syndrome (AS) (n = 12) or high-functioning autism (HFA) (n = 7) according to Diagnostic and Statistical Manual-Revision 4 (DSM-IV R) (American Psychiatric Association, 2000). Patients were recruited from the expert centres (Foundation FondaMental), Chenevier-Mondor Hospital in Créteil (n = 11), France, as well as by Dr Marie-Maude Geoffray, from hospital St Jean de Dieu (n = 8). One patient was rejected after the first scan because he could not stay still. Patients received verbal and performance IQ tests (WAIS-III), (mean IQ = 100.01, range 72-120). Patients were medication-free for at least 2 weeks before and throughout the study. The Autism Diagnosis Interview scores (mean ±sd) were 14.2 ±5.7 for social interaction, 8.4 ±3.2 for language and communication and 3.8 ±2.2 for restricted and repetitive behaviours.

Because food intake can influence serotonin synthesis, participants abstained from food and drink (other than water) for 2 hours before the beginning of the experiment. Subjects were also free from physical exercise, sexual activity, caffeine, tobacco, Coca-Cola, tea, alcohol, chocolate, banana, dry fruits intake for 24 hours preceding the exam. If those conditions were not fulfilled, the subject was excluded from the study.

All subjects gave written informed consent, and were told of their rights to discontinue participation at any time. The study received the agreement of the ethical committee for biomedical research / (Comité de Protection des Personnes SUD EST IV n° 10/040- 2010-019922-15, AFSSAPS: A100727-77).

Protocol

Each participant underwent two PET sessions separated by one week, one in baseline condition, and one with a spray administration. Each scan started at 12:30PM and lasted 60

minutes. Subjects were instructed to lie down and rest without sleeping (the experimenter checked regularly that participants maintained their eyes open).

Baseline session: Subjects arrived at the imaging centre (CERMEP) at 11:50AM. The intravenous catheter, necessary for the injection of the radioligand was placed in a vein of the left forearm at 12h00 PM.

Spray session: Subjects arrived at 11:00AM. The intravenous catheter was placed at 11:10AM. Participants were randomly assigned to the OT or placebo group (9 patients per group), and received 24 IU of OT (Syntocinon Spray; Novartis; three puffs per nostril, with each puff containing 4 IU OT) or a placebo at 11:50AM.

Blood Sampling

During the spray session, five millilitres of blood were sampled twice, and put into dry tube. A first blood sample was collected from the catheter at 11:30AM, before spray administration to serve as a baseline. Then a second blood sample was collected at 12:20PM, 35 minutes after spray administration to assess the drug's effect on serotonin blood concentration.

During the baseline session, one blood sample was also taken at 12:20PM.

Each tube was wrapped into aluminium to protect it from light and brought to NeurobioTec Centre in Lyon Neurological Hospital, which conducted centrifugation and storage of our samples. Blood were centrifuged during 10 minutes at 2000 x g at 4°C, and 2 millilitres of serum were extracted and stored in a freezer at -80°C until assay within an hour after sampling.

Due to technical issue, two controls and two patients could not have one of their sample assessed and were therefore left out from the analysis (one in each drug condition).

Serum serotonin concentration

The HPLC system was an Agilent 1200 with an Alltima Alltech column (150mm). Serotonin was extracted from alkalinized serum in solid phase (C18, Bond Elut, Agilent).

To quantify serotonin concentration, we used inverse phase HPLC. 20 µL of sample were injected on the C18 column, the mobile phase was composed of methanol and water (90% water, 10% methanol). We controlled the procedure by adding an internal standard (5 hydroxymethyltryptamine) to each sample, the CV was found to be <5%.

The quantification of serotonin was made accordingly to a standard curve (0 to 7.5µmol/L).

Anatomical MRI

Subjects underwent an anatomical MRI, performed after one of the two PET-scan exams in a random order across subjects. This structural brain MRI was performed at the CERMEP centre using a 1.5-T Magnetom scanner (Siemens AG) and consisted in a 3 dimensional millimetre anatomic T1-weighted sequence (T1-MRI) covering the whole brain volume (176 slices).

PET-scan acquisition

The radioligand was the 2'-Methoxyphenyl-(N-2'-pyridinyl)-p-18F-fluoro-benzamidoethylpiperazine ([^{18}F]MPPF), obtained by nucleophilic fluoration of a nitro precursor (Le Bars et al., 1998), with a radiochemical yield of 20% - 25 % at the end of the synthesis and a mean specific activity of 140.5 GBq/µmol (range: 42-240).

PET scans were acquired on a Biograph mCT PET/CT tomograph (Siemens). Measures for tissues and head support attenuation were performed with a 1-min low dose CT scan acquired before emission data acquisition. A bolus of [^{18}F]MPPF at 2.7 MBq/kg was injected (mean injected dose, 194 MBq (range: 131-277) for controls and 186 MBq (range: 123-237) for patients). Acquisition mode, dynamic framing of the 60-minutes PET scan and reconstruction parameters were identical to our previous work with OT challenge (Mottolese et al., 2014).

Data processing and ROI definition

For each subject, the T1-MRI image was anatomically segmented into 83 labelled structures using multi-atlas propagation with enhanced registration method (Heckemann et al., 2010). Anatomical T1-MRI were co-registered, with mutual information criteria, to the PET summed image using Statistical Parametric Mapping 8 (SPM8) software (Wellcome Trust Centre of Neuroimaging). The individual 83 structures labelled images were also resampled with nearest neighbour interpolation in the individual PET acquisition space.

A set of regions for the ROI analysis were selected (the amygdala, the hippocampi, the para-hippocampal gyri, the insula, the anterior/medial cingulate area, the orbitofrontal cortex, the subgenual cortex and the dorsal raphe nucleus (DRN), because of their high

concentration of 5-HT_{1A}R, and cerebellar white matter for subsequent analysis (based on MAPER segmentation of white and grey matter). These values were obtained by averaging individuals segmented atlas in the normalized space with the MaxProb method. These ROIs were used for subsequent regional BP_{ND} analysis and to define an inclusive mask for SPM analyses. The DRN was defined on the basis of the PET functional data (Mottolese et al., 2014).

Modelling of [^{18}F]MPPF

Parametric images of Non-Displaceable Binding Potential (BPND) were generated using a three-compartment simplified reference tissue model (SRTM) (Gunn et al., 1998), with cerebellar white matter taken as the reference region (Mottolese et al., 2014).

Deformation field from subject's space to MNI space was determined from the T1-MR image using the "New Segment" function of SPM8, and then applied to the BP_{ND} images. Spatially normalized images were then smoothed using an isotropic Gaussian kernel of 8 mm in full width at half maximum.

Statistical analysis

SPM analysis: To compare Healthy Subjects (HC) and autistic patients (ASD), between groups (HC and ASD) comparison of MPPF BP_{ND} spatially normalized images were performed with a two sample T-test in the voxels comprised in our inclusive mask with SPM8, with a threshold of $p < 0.01$ (uncorrected for multiple comparisons, cluster-forming threshold at voxel-level,) and $pFWE < 0.05$ (Family wised error corrected at the cluster level). We also compared mean MPPF BP_{ND} from our ROIs for each group with two sample Student tests in STATISTICA 8.

To test oxytocin effects in ASD patients, a voxel-based SPM analysis was performed, by using a flexible factorial design with the factors condition (basal x spray) and treatment (oxytocin x placebo), to assess the effect of OT administration or placebo on MPPF BP_{ND} compared with the basal state. SPM of Student *t*-score (SPM-{t}) maps resulting from the contrasts (OT spray – OT basal) and (Placebo spray – Placebo basal) were thresholded at $P < 0.01$ uncorrected for multiple comparisons, similarly to (Mottolese et al., 2014). This analysis was restricted to voxels belonging to our ROI set (amygdala, hippocampus, parahippocampus, insula, anterior/medial cingulate area, orbitofrontal cortex, and DRN; inclusive mask).

ROI analysis: Mean regional MPPF BP_{ND} were extracted with MarsBar toolbox from the regions where oxytocin was shown to have an effect in healthy controls (right amygdala-hippocampus-para-hippocampus complex, right insula, bilateral orbito-frontal cortex, and DRN).

We first compared MPPF BP_{ND} from these regions between HC and ASD patients at basal state, with two sample t tests (STATISTICA 8).

Then, to investigate our drug treatment effects, we submitted MPPF BP_{ND} values to a between-groups (OT × placebo) and within-subjects (basal × spray) ANOVA. Post hoc statistics tested, by region and by treatment (OT or Placebo, if the regional variations of MPPF BP_{ND} between the basal and the spray condition were significantly different from zero (one-sample *t* test performed on the relative difference).

Serotonin concentrations: Spearman's non parametrical correlations between serum free serotonin concentration at basal state and dorsal raphe nucleus MPPF BP_{ND} values were performed.

Because of small sample size and non-Gaussian distribution of serum free serotonin concentration, we used non parametric statistical tests (Wilcoxon signed-rank test, STATISTICA 8) to analyse the effect of drug (placebo or oxytocin) on serum free serotonin concentration before and after spray administration. The threshold used to define statistical significance was corrected for multiple comparisons with Bonferroni's correction for 4 comparisons.

II.2.c. Results

MPPF BP_{ND} of ASD patients compared to healthy controls

At the voxel level, there were no differences between ASD patients and HC at baseline (SPM two sample t test, $p>0.05$). Mean regional MPPF BP_{ND} values from our ROI analysis did not showed differences between groups (Two sample t test, $p>0.05$) (Figure 17 B). The same analysis between controls and patients under placebo did not yield to significant differences.

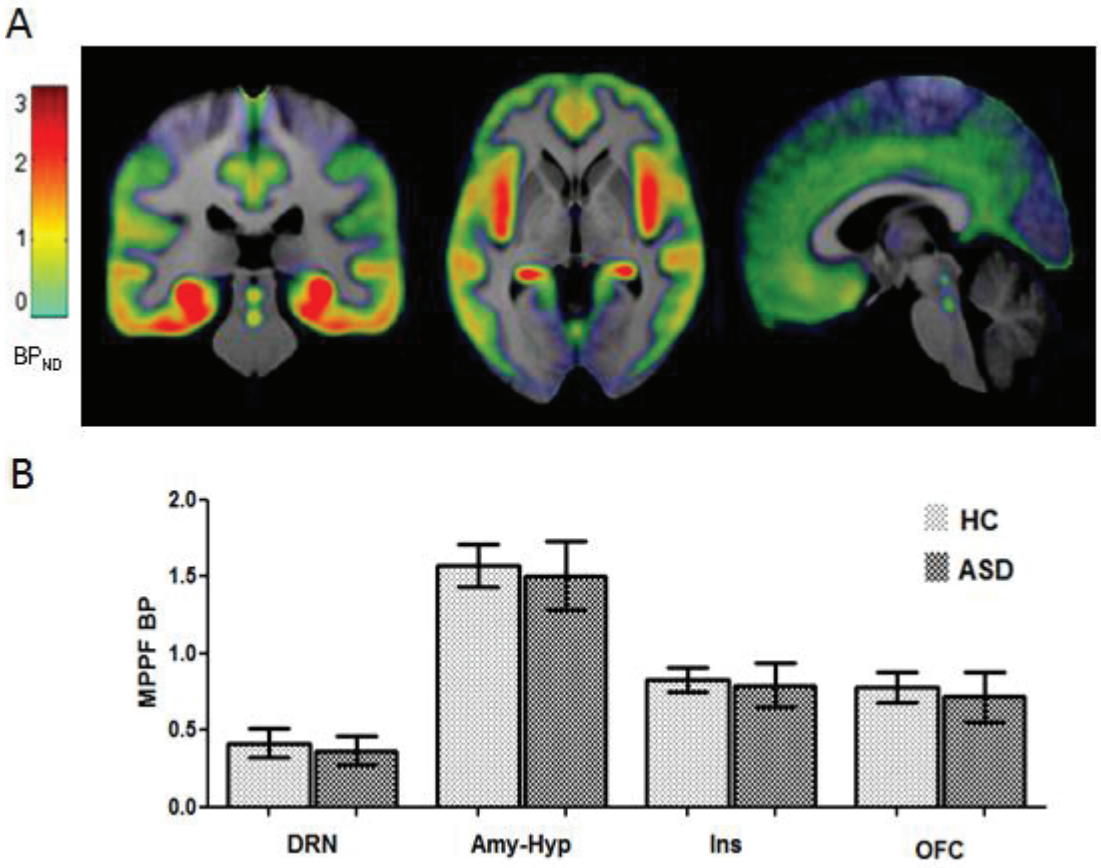


Figure 17. (A) Mean image of patients (n=18) MPPF Binding Potential (BP) at basal state, colour bar = MPPF BP_{ND} value, PET scan functional image is projected on the normalized mean brain of the patients' group. (B) Bar plot of controls and patients BP values (basal state) from each ROI. No statistical differences were found. Error bars indicate S.E.M.

Spray effect on MPPF BP_{ND} of ASD patients

No differences were found at basal state between regional MPPF BP_{ND} of the two groups of ASD patients (oxytocin/placebo; $P > 0.05$, two-sample t test).

In ASD patients, the SPM contrast (OT spray – basal OT) did not show significant differences (all p>0.05) (Figure 18). As in healthy the subjects, the SPM contrast (basal OT – OT spray) did not yielded to any significant differences in ASD patients (all p>0.05).

Moreover, in our ROI analysis, the 2x2 mixed ANOVA across all regions (OT/Pla, basal/spray) did not reached significance (p>0.05), and even direct post hoc comparisons in each ROI failed to reveal an effect of the oxytocin spray in patients (one sample t test vs 0, p>0.05).

The placebo spray did not exert any effects on any of our analyses.

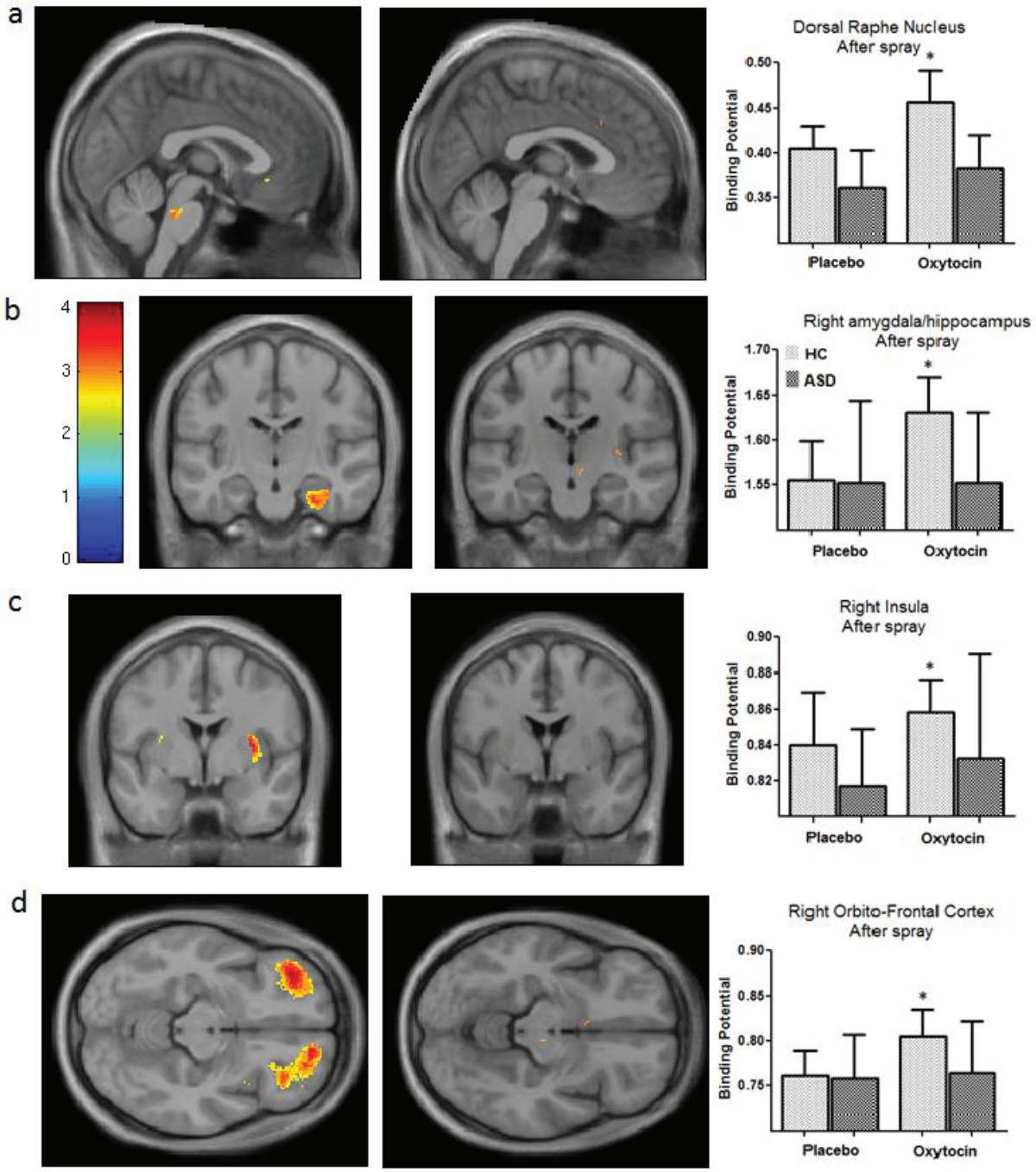


Figure 18. T-map SPM analysis ($P < 0.01$, uncorrected) showing the effect of OXT administration on MPPF BP_{ND} compared with the basal state in (healthy subjects) (left) ((a) DRN, (b) amygdala, (c) insula and (d) OFC) and in ASD patients (middle, no effects). (Right) Binding potential values for each group (light gray = controls, dark gray = patients) in each condition in the DRN, the right amygdalo-hippocampus complex, the right insula and the right OFC in which the spray effect was present in healthy controls. No differences were found under the placebo condition between controls and patients. * indicates a significant difference of the oxytocin induced MPPF BP_{ND} variation (one sample t test $p<0.05$). T-map are projected on the anatomical mean image of each group.

Oxytocin effects on serum free serotonin concentration

Oxytocin spray administration significantly increased serum free serotonin concentration from 0.46 µmol/L before spray to 0.56 µmol/L after (Wilcoxon signed-rank test: Z = 2.67, p corrected = 0.031) in healthy controls. In contrast, placebo did not have any effect on serum free serotonin concentration (Z = 0.44, p > 0.5).

Furthermore, neither oxytocin nor placebo had an impact on serum free serotonin concentration in ASD patients (Z = 0.56, p > 0.5; Z = 0.14, p > 0.5; respectively) (see Figure 19).

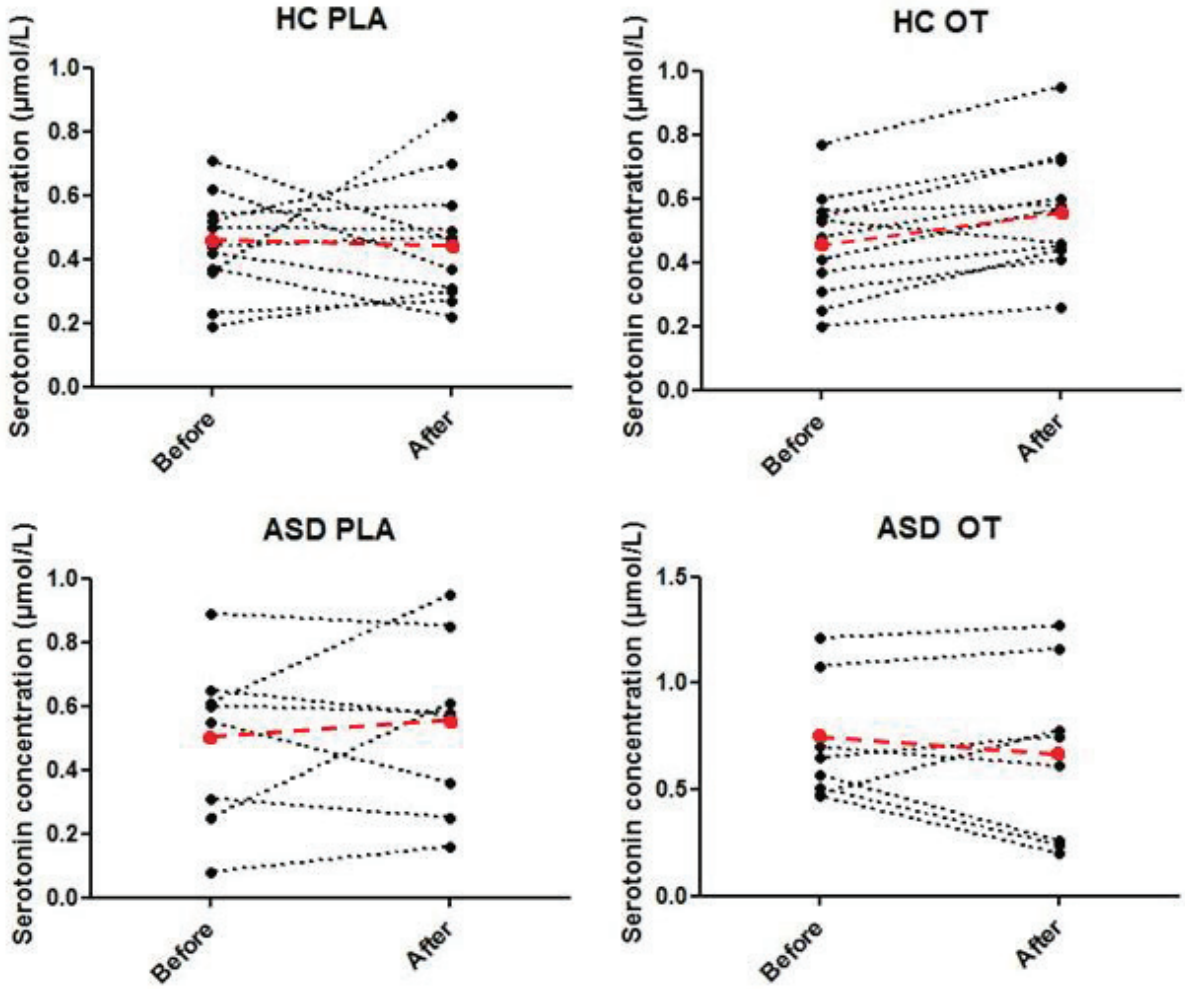


Figure 19. Serum free serotonin concentration before and after spray for each group for each treatment and group, black dotted lines represent individuals. Red dots indicate the mean, red dotted line shows the mean variation.

Serum free serotonin concentration predicts Dorsal Raphe Nucleus MPPF BP_{ND} in both HC and ASD group

We found significant positive correlations between Dorsal Raphe Nucleus (DRN) MPPF BP_{ND} and serum free serotonin concentration during baseline session in both HC and ASD groups (Spearman's correlation: rho = 0.44, p = 0.04, rho = 0.61, p = 0.01, respectively) (see Figure 20). It should be noted that, coherently with these results, oxytocin administration increased MPPF BP_{ND} in the DRN of healthy subjects but not ASD patients.

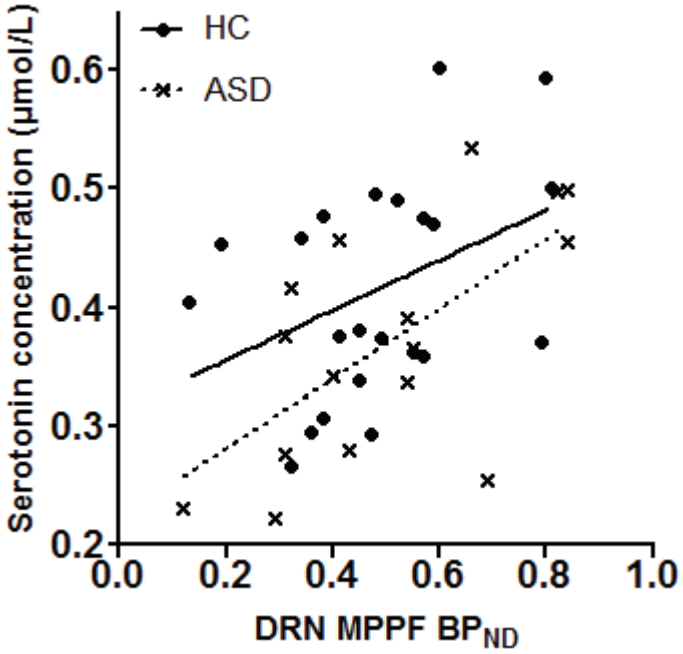


Figure 20. Correlation between serum free serotonin concentration and Dorsal Raphe Nucleus (DRN) MPPF Binding Potential (MPPF BP_{ND}).

II.2.d. Discussion

The present study brings us two main results. First, we compared serotonin 1A receptor (5-HT_{1A}R) in vivo distribution between ASD patients and healthy subjects, without finding any differences; and second, we found that oxytocin administration to ASD patients fails to modulate their serotoninergic neurotransmission, neither on binding potential nor on perfusion rate. These findings are corroborated by our analysis of serum free serotonin, which was increased after oxytocin administration in healthy subjects but not in ASD patients. Taken together, these data show that the 5-HT_{1A}R system in ASD patients is seemingly normal in terms of quantity and distribution, compared to control, but the

absence of oxytocin's effects at both central and peripheral level suggests that it might be functionally altered.

The molecular origins of this dysfunction are rather hard to infer from whole brain imaging data. We know that some autistic mice models have hypersensitive 5-HT_{1A}R, showing greater effects than controls mice after agonistic stimulation, such as hypothermia or dorsal raphe neurons inhibition (Gould et al., 2011; Veenstra-VanderWeele et al., 2012). If that was the case in the patients we tested, we should have observed an increase of [18F]MPPF BP_{ND} even greater than in controls, accounting for the hypersensitivity of the 5-HT_{1A}R. Because we do not find such result, two alternative hypotheses can be proposed. Firstly, this 5-HT_{1A}R oversensitivity may not be present in human ASD patients. This would be in accordance with the fact that some patients are relieved when treated with Buspirone, a partial 5-HT_{1A}R agonist (Vasa et al., 2014). Secondly, it is possible that oxytocin fails to provoke the release of serotonin in ASD patients. To answer this question, we would need to know if the observed modification of 5-HT_{1A}R is provoked by in response to a modification of serotoninergic tone, or by a direct action of oxytocin on this receptor (for instance, *via* heteromerization (Romero-Fernandez et al., 2012)). Although only a rather weak link has been established between oxytocin receptor and ASD pathology (LoParo and Waldman, 2014), we can speculate that while the oxytocin-serotonin interaction is impaired, other oxytocin pathways are intact. This could explain why oxytocin administration in ASD patients still produces positive effects, without fully restoring a normal behaviour. Indeed, recent experiments in non-human primates (Freeman et al., 2014; Freeman et al., 2014) suggest that, as in rodents (Marlin et al., 2015), oxytocin modulates how mammals perceive social stimuli by influencing the activity of neurons located in sensory cortices (Grinevich et al., 2015). Thus, while oxytocin could fail to increase social motivation in patients, it could still exert some actions on social perception. Further experiments on animal models of ASD pathology are highly needed to understand the molecular cascade leading to the effect (or its absence) observed here.

The consequences on ASD patients' behaviour of the altered oxytocin-serotonin interaction are not clear. In animals, it was found necessary for social reward (Dölen et al., 2013), and we know from patients studies that social reward is impaired in ASD (Assaf et al., 2013; Cox et al., 2015). It is thus possible to hypothesize that oxytocin-serotonin interaction is disrupted in ASD patients (at least in some of them), disturbing social reward processing (Dölen, 2015b). Although [18F]MPPF does not allow us to look at the striatum (because of the low concentration of 5-HT_{1A}R in this region), other regions are involved in

the processing reward. For instance, we know that serotonin in the amygdala plays a role in reward signalling (Rygula et al., 2015), thus it is not excluded that oxytocin effects observed in healthy subjects are related to social reward sensitivity. Ultimately, it is now needed to perform behavioural experiments testing at the effect of oxytocin on social reward system in ASD patients, (such as, for instance, (Groppe et al., 2013; Scheele et al., 2013)).

Our study is of importance for pharmaceutical research. Indeed, oxytocin is currently used in multiple clinical trial (Lefevre and Sirigu, 2016), but in a parallel manner, serotoninergic drugs are used in some ASD patients. We suggest that targeting oxytocin and serotonin system simultaneously could potentiate the effects of each drug. At the same time, understanding why the oxytocin-serotonin pathway is disrupted in ASD patients could hopefully lead to mechanism-based drug design.

II.3. Serotonin modulates grey matter volume differently in ASD and HC

The previous study indicated us that the 5-HT_{1A}R is present in normal concentrations and distributions but that it might not be functioning correctly in patient with autism. This is corroborated by the existing animal literature using mouse models of autism (See II.3.a.). Thus in order to go further, I wanted to test another functional aspect of the 5-HT_{1A}R. It has been recently shown that this receptor was correlated with the amount of grey matter in the brain of healthy controls (Kraus et al., 2012). Hence, I used the data from our healthy subjects (Mottolese et al., 2014) to verify the existence of this correlation, and the data from the current study to investigate this relation between 5-HT_{1A}R and grey matter volume in the brain of patients with autism. This section is a paper that will soon be submitted to *Neuroimage*.

5-HT1A-R shapes social personality by sculpting the brain in healthy subjects but not patients with autism

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Abstract:

Serotonin is an important neuromodulator that is notably acting on social behaviour. This system is thought to be altered in patients with autism, a pathology characterized by sociality deficit. Amongst the many functions of central serotonin, there is evidence that it regulates grey matter volume (GMV), and jointly with this fact, patients with autism have been regularly found to have abnormal GMV in various brain regions. Thus we hypothesized that serotonin effect on GMV was linked to social personality in healthy subject but that this relation might be disrupted in patients with autism.

In the present study, we combined anatomical MRI, PET scan with MPPF and NEO PI-R personality questionnaire to investigate the role of serotonin 1a receptor (5-HT_{1A}R) the regulation of GMV and social personality in 24 healthy men controls and 18 male patients with autism.

We found several positive associations between 5-HT_{1A}R and GMV in the neocortex and hippocampi of both healthy subjects and patients with autism. Moreover, we found a bilateral negative correlation between 5-HT_{1A}R and GMV in the posterior putamen of the control group. Moreover, both the 5-HT_{1A}R signal and the GMV in this region was associated with social personality. Critically, these associations were absent in patients with autism, although the amount of receptor was similar to the control group.

This indicates that 5-HT_{1A}R exerts both positive and negative actions on GMV, in a region dependant manner, and that in the putamen, this effect is linked to social personality. However, in patients with autism, our results point to a dysfunction of 5-HT_{1A}R in the striatum of ASD patients.

II.3.a. Introduction

Serotonin is a molecule that has been linked to a wide range of behaviours. Notably, this monoamine neurotransmitter is involved in social behaviour (Crockett, 2009; Harmer et al., 2003), especially in terms of approach and avoidance regulation (Tops et al., 2009). Thus, serotonin has been shown to modulate aggressiveness (Olivier, 2004) and affiliation (Insel and Winslow, 1998) in many different species including humans. Not surprisingly, serotonin deregulation has consequently been suspected to be a potential cause of Autism Spectrum Disorders (ASD) (Chugani et al., 1999). Indeed, serotonin manipulations modulate behaviour in patients (Cook and Leventhal, 1996) and genetic variations of serotonin genes have been associated with autism (Anderson et al., 2009; Huang and Santangelo, 2008; Nyffeler et al., 2014). However, it is still unclear how exactly is the brain serotonergic system is impaired in ASD. This arises from the complexity of the central nervous system and the serotoninergic neurotransmission. Several key points must be considered.

Firstly, because of the numerous serotonin receptors, and other molecules involved in its physiology (e.g., transporter, precursor), we do not know what part(s) of this system is deficient. Among those various actors, the serotonin 1A receptor (5-HT_{1A}R) plays a key role in social behaviours, namely, it has been shown that 5-HT_{1A}R stimulation increases adjacent lying in rats (Thompson et al., 2007) and reduces aggression (Bell and Hobson, 1994; Joppa et al., 1997). Thus, this receptor is an important target for various mental pathologies (Albert et al., 2014). Additionally, studies on autistic animal models have suggested that this receptor could be deregulated in such condition (Gould et al., 2011; Veenstra-VanderWeele et al., 2012).

Secondly, how is 5-HT_{1A}R deregulated is linked to its normal physiological role. 5-HT_{1A}R is a metabotropic receptor that can be coupled to various G protein depending on the brain region (Mannoury la Cour et al., 2006). It has an important self inhibitory action in the raphe nuclei neurons, but less is known about its functions elsewhere in the brain (Hamon et al., 1990). Interestingly, one way through which this receptor could exert effects on social behaviour would be through the modulation of brain morphology, because 5-HT_{1A}R can positively or negatively influences cellular growth factor in a region dependent manner (Azmitia, 2001; Cowen, 2007; Mannoury la Cour et al., 2006). These results from the animal literature have been confirmed in humans as well by a PET scan study which has found associations between 5-HT_{1A}R and grey matter volume (Kraus et al., 2012). Yet, it is unknown if this 5-HT_{1A}R function is relevant to social processing, as the observed deregulations were not put in relation to social outcomes.

Finally, we have recently found that while the 5-HT_{1A}R is normally distributed and in normal concentrations in the brain of patients with autism, it is however not responding to oxytocin, as it does in healthy subjects (Lefevre et al., submitted). Therefore, we can make the hypothesis that 5-HT_{1A}R functioning is impaired in human ASD patients. Hence, because of the role of 5-HT_{1A}R on social behaviour and its impact on neural plasticity, we hypothesized that this function should be linked to sociality in healthy humans but could be impaired in ASD patients.

Thus, to investigate these associations, we used anatomical MRI, PET scan with MPPF radioligand and NEO PI-R questionnaire of personality to assess, respectively, grey matter volume, 5-HT_{1A}R density and social traits. A group of 24 healthy men and 18 ASD patients participated the study.

II.3.b. Methods

Data used in this experiment originate from two previous studies (Mottolese et al., 2014) and (Lefevre et al., *submitted*). All analyses were all performed on basal scans in which subjects had no tasks to do and received no pharmacological treatment.

Participants

Healthy participants (referred to as the "HC group") of this experiment were the same than in our previous publication (Mottolese et 2014). A total of 24 healthy males participated in this study (mean age: 26.3 ± 6.3 y). Subjects affected by chronic diseases or mental disorders, under pharmacological medication or with a history of smoke, drugs or alcohol abuse were excluded. All these criteria were evaluated during the medical exam, before the beginning of the experiment. Eighteen autistic patients (referred to as the "ASD group") were tested identically to the control group (mean age: 34.3 ±7.6 y). They all had a clinical diagnosis of Asperger syndrome (AS) (n = 12) or high-functioning autism (HFA) (n = 6) according to Diagnostic and Statistical Manual-Revision 4 (DSM-IV R) (American Psychiatric Association, 2000) and ASDI (Asperger Syndrome Diagnostic Interview) (Gillberg et al 2001) were recruited from the expert centres (Foundation FondaMental), Chenevier-Mondor Hospital in Créteil (n = 11), as well as by Dr Marie-Maude Geoffray, from hospital St Jean de Dieu (n = 7). Patients received verbal and performance IQ tests (WAIS-III) and all showed average to above average estimates of intelligence (mean IQ = 100.01, range 72-120). Patients were medication-free for at least 2 weeks before and throughout the study.

Because specific foods can influence serotonin synthesis, participants abstained from food and drink (other than water) for 2 hours before the beginning of the experiment and from exercise, sexual relationship, caffeine, tobacco, Coca-Cola, tea, alcohol, chocolate, banana, dry fruits during the 24 hours preceding the exam. If those conditions were not followed, the subject was excluded from the study.

All subjects gave written, informed consent and were told of their rights to discontinue participation at any time. The study received the agreement of the ethical committee for biomedical research / (Comité de Protection des Personnes SUD EST IV n° 10/040- 2010-019922-15, AFSSAPS: A100727-77).

Behavioural assessment

All subjects filled the NEO PI-R (Costa and McCrae, 1995) which assesses 5 core personality dimensions: "extraversion" (tendency to enjoy human interactions, enjoy time spent with people, and find less reward in time spent alone), "Neuroticism" (tendency to experience negative emotions, emotional instability), "Openness" (active imagination, aesthetic sensitivity, and intellectual curiosity), "Agreeableness" (tendency to be compassionate and cooperative), and "Conscientiousness" (tendency to show self-discipline, act dutifully, and aim for achievement). It is composed of 240 affirmations to which the subject has to answer on a scale from 0 (completely disagree) to 4 (completely agree). Each of the 5 dimensions are subdivided in 6 facets.

Anatomical MRI

Participants were scanned using a 1.5-T magnetic resonance imaging scanner (Siemens Magnetom Sonata) located at the nearby Imagery Center (CERMEP Lyon). Images were acquired using a sagittal 3-dimensional T1-weighted MPRAGE sequence covering the whole brain volume with 1 mm cubic voxel size (field of view 256 mm, matrix 256 × 256, repetition time/echo time/flip angle 1970 ms/3.93 ms/20°, slice thickness 1 mm).

MRI processing

The anatomical images were processed using SPM8 (Wellcome Trust Centre for Neuroimaging, London http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) and the voxel-based morphometry toolbox version 8 (VBM8;http://dbm.neuro.uni-jena.de/vbm/). The native T1 images were segmented into grey matter, white matter and cerebrospinal fluid tissue classifications using the adaptive Maximum A Posteriori technique employed in the

VBM8 toolbox (Rajapakse et al., 1997). Partial volume of the mixed grey matter-white matter and grey matter-cerebral spinal classes was estimated (Tohka et al., 2004) and a spatially adaptive non-local means denoising filter was applied to the data (Manjón et al., 2010). Then, the DARTEL (Diffeomorphic Anatomical Registration using Exponentiated Lie algebra) algorithm (Ashburner, 2007) was used to determine the nonlinear deformations for warping all the grey and white matter images so that they match each other and to produce a customized average template data, to which the data were iteratively aligned. Finally, gray matter volume (GMV) maps (Ashburner and Friston, 2009), spatially normalised in the standard Montreal Neurologic Institut (MNI) space at a voxel size of 1.5x1.5x1.5 mm3, were generated using the deformations estimated in the previous step. To correct for nonlinear spatial normalization, images were modulated by multiplication with the Jacobian determinants of the deformation fields in order to preserve the actual amount of gray matter within each structure before normalization. In a final step, images were smoothed with an 8-mm full-width at half-maximum Gaussian kernel. The obtained images contain a value of gray matter quantity for each voxel and are in the same MNI space, allowing GMV comparisons.

PET-scan acquisition

Subjects were conducted to the imagery centre (CERMEP) at 11:50AM, PET-scan session always began at 12:30PM. During the 60 minutes PET acquisition subjects were laying at rest in the machine. They were quietly installed in the machine and then the intravenous catheter, necessary for the injection of the radioligand was placed in a vein of the left forearm at around 12h00 PM.

The radioligand was 18F-MPPF PET: 2'-Methoxyphenyl-(N-2'-pyridinyl)-p-18F-fluoro-benzamidoethylpiperazine ([18F]MPPF) which was obtained by nucleophilic fluoration of a nitro precursor with a radiochemical yield of 20% - 25 % at the end of the synthesis and a specific activity of 32-76 GBq/mmol (Le Bars et al., 1998).

PET scans were obtained on a Biograph mCT PET-CT tomograph (Siemens). Measures for tissues and head support attenuation were performed with a 1-min low dose CT scan acquired before emission data acquisition. A bolus of [18F]MPPF at 2,7 MBq/kg was injected through an intravenous catheter placed in a vein of the left forearm (mean injected dose, 192 MBq for controls and 184 MBq for patients). A dynamic emission scan was acquired in List-mode during 60 min post-injection. 35 frames images were reconstructed using 3D-OP-OSEM iterative algorithm incorporating PSF and Time of Flight (with a Gaussian filter of 3mm) after correction for scatter and attenuation (128x128 voxels in-plane (2.12mm²)

and 109 slices (2.03mm thickness). The resolution for the reconstructed images was about 2.6 mm in full width at half maximum in the axial direction and 3.1 mm in full width at half maximum in the transaxial direction for a source located at 1 cm from the field of view.

Pet-scan processing

PET images were co-registered, with mutual information criteria, to the T1-MR anatomical image using SPM8 software. Parametric images of Non-Displaceable Binding Potential (BPND) were generated using a three-compartment simplified reference tissue model (SRTM) (Gunn et al. 1998). In this model, the assessment of free and nonspecific ligands kinetics is based on the time-activity curve (TAC) of a reference region (i.e., cerebellar white matter) that is devoid of specific 5-HT1A receptor binding. Regional TACs were extracted using segmented images previously created from anatomical images using the MAPER method (Heckemann et al., 2010) based on a maximum probability brain atlas defining 83 regions (Gousias et al., 2008; Hammers et al., 2003). BPND images were then spatially normalized into the MNI space with SPM8 using the deformation fields from subject's space to MNI space previously computed from structural image using SPM8 and the VBM8 toolbox. Normalized images were smoothed using an isotropic Gaussian kernel of 8 mm in full width at half maximum.

Multimodal and statistical analysis

Each subjects' MRI and PET scan images were co registered together. We used SPM8 to apply the transformation matrix of the structural scans obtained during normalization to the PET images. As the structural scans were already normalized to standard MNI space, this step also brought the PET data to MNI space.

A voxel-by-voxel regression model between GMV and BPND maps, both previously normalized in the MNI space, was created using the Biological Parametric Mapping (BPM) toolbox for SPM8 (Casanova et al., 2007), which is designed to calculate voxel-by-voxel statistics for multiple imaging modalities. Thus, a multiple regression was calculated in each voxel with BPND as the independent variable and with GMV, brain total GMV and age as dependant and controlling variables. We used a level of statistical significance of $p < 0.001$ (cluster-forming threshold at voxel-level) and correction for multiple comparisons at cluster level with a threshold of FWE $p < 0.05$.

We extracted the mean and peak values of GMV and BP_{ND} for each significant cluster using Matlab.

Between group (HC and ASD) comparison were performed with standard T-test. Pearson's correlations between extraversion scores, GMV and BPND values (mean or peak of each clusters) were performed thanks to STATISTICA 8.

Correlations between BPND and Extraversion scale at the whole brain level were performed with SPM8. All SPM results are reported with a cluster forming threshold at voxel level of $p < 0.001$ uncorrected and a cluster level of $p < 0.05$ corrected with FWE.

II.3.c. Results

Serotonin 1a receptor density is positively correlated to grey matter volume in the hippocampus and the cortex of both HC and ASD

We first looked at positive correlations between 5-$HT_{1A}R$ density (MPPF Non Displaceable Binding Potential (BP_{ND})) and Grey Matter Volume (GMV) (measured with VBM) in healthy subjects and ASD patients and found similar regions than Kraus and colleagues (Kraus et al., 2012). For both groups, there were significant clusters in the hippocampus and intracalcarine cortex (see Table 3 and 4 and Figure 21 b, c, e and f). However, in the Healthy Controls (HC) group, there were also positive associations in the frontal and occipital cortex that could not be found in ASD patients (Figure 21 a and d, Table 3 and 4). Note that none of these regions were associated with personality traits (all $p > 0.1$).

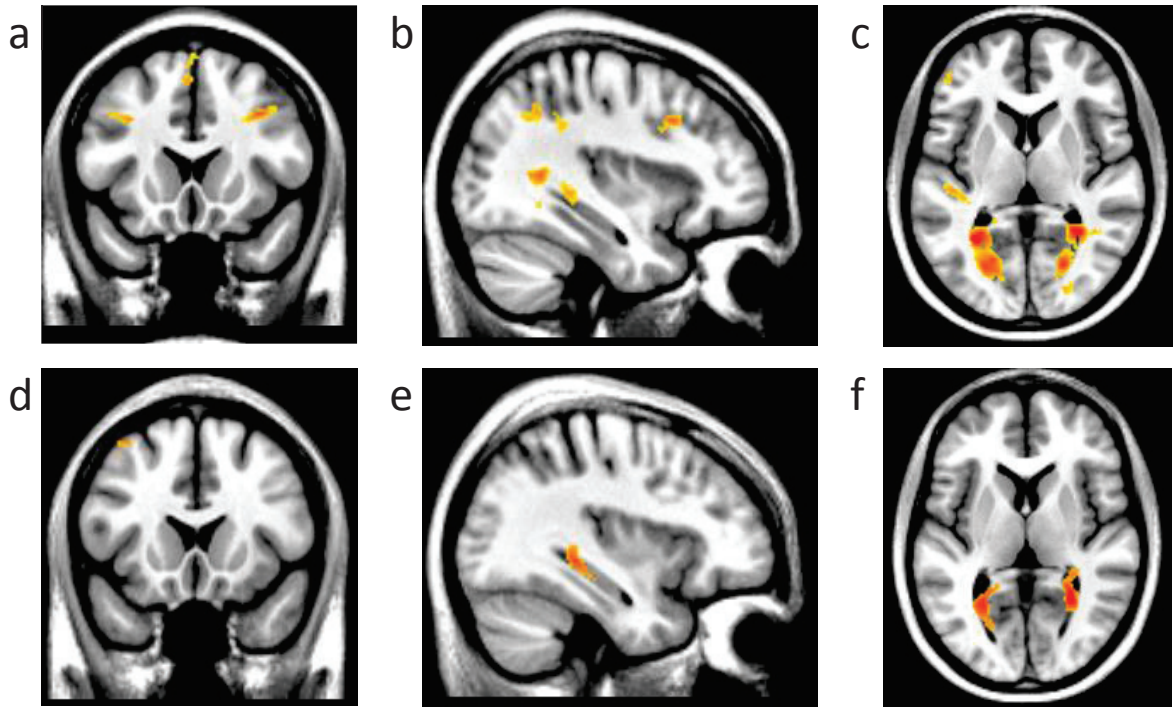

Figure 21. Positive correlations between MPPF BP_{ND} and GMV in HC (upper) and patients with ASD (lower). Significant clusters were found in the frontal cortex of HC but not ASD (a and d, y = 12), in the hippocampi for both groups (b and e, x = 33) and in the intracalcarine area (c and f, z = 7).

Region	Peak				Cluster		
	x	y	z	Z	p-FWE	Voxels	R^2
Negative correlations							
Left Posterior Putamen	-28	-7	-1	5.84	<0.001	289	0.66
Right Posterior Putamen	30	-13	3	5.03	<0.001	265	0.75
Left Lingual Gyrus	-22	-43	-17	4.18	<0.001	158	0.59
Positive correlations							
Right Hippocampus	34	-36	0	4.21	<0.001	133	
Superior frontal	2	5	69	5.03	<0.001	1007	
Left inferior orbitofrontal	-50	35	-3	4.89	<0.001	289	
Left middle frontal	-36	8	-28	5.49	<0.001	184	
Right middle frontal	34	15	36	5.04	<0.001	218	
Left intracalcarine	-30	-54	-7	6.79	<0.001	870	
Right intracalcarine	28	-49	6	6.07	<0.001	669	
Right superior parietal	27	-31	43	5.29	<0.001	151	
Left superior parietal	-38	-36	40	4.45	<0.001	182	
Left Medial Temporal	-44	-25	4	4.55	<0.001	107	
left superior occipital	-18	-76	36	4.83	<0.001	438	
Right superior occipital	21	-57	39	4.62	<0.001	463	
Right medial occipital	15	-90	12	4.86	<0.001	126	
Left medial occipital	-12	-87	15	4.37	<0.001	125	
Right inferior occipital	42	-66	-8	5.18	<0.001	441	

Table 3. Significant SPM clusters for positive and negative correlations between MPPF BP_{ND} and GMV in healthy subjects.

Region	Peak				Cluster		
	x	y	z	Z	p-FWE	Voxels	R^2
Negative correlations							
Left Posterior Putamen	-28	-7	0	3.62	0.10	29	0.29
Positive correlations							
Right intracalcarine	27	-52	6	5.27	<0.001	719	
Right hippocampus	33	-36	-5	4.61			
Left intracalcarine	-26	-57	6	4.63	<0.001	312	
Left hippocampus	-27	-43	-3	4.72	<0.001	424	
Left middle frontal	-45	26	28	4.02	<0.001	117	
Subcallosal cortex	-6	33	-8	3.84	<0.001	157	

Table 4. Significant SPM clusters for positive and negative correlations between MPPF BP_{ND} and GMV in patients with autism (for the hippocampus cluster, p value and voxel size is the same as intracalcarine because both regions were in the same big cluster).

Serotonin 1a receptor density is negatively correlated to posterior putamen size in healthy subjects but not in ASD patients

We computed a whole brain voxel-by-voxel parametric map of negative correlations between MPPF BP_{ND} and GMV. In healthy subjects, significant associations were found bilaterally in the posterior putamen (left: x = -28, y = -7, z = -1, k = 289, Z = 5.84 and right: x = 30, y = -13, z = 3, k = 265, Z = 5.03) and in the left lingual gyrus (x = -22, y = -43, z = -17, k =158, Z = 4.18) (See Figure 22 a, b, c, f, g and Table 3). This means that in these regions, the more 5-HT_{1A}R there are, the less grey matter there is.

We performed the same whole brain analysis on ASD patients, but none of the clusters resisted statistical correction (all p > 0.1 Figure 22 h, i and j, Table 4). We extracted mean and peak values of GMV and MPPF BP_{ND} of ASD patients' left and right putamen from the cluster found in healthy subjects and found negative correlations (left: r = -0.54; p = 0.02, right: r = -0.48; p = 0.04). These correlations were however significantly weaker compared to healthy subjects (right: p = 0.01, left: p = 0.07, Figure 22 f and g). Critically, this cannot be explained by sample size difference because the same analysis performed on 18 randomly selected healthy subjects led to similar results (significant bilateral clusters in healthy subjects after statistical correction) (analysis not shown).

To investigate further this difference, we performed Student's two sample t tests on left and right posterior putamen MPPF BP_{ND} and GMV values (ASD vs HC), which showed no differences between groups on MPPF BP_{ND} values (left: t = 0.30, p > 0.1, right: t = 0.72, p > 0.1) but significantly smaller posterior putamen volume in ASD patients (left: t = 3.17, p <

0.01, right: t = 2.87, p < 0.01) (Figure 22 d and e). Moreover, this difference of posterior putamen size was confirmed with a whole brain analysis showing that patients hac significantly smaller grey matter volume in this area (note that we also reproduced the well described difference in the cerebellum) (SPM T test p < 0.001) (see figure S1).

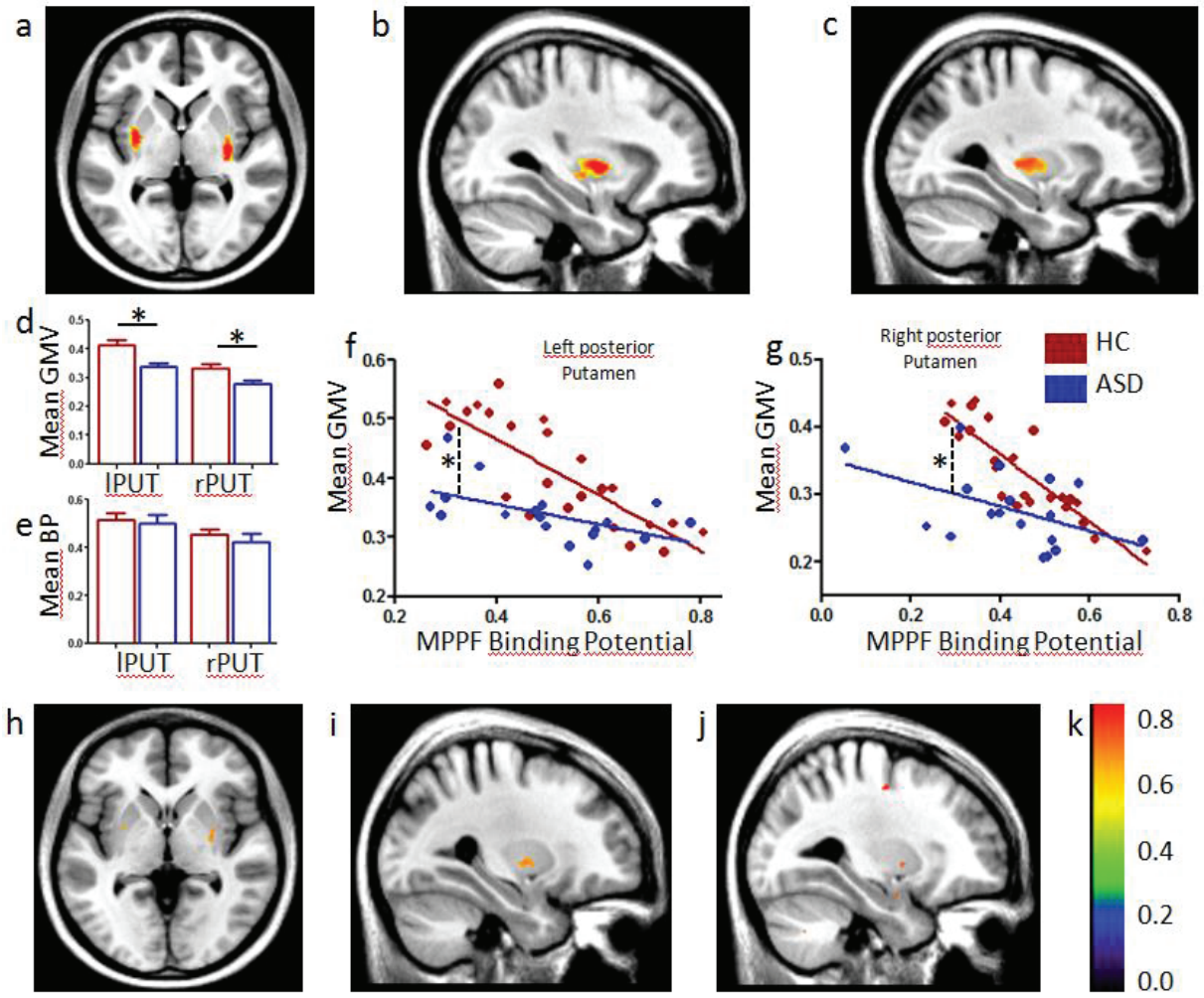

Figure 22. 5-HT$_{1A}$R is negatively associated to grey matter in posterior putamen of HC but not in ASD patients. a-c, Parametric maps showing significant negative correlations between MPPF BP$_{ND}$ and GMV in left and right posterior putamen of HC, overlaid on the mean anatomy of subjects. (a) Transversal slice (z = 1), (b) left hemisphere (x = -29), and (c) right hemisphere (x = 30). (d, e) Histograms depicting GMV (d) and MPPF BP$_{ND}$ (e) in HC (red) and ASD patients (blue) (lPUT = left putamen, rPUT = right putamen, *: p<0.01 Student's two sample t test). (f, g) Correlations between MPPF BP$_{ND}$ and GMV in left and right posterior putamen of HC (red) and ASD patients (blue); *: significant difference between correlation strength. (h-j) Parametric maps showing negative correlations between MPPF BP$_{ND}$ and GMV of ASD patients, overlaid on the mean anatomy of patients. None of

the clusters resisted statistical correction at cluster level. (h) Transversal slice (z = 1), (i) left hemisphere (x = -29), and (j) right hemisphere (x = 30). (k) C-score scale.

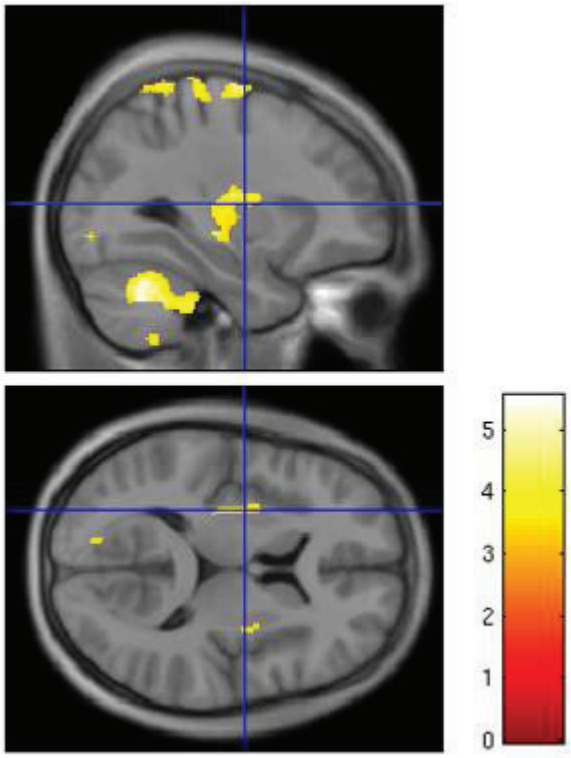

Figure S1. SPM T test healthy controls > ASD patients ($p < 0.001$ uncorrected). Scale bar indicates the T statistic. The cluster was bilateral. The opposite contrast (ASD patients > healthy controls) did not lead to significant clusters.

Serotonin 1a receptor in posterior putamen is negatively correlated to extraversion in healthy subjects but not in ASD patients

We next wanted to investigate whether the above relation between 5-HT_{1A}R and GMV in the putamen is linked to sociality.

Autism being characterized by social deficit, we coherently found that our ASD patients group had significantly lower levels on the NEO PI-R Extraversion dimension (which represents social personality) (Student's t test: $T = 3.08$, $p < 0.01$) (see Figure 23 i). Furthermore, they showed diminished levels in several facets of Extraversion: warmth, gregariousness, assertiveness and activity (respectively: $T = 3.17$, 3.27, 3.00 and 2.22, all $p < 0.05$). They also displayed lower scores in Openness dimension ($T = 2.83$, $p < 0.01$) as it has been previously observed (Strunz et al, 2014).

In healthy subjects, Extraversion scores were negatively correlated to MPPF BPND values extracted from the left posterior putamen (Peak: $r = -0.62$, $p < 0.01$; Cluster: $r = -0.42$, $p < 0.05$) (see Figure 23 a), but not with right posterior putamen neither with any GMV

values (all $p > 0.05$). Because such association is likely driven by a specific aspect of social personality, we tested if Extraversion facets correlated with MPPF BPND from the left posterior putamen. Only the Activity facet (a facet representing pace of living, social activity) was correlated to MPPF BP_{ND} Peak values after Bonferroni correction for multiple comparisons ($r = -0.65$, $p_{corrected} < 0.01$) (see Figure 23 b). In addition, Activity facet scores were also correlated to GMV peak values from the left posterior putamen ($r = 0.52$, $p_{corrected} < 0.01$; note that the positive direction of this correlation was expected since GMV negatively correlated to MPPF BP_{ND}) (see Figure 23 c). Because this Activity trait correlated with the Peak value of our posterior putamen cluster, we went back to a whole brain analysis to identify the precise location of this correlation and found a cluster in the left posterior putamen ($x = -33$, $y = -6$, $z = -2$, $k = 33$, $Z = 3.98$, $p_{uncorrected} = 0.02$, see Figure 23 d-f), however this cluster did not resist FWE correction, but it was bigger than the expected number of voxels ($k = 5.6$).

Critically, we did not expect to find this association between social personality and 5-$HT_{1A}R$ in ASD patients since they do not show the association between 5-$HT_{1A}R$ and GMV in the posterior putamen and this was indeed the case (all $r < 0.5$, all $p > 0.1$, see Fig. 23 j, k).

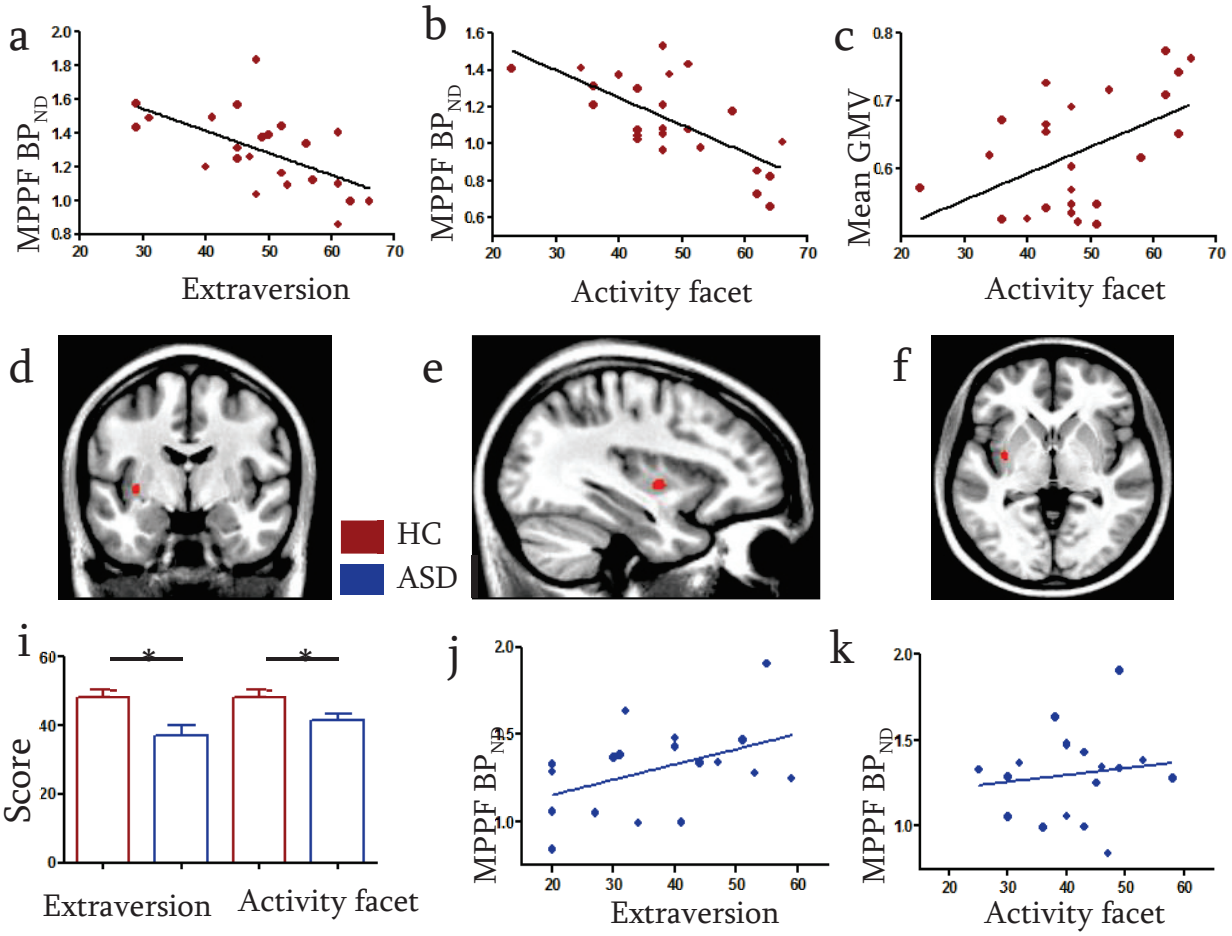

Figure 23. Left posterior putamen 5-HT$_{1A}$R density is negatively correlated to social activity. a-c Correlations between social personality traits and left posterior putamen 5-HT$_{1A}$R density and GMV: Extraversion (a) and Activity (b) negatively correlates with Peak MPPF BP$_{ND}$ and Activity also correlates with Peak GMV (c). (d-f) The significant association between Activity scale and MPPF BP$_{ND}$ in left posterior putamen of HC. (d) Coronal slice (y = -6), (e) sagittal slice (x = -33), and (f) transversal slice (z = -2). (i) Histogram depicting differences in Extraversion and Activity scores between HC (red) and ASD patients (blue). (i, j) Absence of correlation between social personality traits ((j) Extraversion and (k) Activity) and MPPF BP$_{ND}$ from left posterior putamen of patients with ASD.

II.3.d. Discussion

We have found evidence of a positive correlation between the serotonin 1A receptor (5-HT_{1A}R) density and grey matter volume in both the left and right hemisphere of healthy subjects. We obtained similar results in ASD patients for most but not all of these positive associations. Moreover, we found in healthy subjects, a very strong negative correlation between MPPF BP_{ND} and GMV in the left and right posterior putamen. Importantly, this relation is altered in ASD patients. Finally, we found a significant association between 5-HT_{1A}R density of the left posterior putamen and the degree of extraversion; again, this was true in controls but not in patients.

The positive correlations between GMV and 5-HT_{1A}R cannot be explained by a simple link between the number of neurons and the number of receptors, because we only observed these correlations in some regions and not in each areas containing 5-HT_{1A}R (e.g. the amygdala contains a lot of 5-HT_{1A}R but they are not associated with GMV). This suggests that the receptor is acting on grey matter volume in the significant clusters we found. Several clues support this hypothesis, first, MRI measured volume of grey matter is representative of neuronal density (la Fougère et al., 2011), second, it is known that the 5-HT_{1A}R is important for neuronal morphology in neurons and glial cells (Cowen, 2007). For instance, it was found in rats that 5-HT_{1A}R is important for the regulation spine density in the hippocampus (Yan et al., 1997). Thus the 5-HT_{1A}R can modulate the molecular pathways acting on neuronal growth (notably, protein kinase B and extra-cellular regulated kinase) and these changes can be observed by MRI. Importantly, some of the positive correlations were not found in patients with autism, suggesting a potential dysfunction of the 5-HT_{1A}R. It is interesting to remark that the morphologic role of 5-HT_{1A}R starts early in life (Daubert and Condron, 2010), and it is possible that the associations observed in the present study are originating from the firsts stage of brain development. This could explain why some of the clusters were not found in patients, as ASD is a developmental pathology. It is however unclear what the role of 5-HT_{1A}R would be at the adult stage in this regions. Longitudinal studies are needed to answer this question.

A more intriguing result is the bilateral negative correlation we found in the posterior putamen. In the context of autism and social behaviour, the striatum is of particular interest because serotonin has been shown to trigger social reward *via* a context dependent modulation of ventral striatum activity (Dölen et al., 2013). Another recent experiment has added evidence for a role of striatal serotonin in social behaviour, indeed, Noonan and

colleagues (Noonan et al., 2014) have found that the size of the posterior putamen (grey matter volume) is negatively correlated with the degree of social dominance in macaques monkeys, and critically they showed that the raphe nuclei volume is positively correlated with this same trait. Taken together, these results indicate that putamen function could be regulated by serotoninergic transmission whilst processing social information.

This negative relation suggests that 5-HT_{1A}R negatively modulate the plasticity in the posterior putamen. This is in line with *in vitro* work demonstrating that 5-HT_{1A}R stimulation inhibits neurite outgrowth (Anelli et al., 2013). Moreover, a post mortem study has found that suicide patients had more 5ht1a-r but diminished neuron density than controls in the prefrontal cortex (Underwood et al., 2012). It is also to note that negative correlations between grey matter volume and PET scan binding potential has already been identified in humans (Woodward et al., 2009).

Additionally, the MPPF BP_{ND} in the left posterior putamen was negatively correlated with extraversion, indicating a functional consequence of the 5-HT_{1A}R morphologic role in this region. In men, the posterior putamen activity has been linked to motivation for reward (Miller et al., 2014) and affiliation motives (Acevedo et al., 2012; Quirin et al., 2013). This posterior part is also known to be functionally connected with frontal and temporal cortex as well as more limbic regions (Tziortzi et al., 2013), suggesting a possible way through which putamen modulates social brain regions. In line with this is, it has been recently shown that extraversion degree of healthy subjects was correlated with the strength of amygdala-putamen connectivity (Aghajani et al., 2014). Our results therefore suggest that the grey matter volume modulation induced by the serotonin 1A receptor in the posterior putamen shapes the social personality. Thus, the more 5-HT_{1A}R there are in the posterior putamen and the less grey matter volume there is in this region, which leads to diminished extraversion levels.

Critically, none of these associations were found in ASD patients, who had reduced grey matter volume but similar MPPF binding potential in the posterior putamen. This is in line with animal models of autism in which the serotonin system is altered. Indeed, in such mice, the number of 5-HT_{1A}R is similar to controls, but their functionality is altered (Gould et al., 2011; Veenstra-VanderWeele et al., 2012). This could explain why patients who show low levels of MPPF binding potential still have small volumes of grey matter, as an over functioning 5-HT_{1A}R system would inhibit GMV even at low density.

Because the activity facet of extraversion was the most associated to MPPF BP_{ND}, we can hypothesize that 5-HT_{1A}R deregulation in the posterior putamen is involved in patients' social motivation deficit. In addition to a potential disruption of 5-HT_{1A}R function, it has been found that the putamen function is generally altered in autistic patients. Notably, they exhibit reduced levels of glutamate (Horder et al., 2013) as well as lower glucose metabolism (Haznedar et al., 2006). Finally, a study in patients with ASD found that tryptophan depletion modulated the BOLD activity in various brain regions, including the striatum, in an opposite manner between healthy controls and ASD patients, further suggesting altered functioning of serotonin receptors in this pathology (Daly et al., 2012).

The present study is limited by the fact that ASD patients involved were all high functioning and it is unclear to which extent our results apply to other categories of patients. Moreover, we performed our analysis with the Activity facet of the Extraversion domain of the NEO PI-R, meaning that the present associations represent only a small sub part of social behaviour. Also, one critical point to keep in mind is that all results are correlations, preventing us to conclude to a causal effect of the 5-HT_{1A}R.

A precedent article did not find the negative correlation we observed in the putamen (but they found a cluster that failed to reach significance, unpublished observations) (Kraus et al., 2012), however, they had a lower spatial resolution (voxel size: 4.36 mm vs 3.1 mm), mixed sex group and most critically used a different radioligand (WAY100635, a radioligand that is marking more receptors as it penetrates cells' membranes). It would be interesting to look if the present findings are specific to men. It is known from other PET scan studies that the concentration of 5-HT_{1A}R in the putamen is rather low (Savli et al., 2012). In average we found still found a MPPF BP_{ND} over 0.5, which is low but physiologically significant. Importantly, this value was not correlated to the values from the neighbouring insula area, thus ruling out a partial volume effect. Moreover, the clusters precisely follow the shape of the putamen grey matter. Ultimately, it could be argued that a high MPPF BP_{ND} in the insula is linked to a high density of fibres, and thus we would in fact be observing a positive correlation between white matter volume and 5-HT_{1A}R density. However, if this was the case we should have observed a correlation with the BP_{ND} in the insula. Another similar explanation would be that the BP_{ND} in our clusters are due to the serotonergic fibres passing by this area toward the insula, but the presence of 5-HT_{1A}R in the middle of these fibres is unlikely.

On a final note, the present results should be interpreted with caution because the grey matter that we measured contains not only neurons, but also glial cells. Hence, as we mentioned earlier that 5-HT_{1A}R can also act on such cells (Cowen, 2007), the present

correlations may not necessarily implicate neuronal cells, although the functional link with behaviour supposes an effect of 5-HT_{1A}R on neurons.

Taken together our results argue in favour of a role of the 5-HT_{1A}R on grey matter volume of various regions in the neocortex, the hippocampus and the putamen. It seems that the 5-HT_{1A}R can both positively and negatively regulates brain morphology. Only in the putamen, 5-HT_{1A}R density was found associated with social personality through a structural effect on the posterior putamen. Importantly, because serotonin is malfunctioning in ASD patients (Nakamura, 2010), this could lead to the observed altered putamen morphology (Qiu et al., 2010) and altered Activity social trait.

III. Chapter 3 – Experiment 2: Neural mechanisms of oxytocin and serotonin interaction in the non-human primate

III.1. From humans to macaques: exploring the synapse

Following our findings of OT modulation of the serotonergic system in humans, the next obvious question was to investigate this mechanism. The core of my PhD was thus an experiment on macaque monkeys, which allowed us several key advantages such as the possibility to use repeatedly different radiotracers in the same individual, and the possibility to administer OT directly into the brain. Moreover, it also provided us the opportunity to link our *in vivo* results to *post mortem* complementary experiments. The next section is a paper currently in preparation for publication.

Neural mechanisms of oxytocin and serotonin interaction in non-human primates

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Abstract

Oxytocin is increasingly studied for its therapeutic potential in psychiatric disorders which are associated with the deregulation of several neurotransmission systems. Hence investigating neurotransmitters' interaction is a relevant step towards mechanism-based treatment. Studies in rodents demonstrated that the interaction between oxytocin (OXT) and serotonin (5-HT) is critical for several aspects of social behavior. Using PET-scan in humans we have recently found that 5-HT 1A receptor (5-HT_{1A}R) function is modified after intra-nasal oxytocin intake. However, whether OXT modulates 5-HT_{1A}R receptors through a modulation of 5-HT release, receptor externalization or via a more direct action on this receptor is still unclear.

To understand these mechanisms we tested 3 macaque monkeys using both [18F]MPPF and [11C]DASB, PET radiotracers, two markers of the 5-HT_{1A}R and the serotonin transporter, respectively. Oxytocin (1IU in 20µL of artificial cerebro-spinal fluid) or placebo was injected into the lateral ventricle 45 minutes before scans.

Compared to placebo, OXT significantly reduced [^{11}C]DASB binding potential in right amygdala, insula and hippocampus whereas [^{18}F]MPPF binding potential increased in right amygdala and insula.

Our results show that oxytocin administration in non-human primates influences serotoninergic neurotransmission *via* at least two ways: first by provoking a release of serotonin in key limbic regions and second, by increasing the availability of 5-HT_{1A}R receptors in limbic and cortical areas. Because these two molecules are extremely important for social behavior, further studies on the precise nature of their interaction will help to develop new mechanisms based therapies.

III.2. Neural mechanisms of oxytocin and serotonin interaction in non-human primates

III.2.a. Introduction

Oxytocin (OT) is a fascinating neurohormone because of the very wide range of actions it exerts at both the peripheral and the central level (e.g., (Eliava et al., 2016)). As a consequence, this nonapeptide is being studied as a potential therapeutic molecule in various diseases (Altirriba et al., 2015; Feifel et al., 2015; Lefevre and Sirigu, 2016). One way through which OT is able to influence so many process is by its modulatory effects on other neurotransmission systems. It has been found for instance that OT influence the dopaminergic system (Baskerville and Douglas, 2010; Young and Wang, 2004), or the corticotrophin releasing factor (Bosch et al., 2015; Dabrowska et al., 2011). These interactions have very different behavioural consequences, the OT-dopaminergic pathway being a regulator of reward (Love, 2013) while the OT-CRF is involved in stress and anxiety (Dabrowska et al., 2013; Windle et al., 2004).

Importantly, we know from animal experiments that OT also modulates serotonin (5-HT), provoking its release (Dölen et al., 2013; Pagani et al., 2015; Yoshida et al., 2009). In this line, we recently demonstrated that this OT/5-HT interaction also occurs in brain regions important for social cognition and emotions such as the amygdala, the insula and the orbitofrontal cortex in humans (Mottolese et al., 2014), a finding of importance for clinical research as 5-HT is also a therapeutic target for psychiatric disease (Bandelow et al., 2002; Celada et al., 2013; Vasa et al., 2014). The effect of intra nasal OT administration was an increase of MPPF (a serotonin 1A receptor ($5\text{-}HT_{1A}$-R) radiotracer) non-displaceable Binding Potential (BP_{ND}), which suggest either a decreased serotonin concentration or an increased density of $5\text{-}HT_{1A}$ receptors. Because OT has been found to increase serotonin concentration (Dölen et al., 2013), we suggested that what we observed was an externalization of $5\text{-}HT_{1A}$-R. However, using only one radiotracer in this study (Mottolese et al., 2014), we were not able to answer this question.

In order to test this hypothesis, we decided to use macaque monkeys for several reasons. First, this will permit us to administer OT directly into the brain, thus avoiding critics regarding intra-nasal OT (Leng and Ludwig, 2015). Moreover, intracerebroventricular (icv) is a method that shows consistent effects, lasting several dozens of minutes, both on behaviour (Pedersen et al., 1982) and brain activity (Febo and Ferris, 2014). It also offers complete control over the dose that is delivered to the brain. Second, unlike in humans it is possible to repeat PET scans several times in macaque monkeys, as their life span of 15 to 20 years reduces the impact of radiations.

To further investigate the effects of OT on 5-HT neurotransmission, we chose to reproduce and extend our previous PET scan experiment (Mottolese et al., 2014). We combined two radiotracers, MPPF, the 5-HT_{1A}-R marker, and DASB, a molecule binding to the serotonin transporter. Thus, the aim was to track changes at both the 5-HT_{1A} receptors (MPPF) and the serotonin concentration (DASB). This design would give us a full picture of the OT/5-HT interaction in the primate brain.

We expected to reproduce our previous results (i.e., an increase of MPPF BP_{ND} in limbic regions associated with socio emotional behaviours), and to extend them by showing that OT modulates serotonin release in these same regions (i.e. a decrease of DASB BP_{ND} indicating a decrease of 5-HT concentration (Lundquist et al., 2007)). Our regions of interest (ROI) were thus the regions in which we previously found an effect of OT on 5-HT neurotransmission: amygdala, hippocampus, insula and orbitofrontal cortex (Mottolese et al., 2014). Moreover, we predict that in macaques, as in humans, the effect could be localized in the right hemisphere.

III.2.b. Methods

Animals

This study was approved by our local animal experimentation ethics committee (CELYNE) and used experimental procedures complying with the recommendations of the local authorities on animal care (Direction Départementale des Services Vétérinaires, Lyon, France) and the European Community standards for the care and use of laboratory animals. Three rhesus macaques (monkey V, P and J) were housed together at the Centre de Neuroscience Cognitive in Bron, France. Subjects were all males (mean age = 4.1 years, mean weight = 5.8kg), obtained from SILABE (Niederhausbergen, France). These monkeys were kept under standard conditions (12-h light cycles, 23°C and 50% humidity), were fed with monkey chow, vegetables and fresh fruits, had *ad libitum* access to water and enrichments were regularly offered (boxes and puzzles containing dry fruit, at least once per week) following recommendations from our own laboratory animal welfare committee. Daily clicker training ensured monkeys' cooperation for various procedures (going in the contention chair, head fixation habituation, anaesthesia procedure, etc…).

Surgical procedure

Each monkey underwent two surgeries. Both were performed in a fully sterile environment. Anaesthesia was induced with Ketamine (Virbac 10mg/kg) and maintained

with Isoflurane (1-2%). After each surgery monkeys received appropriate antibiotic coverage and pain-relievers as needed (buprenorphine), at least one month was given to the monkey to fully recover.

During the first surgery, animals were implanted with an MRI-compatible head-restraint post using standard techniques (dental acrylic, titan and ceramic screws). In the second procedure, once the monkey was habituated to head fixation, a chronic injection chamber (plastic) was implanted, to allow descending an injection needle into the brain. This chamber was cleaned with oxygenated water, betadine and physiologic serum at least twice per week in a contention chair with the head fixed.

Anatomical MRI

Each monkey underwent at least two anatomical MRI, one before the chamber implantation, to precisely localize the right lateral ventricle, and one after the surgery, to verify the position of the chamber and estimate the depth that needed to be reached. Additionally, monkeys V and J underwent a third anatomical MRI to check the path of the injection needle after the end of experiments.

The anatomical scans were performed at the imaging centre (CERMEP, Bron) on a (1.5-T MR scanner Sonata; Siemens) with a radial receive-only surface coil (10 cm diameter) placed around the monkey's head plot, and consisted in a T1-3D MPRAGE sequence (repetition time [TR] 2160ms; echo time [TE] 2.89ms; inversion time [TI] 1100ms; 176 sagittal slices; 0.6×0.6×0.6 mm voxels).

Intracerebroventricular injections

For each monkey, we first precisely localized the right lateral ventricle, guided by structural MR images, by sampling 200µL of Cerebro Spinal Fluid (CSF) as such amount of liquid can only be obtained at this place. This procedure was done in awoken animals under head restraint conditions. These samples were used in another study (Lefevre et al., *In prep*).

On the day of scanning, a 23Gauge needle (Terumo), already filled with Placebo (artificial CSF or Oxytocin diluted in aCSF (Sigma Aldrich), attached to a 100µL microsyringe (Hamilton) was descended at 50µm/s to the location previously identified as the right lateral ventricle, with a micro descender (Narishige). The repeatability of this manipulation was ensured with a grid oriented in the exact same manner every time, for each monkey, the same grid's hole was used throughout the experiment. Once the correct depth was reached, 20µL of solution were manually injected over 5 minutes, to allow the

ventricle to adapt to the incoming liquid. OT and Placebo were injected in a random order in each monkey.

Anatomical MR images were used to check the path of the needle (supplementary figure 1).

Procedure

Because of the existence of a diurnal rhythm of OT concentration in the CSF (Amico et al., 1989), PET scans always occurred between 12 am and 4 pm. No more than one scan per week was performed on the same monkey and a strict minimum of 5 days was observed between two scans.

Monkeys were isolated from cage mates and deprived of food on the evening before the scan, but still had *ad libitum* access to water. They were anaesthetized with Zoletil (Tiletamine/Zolazepam, Virbac 15mg/kg) approximatively 90 minutes (86.7 ±16.6 min) before the beginning of the scan. It should be noted that Zoletil does not alter serotonergic PET scan binding, at least for the transporter (no studies so far on the 5-HT_{1A}-R) (Elfving et al., 2003; Yamanaka et al., 2014). The consciousness state of the monkey was monitored by a trained experimenter during the whole experiment and an additional zoletil dose was administered when required (usually just before the beginning of the scan, mean total dose = 130mg). A catheter was installed on the saphenous vein and Ringer liquid was administered throughout the experiment. A first blood sample was withdrawn from the saphenous vein (on the leg without the catheter), 20 minutes before the icv injection. The chamber was cleaned and lidocaine was sprayed on the tissue. After rinsing with physiological serum, OT or placebo was injected in the right lateral ventricle about 45 minutes prior to the PET scan exam (mean = 47.6 ±6.9 min). This delay was similar to our previous experiment in humans, and we chose to inject in the right hemisphere because the OT effects were found lateralized in humans (Mottolese et al., 2014). The second blood sample was collected 5 minutes after the icv injection. Then, the animal was brought to the imagery centre (CERMEP, Bron) and installed in a stereotaxic frame (lidocaine and ocular gel were applied to ears and eyes to prevent any discomfort), the cardiac rhythm and O_2 saturation were monitored during the scanning and wool covers prevented body temperature to diminish. A 1-min low dose CT scan was performed to measure tissue and head support attenuation, then the third blood sample was collected just before the radioligand was injected and the PET scan started. At the end of the scan, the monkey was brought back to the lab where a fourth blood sample was collected, and then put back to his home cage with a heat lamp, isolated for the night from his congeners (see Figure 24).

Depending on their state, they were fed or not before the lights turned off (8 pm). They were reunited to their cage mates on the morning after.

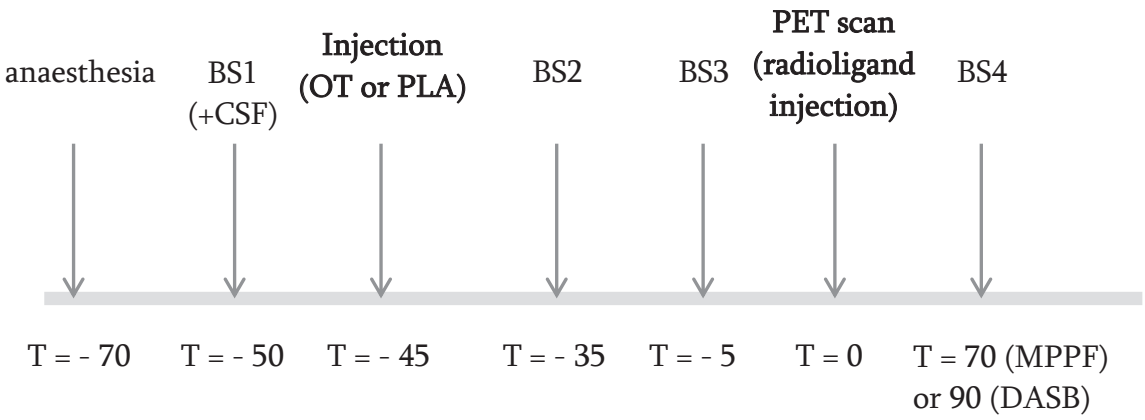

Figure 24. Protocol of each PET scan session. BS indicates a blood sample, T = time in minutes.

Oxytocin concentration analysis

Within 20 minutes, blood samples (1mL in an EDTA tube) were centrifuged at 2000g for 10 minutes at 4°C. The plasma obtained was then immediately stored at -80°C. Before analysis, plasma was extracted accordingly to manufacturer's recommendations. Briefly, 250µL of 0.1% trifluoroacetic acid (TFA-H_2O) and 250µL of plasma were centrifuged at 16000g for 15 minutes at 4°C. The supernatant was applied to a 200mg Sep Pak column (previously equilibrated with 1mL of acetonitrile and 15mL of 0.1% TFA- H_2O) and washed with another 15mL of 0.1% TFA- H_2O. The sample was then eluted with 3mL of 95% acetonitrile / 5% of 0.1% TFA- H_2O, and the eluate (kept cold in an ice bath) was evaporated with compressed nitrogen gas and stored at -20°C until assay.

OT concentrations were assayed with a commercially available enzyme immunoassay (Enzo Life Sciences, Farmingdale, USA). Prior to assay, samples were reconstituted with 250µL of assay buffer. The inter assay variability was not calculated here but of 20.9% according to manufacturer and the intra assay variability was less than 3% for the 3 plates used. One sample was spike with 500 pg, and found to have a recovery rate of 102.5%.

Autoradiography

Adjacent sagittal macaque brain slices from the CERMEP stock were defrosted. They were then incubated for 20 min in Tris phosphate-buffered saline (TBS) buffer (Sigma, with

Ca^{2+}, pH adjusted to 7.5) containing 1 µCi/mL of [^{18}F]MPPF or [^{11}C]DASB. For MPPF, increasing amounts of OT (Sigma Aldrich) were then added (0, 5, 100, 2000ng), and for DASB, different physiologic concentrations of 5-HT (Sigma) were added (0, 5, 25, 75, 150nM).

After incubation, slices were rinsed in TBS+Ca^{2+} for 1.5min and purified water for 1.5min, then dried and juxtaposed to a phosphor imaging plate for 60 min (BAS-5000, Fujifilm). All films were analyzed by a computer-assisted image analysis system (MultiGauge, Fujifilm), and regions of interest were drawn manually, according to a macaque brain atlas (Paxinos). Quantification of labelling was done by measuring photo stimulated luminescence (PSL), in the caudate nucleus. All conditions were run in duplicate.

PET scan

PET scans were acquired on a Biograph mCT PET/CT tomograph (Siemens) at the imagery centre (CERMEP, Bron). We used MPPF to map the 5-HT_{1A}-R

A total of 30 scans were performed (monkeys V and J: 3 MPPF under OT, 3 MPPF under placebo, 3 DASB under OT, 3 DASB under placebo; monkey P: 2 MPPF under OT, 2 MPPF under placebo, 1 DASB under OT, 1 DASB under placebo).

A dynamic emission scan was acquired in list mode during 90min for DASB, and 70min for MPPF, after radiotracer injection. A total of 30 (DASB) or 24 (MPPF) frame images were reconstructed by using the 3D-ordinary Poisson-ordered subset expectation maximization iterative algorithm incorporating PSF and time of flight (with an All Pass filter) after correction for scatter and attenuation as well as a transversal zoom factor of eight [256 × 256 voxels in-plane (0.4 mm^2) and 109 slices (2.03-mm thickness)]. The resolutions for reconstructed images were approximately 2.6 mm in full width at half maximum in the axial direction and 3.1 mm in full width at half maximum in the transaxial direction for a source located 1 cm from the field of view.

- MPPF:

2'-Methoxyphenyl-(N-2'-pyridinyl)-p-18F-fluoro-benzamidoethylpiperazine ([^{18}F]MPPF) was obtained by nucleophilic fluoration of a nitro precursor (Le Bars et al., 1998), with a radiochemical yield of 20% - 25 % at the end of the synthesis and a mean specific activity of 4.41 ±1.86 Ci/µmol. A bolus of [^{18}F]MPPF was injected (mean injected dose, 4.16 ±0.52 mCi). It is an antagonist to 5-HT_{1A}-R with a binding affinity of 2.8nM.

- DASB:

[11C]*N*,*N*-dimethyl-2-(2-amino-4-cyanophenylthio)benzylamine ([^{11}C]DASB) was synthetized on site, with a mean specific activity of 1.22 ±0.66 Ci/µmol. A bolus of [^{11}C]DASB was injected (mean injected dose, 4.31 ±0.45 mCi).

Data processing

For each monkey respectively, PET scans and anatomical MRI were registered linearly using the Minc ToolKit (http://bic-mni.github.io/) (Collins et al., 1994). For each PET scan, the frames were summed to obtain one image per session. These images were registered for each radiotracer on a reference chosen for its high raw activity. Then, the mean PET, per monkey and per radiotracer, was computed and a second registration of each PET on this average was done. The mean images of both radiotracers were registered on each monkey anatomical MRI.

To perform comparisons between our three monkeys and to overlap ROIs provided by the atlas (Ballanger et al., 2013) with our scans, the transformation between each monkey space and a common macaque brain template (Ballanger et al., 2013) was also computed. Individual anatomical MRI were non-linearly registered on the template using FNIRT (FSL, http://fsl.fmrib.ox.ac.uk/fsl/).

We used a simplified reference tissue model to compute non-displaceable Binding Potential (BP_{ND}), with cerebellum (minus the vermis) as the reference region for DASB and white matter of the cerebellum as the reference region for MPPF. These regions were defined from the atlas registered on the template (Ballanger et al., 2013). Regional parametric values were obtained by modelling of the mean regional kinetics, extracted in the native PET spaces inside ROIs from the atlas registered to each monkey space using the inverse of non-linear transformation computed previously, these ROI values were used for the inter regions correlations. Whole brain parametric images were obtained by modelling the voxel kinetics. Resulting parametric images were then non-linearly transformed to the common template space for further voxel-based SPM analyses.

Statistical analyses

If not otherwise specified, all analyses were performed with SPM12 and STATISTICA 8.

- PET scan data:

For MPPF, we reproduced the same analysis than in our human study (Mottolese et al., 2014). A flexible factorial design ($p<0.01$, uncorrected), with a subject factor, testing the

effects of treatment (OT vs placebo) on MPPF BP_{ND} with an ANCOVA by subject to account for the observed inter subject variability and restricted to our ROI by an inclusive mask containing (amygdala, hippocampus, insula and prefrontal cortex, same mask as in (Mottolese et al., 2014)). We also computed raw BP variations from the clusters (SPM12, extracted from SPM, http://www.fil.ion.ucl.ac.uk/spm/), values were divided by the monkey mean value to account for inter individual variability, and transformed in percentage to compare with the variations obtained in humans (Mottolese et al., 2014). Moreover, this contrast was limited to voxels in which the binding potential was superior to 0.2 (a BP_{ND} lower than 0.2 does not represent a significant concentration of 5-HT_{1A}-R).

To further reproduce our previous results obtained in humans (Mottolese et al., 2014), we performed correlation tests between the mean amygdala BP_{ND} (ROI extracted from the atlas) and the regions that correlated with it after OT in humans: hippocampus, insula, orbitofrontal cortex and anterior cingulate gyrus (in our atlas, this region integrated the subgenual cortex). We tested correlations with Spearman's rank tests, corrected for multiple comparisons with Bonferroni's correction ($p_{corrected}<0.0125$).

For DASB, we also used a flexible factorial design, with a subject factor, to test the effects of treatment (OT vs placebo) on DASB BP_{ND}, but with a proportional scaling to account for the observed inter scans variability and a more conservative statistical threshold (since it was not based on an existing result) of $p<0.0001$, uncorrected. This design was not restricted to our ROI but applied to the whole brain as we know there are differences between the distribution of serotonin transporter and 5-HT_{1A}-R (Savli et al., 2012). Moreover, this contrast was limited to voxels in which the binding potential was superior to 0.2 (a BP_{ND} lower than 0.2 does not represent a significant concentration of serotonin transporter).

III.2.c. Results

Oxytocin modulates MPPF Binding Potential

Using a voxel based analysis, we found a significant effect of treatment (OT > Placebo) on MPPF BP_{ND}, in two clusters located in the right amygdala (k=76) and in the right insula (k=491) (Figure 25). The mean BP_{ND} values from these clusters indicated that OT increased MPPF BP_{ND} by 33.3% in the amygdala and by 32.8% in the insula (Figure 25). There were no effects of anaesthesia (zoletil dose) or scanning starting time.

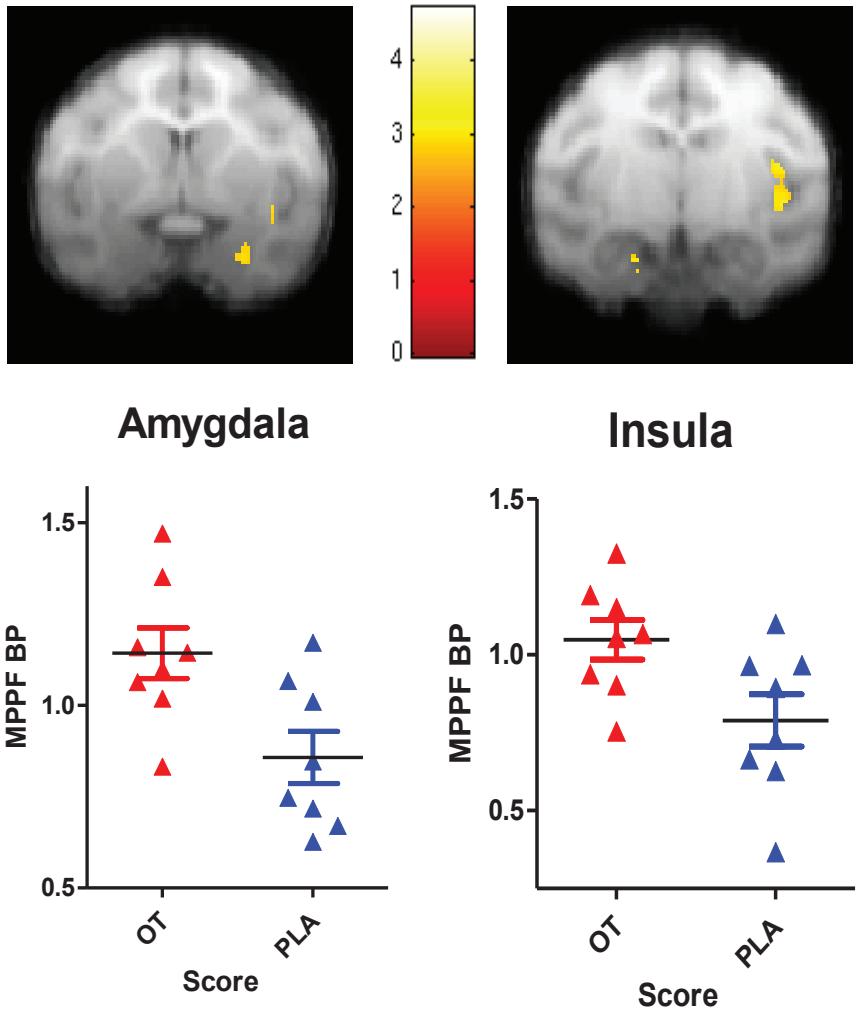

Figure 25. (*Upper*) T-map SPM analysis (voxel significance level p<0.01, uncorrected) showing the effects of oxytocin on MPPF BP_{ND} compared to placebo (OT > Placebo). Effects were localized in the right amygdala (left) and the right insula (right). Scale bar (middle) represents T score.

(*Lower*) Mean BP inside amygdala and insula clusters, for each scan (n=16, extracted from SPM and normalized per individual). The average increase of BP_{ND} after OT is 33.3% in the amygdala and 32.8% in the insula.

Between region correlations after OT

Similarly to (Mottolese et al., 2014), we found no significant correlations between the right amygdala and our ROIs under placebo (all p>0.0125, see Table 5), however, after OT treatment, all these regions, excepted the hippocampus (p>0.0125) were found to be significantly correlated (all p<0.0125, see Table 5).

	Placebo	Oxytocin
Hippocampus	0,76	0,79
Insula	0,62	1,00 *
OFC	0,60	1,00 *
ACC	0,58	0,90 *

Table 5. Coefficients of correlation between MPPF BP_{ND} in the amygdala and in our other ROI (Spearman's rank correlation tests). * indicates significant p-values after correction for multiple comparisons.

Oxytocin modulates DASB Binding Potential

Using a voxel based analysis, we found a significant effect of treatment (OT < Placebo) on DASB BP_{ND}, in several clusters located in the right amygdala, the right insula, the right hippocampus and the temporal cortex (Figure 26). Moreover, we found a bilateral decrease of BP_{ND} in the ventral striatum (Figure 26). All these clusters resisted FWE correction ($p_{FWE}<0.05$). No significant clusters were found in the left hemisphere. There were no effects of anaesthesia (zoletil dose) or scanning starting time.

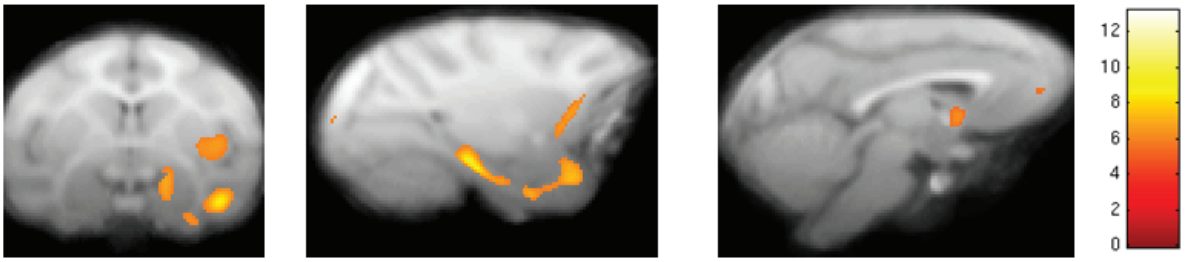

Figure 26. T-map SPM analysis (voxel significance level p<0.0001, uncorrected) showing the effects of oxytocin on DASB BP_{ND} compared to placebo (Placebo > OT). Effects were localized in the right amygdala (Upper, and Lower), the right insula (Middle), the right hippocampus (Lower) and in the temporal cortex (Lower). Scale bar (Middle) represents T score.

In vitro modulation of MPPF and DASB Binding Potential

We tested if OT could act directly on the 5-HT$_{1A}$-receptor by incubating brain slices with MPPF and OT, up to a dose of 2µg, which is the dose we injected icv. We did not find any MPPF labelling differences between the control slice (no oxytocin) and any of the oxytocin conditions (5ng, 100ng, 2µg) (Figure 27), contrarily to what we observed *in vivo* with PET scan. There were no variations of MPPF labelling between duplicate slices. Photo Stimulated Luminescence (PSL) values were similar between conditions (not shown).

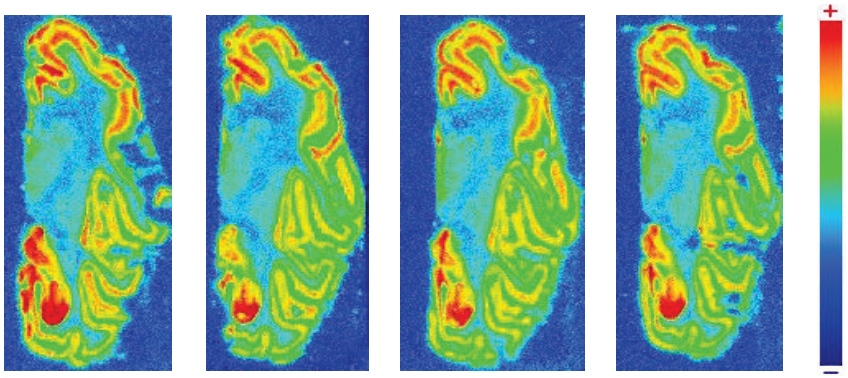

Figure 27. Adjacent macaque sagittal slices incubated with MPPF and increasing concentrations of OT did not show any effects of OT on 5-HT$_{1A}$-r MPPF labelling.

We also tested if DASB labelling of the serotonin transporter was susceptible to serotonin concentration. We found a dose dependent effect of serotonin on DASB labelling, which decreased proportionally to the amount of serotonin present during incubation (Figure 28). This result is similar to what we observed *in vivo* with PET scan. Moreover, the PSL values of caudate nucleus, a region rich in serotonin transporter, were also found to decrease according to the serotonin dose. More precisely, the 5nM serotonin dose, which represent baseline levels, did not affect DASB labelling, but higher doses, which are in the range of *in vivo* endogenous serotonin release, reduced PSL (figure 28). There were no variations of DASB labelling between duplicate slices.

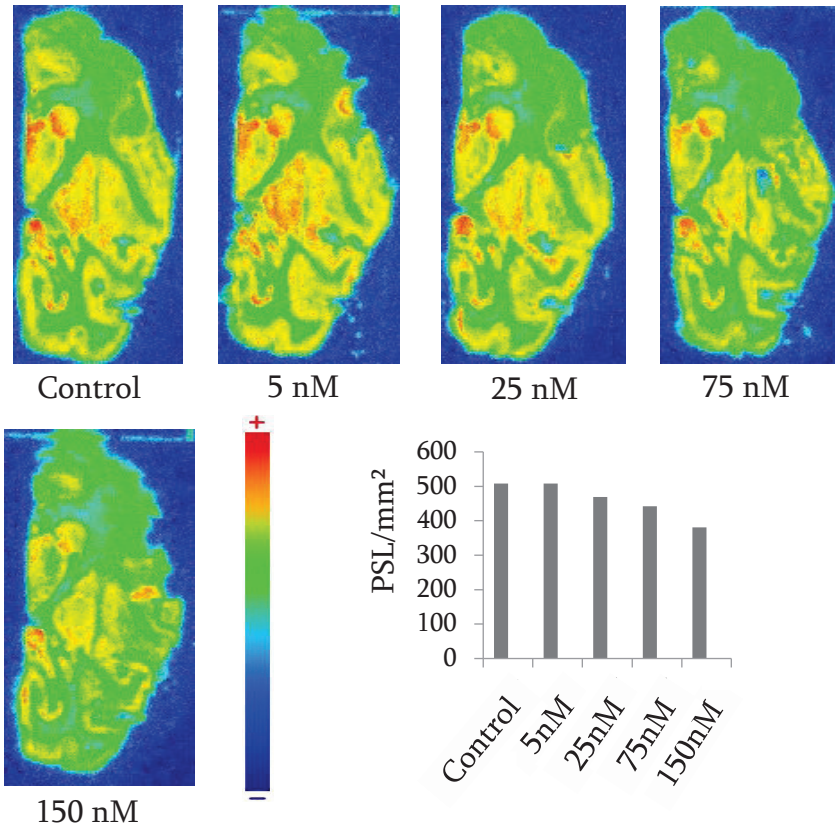

Figure 28. Adjacent macaque sagittal slices incubated with DASB and increasing concentrations of 5-HT, which dose dependently reduced DASB labelling of the serotonin transporter. Graph shows PSL values of the caudate nucleus.

Effects of icv OT on plasma concentration

We analysed 67 blood samples (it often happens that the last blood sample could not be taken as the monkey was too awake, and for one monkey, we decided not to perform blood sampling as we had enough already).

We did not find any effects of treatment (OT or placebo) or time (sample 1, 2, 3 or 4, see figure 29) on plasma concentration of OT (all $p > 0.1$, repeated measure ANOVA).

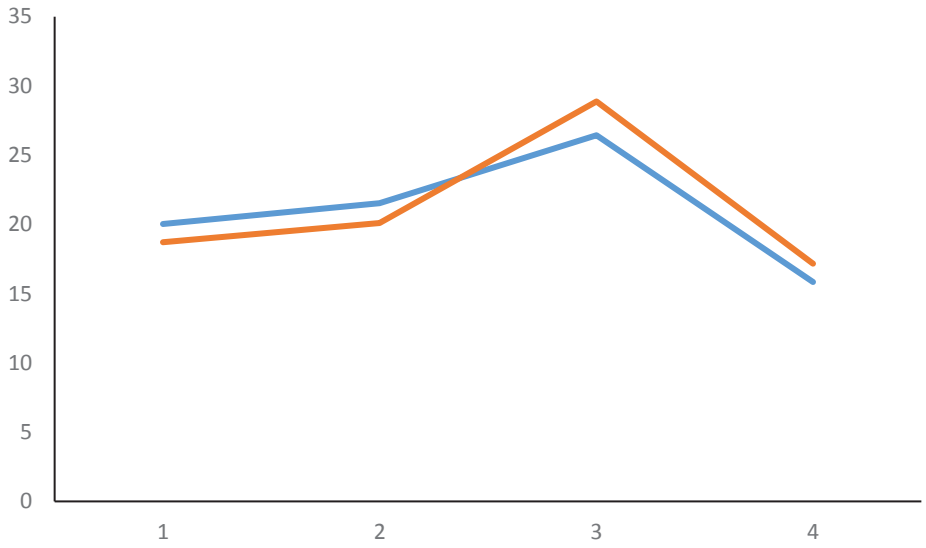

Figure 29. OT concentration in the plasma during the course of the protocol (1 = 5 min before injection, 2 = 10 min after injection, 3 = just before PET scan beginning and 4 = at the end of the PET scan). Note that an non significant raise occurs at 3 for both OT (blue) and Placebo (orange), this could be linked to longer plasma extraction as samples had to be brought from the imagery facilities back to the laboratory.

III.2.d. Discussion

Using macaque monkeys, we found that oxytocin (OT) directly injected into the lateral ventricle increased MPPF BP_{ND} and decreased DASB BP_{ND}, which are respectively marking the 5-HT_{1A}-R and the SERT, in regions important for socio-emotional functioning: the amygdala, insula, hippocampus as well as other areas like the temporal cortex and the ventral striatum. Moreover, we found that OT did not act directly on MPPF BP_{ND} on *in vitro* brain slices, but that serotonin decreased DASB BP_{ND} on the same slices. Thus the present experiment has brought new and clear evidence that OT is modulating the serotonergic system in primates.

It should first be noted that we observed effects in regions which have already been seen to be affected by exogenous OT in several human fMRI studies. A recent systematic review of fMRI studies showed that OT consistently modulates the amygdala and the insula (Wigton et al., 2015). In addition, other studies have found effects of OT in the ventral striatum Moreover, these regions have also been found to be modulated by OT in experiments on rodents, notably in the amygdala where OT triggers GABA neurons activity (Knobloch et al., 2012; Viviani et al., 2011) and in the nucleus accumbens (Dölen et al., 2013; Herisson et al., 2016; Young and Wang, 2004). Thus our results are coherent with the

literature from both animal and human experiments in regard of their localization in the brain.

Displacement of Binding Potential should be carefully interpreted as many factors can impact this measure (Paterson et al., 2010). For DASB, a decrease of BP_{ND} could mean either an increase of synaptic serotonin (higher competition for SERT sites) or a decrease of SERT concentration. We know that OT can provoke the release of 5-HT (Dölen et al., 2013). Therefore, a straightforward interpretation would be that OT has increased serotonin concentration. This is in accordance with our *in vitro* results which suggest that DASB labelling is sensitive to endogenous serotonin concentration. However, given that OT was administered 45 minutes before the scan, the SERT internalisation seems more likely as SERT undergoes internalisation after agonistic stimulation (Jørgensen et al., 2014). Thus, we conclude that OT has indeed released serotonin in the amygdala, insula and nucleus accumbens but what we observed with PET scan could be the subsequent down regulation of SERT.

Regarding the increased MPPF BP_{ND}, it could be either due to a decrease of serotonin concentration or an increase of 5-HT_{1A}-R. The decrease of serotonin is unlikely for several reasons. First, it is in contradiction with the literature (Dölen et al., 2013) and our results with DASB, second, MPPF radiotracer is only capable to detect huge (non-physiologic) releases of serotonin (Zimmer et al., 2002), but not endogenous modifications in primates (Praschak-Rieder et al., 2004; Udo de Haes et al., 2006). This is likely because MPPF has a higher affinity for 5-HT_{1A}-R than serotonin and because MPPF is an antagonist and thus binds to both low and high affinity (free and G protein coupled 5-HT_{1A}-R) whereas serotonin only binds to high affinity receptors (Kumar et al., 2012). Thus, we conclude that the present increase of MPPF BP_{ND} is due to an externalisation of 5-HT_{1A}-R, which would be a consequence of the serotonin release induced by OT. Note that although 5-HT_{1A}-R are known to internalise following agonistic stimulation, this phenomenon only happens in the raphe nuclei and not in other regions (Kennett et al., 1987; Riad et al., 2001).

Other interpretations are possible, for instance, OT could directly act on the 5-HT_{1A}-R binding properties, but our *in vitro* results do not suggest this is possible. Another option would be the formation of OTR-5-HT_{1A}-R heteromers, as such receptors complexes can change the affinity and trafficking of receptors (Bouvier, 2001; Ferré et al., 2009). Both OTR and 5-HT_{1A}-R have been found to undergo heteromerization (Łukasiewicz et al., 2016; Romero-Fernandez et al., 2012), but not together yet. This hypothesis could explain OT-unique type of action on the 5-HT_{1A}-R system.

There are several limitations to the present study, for instance the timing (OT was administered 45 minutes before scans) did not allow us to study short term effects of OT.

However, studies generally found OT effects for dozens of minutes to hours, indicating that midterm action of OT could be responsible for these changes. Another concern could be the use of anaesthetics, but the molecules we used (Tiletamine, Zolazepam) do not seem to influence the serotonergic transporter system (Elfving et al., 2003; Yamanaka et al., 2014). Finally, we did not observe changes in the dorsal raphe nuclei. This is in contradiction to our previous study in humans (Mottolese et al., 2014), and could be explained by the difficulty to delimitate this small structure, especially in macaque monkeys. Notably, the atlas we used included the median raphe nucleus as the resolution of PET scan cannot distinguish between these two regions.

In sum, the present work brings new evidence that OT modulates the serotonergic system *via* two pathways in the limbic structures important for social behaviours. First by provoking the release of serotonin and second by enhancing 5-HT_{1A}-R functioning. This finding could have an important impact for pharmaceutic research, as OT, 5-HT_{1A}-R and SERT are all important targets in several pathologies, including depression, autism and general anxiety (Bandelow et al., 2002; Celada et al., 2013; Vasa et al., 2014). Thus, studying interaction between these systems could be a critical step towards improved treatments (Lefevre et al., *submitted*).

IV. Chapter 4 – Discussion

IV.1. Significance and implications

Summary and Interpretation: Taken together, the experiments realized during this PhD have shown several important results. First, we confirmed that in non-human primates, as in humans, oxytocin (OT) is acting directly, at the middle term (several dozens of minutes to a few hours) on the serotonin 1A receptor (5-HT_{1A}-R), moreover, we showed that OT provokes the release of serotonin (5-HT) in several regions implicated in social behaviour regulation. As OT did not seem to act directly on the 5-HT_{1A}-R, we think this effect is linked to the increased 5-HT concentrations (see Figure 30). In parallel, we found that this modulation of the 5-HT_{1A}-R following administration of OT was absent in patients with Autism Spectrum Disorders (ASD), and that the 5-HT_{1A}-R was not associated to grey matter volume and social personality in the same way than in healthy controls.

To interpret our findings, I propose a model of OT actions on the serotonergic system (Figure 28) that opens several questions. First, how OT reaches the limbic areas such as the amygdala or insula is unclear, as it has been discussed in part I.1.c. but remains an important interrogation. Second, the cellular localisation of receptors is still unclear. For OTR, it has been reported that OTR can be located at both pre and post synaptic levels as well as outside the synapse (Mitre et al., 2016). Although this was found in cortical regions, we can speculate a similar repartition in the limbic areas where we found our effect. This is because in these regions, OT has been both found at the postsynaptic level (Huber et al., 2005; Knobloch et al., 2012; Viviani et al., 2011) where it was acting on GABAergic neurons, and at the presynaptic level (Dölen et al., 2013) where it was modulating the release of 5-HT. Thus in our case, I hypothesized that the effects we observed after exogenous OT were linked to the activation of presynaptic OTR, located on the axonal terminations of raphe nuclei projections. Note that localization of OTR was also described at excitatory synapses in the cortex and this could also be the case in limbic regions, complicating the interpretation.

On the other hand, outside of raphe nuclei the localization of 5-HT_{1A}-R is also uncertain. To my knowledge, these receptors could be situated, as OTR at both pre- and post-synaptic levels. In the model I propose, the modulation of MPPF BP_{ND} should be linked to postsynaptic receptors since OTR would already be located at the presynaptic level and because we think the 5-HT_{1A}-R modulation is a reaction to the serotonin release.

Another point is the temporality of OT action. It is often found to have lasting actions, both *in vivo* and *in vitro*, usually starting five or ten minutes after the injection if administered directly into the brain. For the serotonin transporter (SERT), we recorded the effects of OT during 90 minutes 45 minutes after injection, which could mean to similar phenomenon, either an increase of 5-HT or a decrease of SERT. As these two interpretations are coherent, I chose to represent the release of 5-HT because it is supposed to be the direct consequence of OTR stimulation whereas SERT internalisation is following this 5-HT increase. Although it should be noted that what we measured with PET scan was probably linked to the SERT concentration modification, given our experimental design. Moreover, this would be in line with the fact that we observed an increased concentrations of 5-HT_{1A}-R. If both 5-HT and 5-HT_{1A}-R were upregulated at the same time, we might not have been able to see our effects of MPPF BP_{ND}.

The impairment we found in patients with ASD could be due to a problem at the OTR itself (e.g., lack of OT in these regions, lack of OTR on serotonergic projections, disturbed OTR functioning), or linked to the serotonergic system (e.g., altered serotonergic system or deregulation of 5-HT_{1A}-R). As both abnormalities on the OTR and 5-HT_{1A}-R systems were reported in patients, we cannot conclude on the origin of the observed alteration. However, we can say that patients with ASD have an impairment at the functional level, since we did not found differences of concentration or distribution of 5-HT_{1A}-R.

In conclusion, I tend to think that it is the 5-HT_{1A}-R system that is deregulated in the autism pathology, because OT administration have produced positive results at both behavioural and biological levels in other studies. In addition, we failed to find a link between 5-HT_{1A}-R and grey matter volume, further suggesting an abnormal functioning of the 5-HT_{1A}-R, as it has been found in rodent models of ASD.

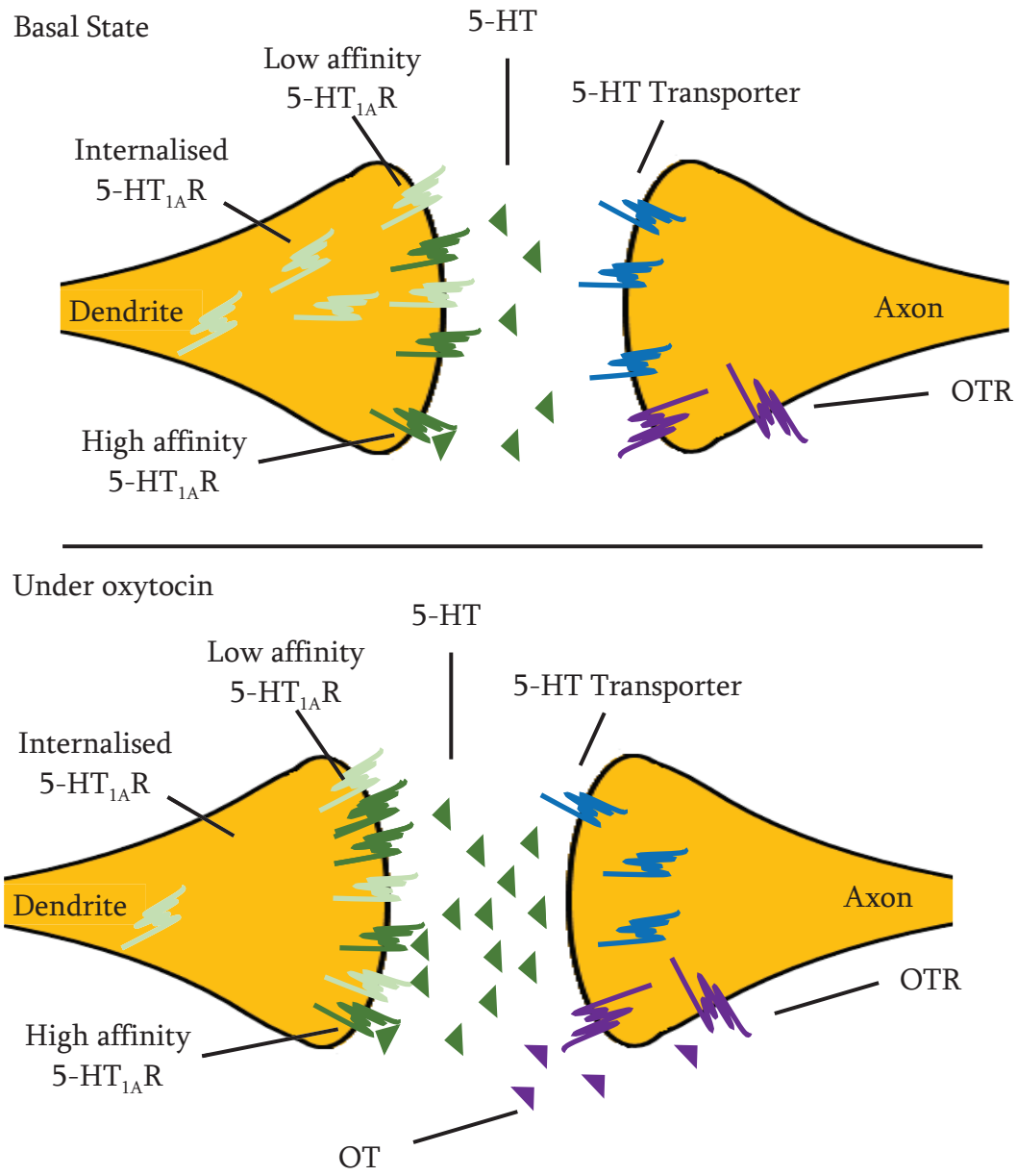

Figure 30. Model of OT action on the serotonergic system. Axons represents projections from the raphe nuclei. (Upper) Under basal state, and (Lower) after an increase of OT concentration (whether it is from endogenous or exogenous source should not influence this model), that provokes the release of serotonin which causes an upregulation of the 5-HT1A-R, and a down regulation of the 5-HT transporter.

Limitations: These results are of course limited by some factors. First, the patients recruited in this study were all males, as ASD are mainly affecting men. However, this decision also increased our statistical power, by suppressing the sex factor which is known to be sexually dimorphic in both rodents (Dumais et al., 2013, 2015; Dumais and Veenema,

2015) and humans, including patients with ASD (Miller et al., 2013; Rilling et al., 2013). Another fact is that our patients were all high functioning autists (n=6) or Aspergers (n=12), with an Intellectual Quotient superior to 70. Note that our patients were recruited from two different centres (11 from Hopital Chenevier-Mondor in Créteil and 7 from Hôpital Saint Jeand De Dieu in Lyon), thus limiting a potential selection bias. It is not possible to scan patients with more severe forms of ASD without medication, and this also represents an ethical limit. Nonetheless, as for all studies involving scanners or EEG on patients with ASD, it is unknown how our results translate to more impaired patients. This brings down to the question if patients with different levels of behavioural impairments express different neurobiological abnormalities or if only the degree of affection differs.

Another critic that could be made is that we did not use a task during the scan sessions. There were several reasons for that. First, in order to compare to healthy controls, we had to use the exact same design (i.e., resting state and spray), and we also knew that we were going to scan monkeys under anaesthesia, as having them awake and performing a task was to ambitious. It is to note though, that 11 patients had been involved in a previous experiment in our team, which showed the beneficial effects of intra nasal OT on their social behaviour (Andari et al., 2010). Although 4 years separated the two studies, we can still argue that OT had a behavioural impact on them. Moreover, we did not want our subjects to be processing social information, as this could have recruit their endogenous oxytocinergic system and biased our pharmacologic manipulation. We did not found any correlation or link between MPPF BP_{ND} and the previous behavioural result. This means that the effects of OT in patients with ASD are not mediated by serotonin, or at least the 5-HT_{1A}-R.

Finally, from a methodological point of view, we over sampled dynamic PET scan images, this could induce a small bias in term of le localization of our clusters, especially in the non-human primate study, however the localization of significant voxels matched our *a priori* hypothesis

Significance: There are two levels of implication of our research. First, from a fundamental point of view, our findings are an important step that validates the rodent experiments which have shown that OT modulates brain serotonergic system (Dölen et al., 2013; Pagani et al., 2015; Yoshida et al., 2009). At the behavioural level, the release we found in the nucleus accumbens could indicate an effect of OT on long term social reward (see part I.2.e.). The other two studies are however linked to another interesting aspect of OT: its role in anxiety. This point must however be considered carefully, as although it is demonstrated that administration of OT leads to general anxiolytic effect, it is less clear if

OT is released and exert such effect in response to any stress or only in the case of social traits. Thus all studies which look at the consequences of an OT increase (whether it is an injection, or an optogenetic stimulation) are potentially acting on OTR in a situation that would normally not recruit them. Some evidence points to a release of OT in non-social stress context, for instance when put in a Morris water maze, rats will release OT at both central and peripheral levels (Wotjak et al., 1998), indicating a magnocellular activity in both PVN and SON. Importantly, as suggested previously, OT effects on anxiety could recruit the serotonergic system. In this context, it is reasonable to think that 5-HT_{1A}-R are involved, as they are important for the regulation of anxiety, and mood in general. The consequence is that OT could act on mood regulation to adapt it in social contexts. While highly hypothetical, this interaction could be partly responsible for OT actions on cooperation between individuals (*via* a reduction of social anxiety).

Thus, we can identify at least two mechanisms of OT and 5-HT interaction. First, in the amygdala, it would be involved in the regulation of emotions and mood, notably *via* the 5-HT_{1A}-R. Second, in the nucleus accumbens it would be implicated in social reward processing, recruiting another serotonergic receptor (1B being the best candidate so far). Interestingly, we have found modulation of 5-HT_{1A}-R signal in regions outside the amygdala, such as the hippocampus, the insula and the orbitofrontal cortex. While these regions are all involved in socio-emotional regulation, the neurobiology of OT / 5-HT interaction has not been described yet. Therefore, we cannot say if this similar upregulation of 5-HT_{1A}-R across these brain regions is underlying the same behavioural effects.

Second from a clinical point of view, the evidence that OT provokes the release of 5-HT in healthy subjects but not in patients with autism is an interesting result that opens ways to new pharmacologic strategies. As mentioned in part II.1.a., drugs targeting the 5-HT_{1A}-R are already used in some patients and their combination with OT could lead to desirable effects on social behaviour. Although they are mostly directed at comorbid symptoms of autism, such as depression and anxiety, the large amount of available molecules targeting other systems of 5-HT receptors gives many opportunities to explore combined OT and 5-HT treatments, especially in animal models of autism.

It should also be noted that our model is coherent with an over functioning 5-HT_{1A}-R. Indeed, we found that in patients with autism, even a low MPPF BP_{ND} was linked to a small volume of grey matter in the posterior putamen, which would be expected if the 5-HT_{1A}-R inhibits cellular growth and is over functioning. However, drugs actually in use are mostly partial agonists to the 5-HT_{1A}-R, which is in contradiction with an over functioning 5-HT_{1A}-R. However, it is currently too early to conclude on this issue as their mechanism of action

is not yet completely clear. Indeed, these molecules often show agonism and antagonism to dopamine receptors as well, complicating the comprehensibility of their effects on the brain. Another problem for combined OT / 5-HT treatment is the natural relationship between the two systems. As stated in part I.1.e., OT release itself is stimulated by 5-HT, and provokes the release of 5-HT, therefore, it is likely that the co administration of these molecules will not result in the simple addition of their individual effects, but in a more complex interaction. In spite of this, the current absence of pharmacological therapy and the lack of efficiency of OT alone should push further research in this way.

IV.2. Conclusion and perspectives

Hypotheses and models of OT: To conclude, I would like to present an overview of the current theories of OT behavioural impact. One of the reason this hormone is fascinating scientist is probably linked to its wide range of effects, that have not been yet encompassed in a general model. Indeed, several views have been presented, but they often fail to explain all the spectrum of OT actions.

Three main categories can be outlined. First, OT would modulate the saliency of social stimuli in particular (see for instance (Shamay-Tsoory and Abu-Akel, 2015)), through an effect of OT on the processing of stimuli (sensory cortices, amygdala and insula, see part I.2.c. and Figure 31 green part) and a modulation of the "wanting" part of the reward system (i.e. the dopaminergic meso-limbic pathway, see part I.2.e. Figure 31 red part). Second, OT would increase social reward, decision making and learning, through an action on the nucleus accumbens and prefrontal cortex (see part I.2.d. and e) (Dölen, 2015a, 2015b). This aspect of OT's role in behaviour is also linked to approach / avoidance model such as the GAAO (Figure 31 red part) (Harari-Dahan and Bernstein, 2014). Finally, OT is also an anxiolytic hormone, and several theories have claimed that the observed behavioural effects we found were all underlined by a general anxiolytic action of OT (Figure 31 blue part) (Churchland and Winkielman, 2012; Neumann and Landgraf, 2012; Neumann and Slattery, 2015). This reduction of anxiety would depend on OT effects in the amygdala, septum and BNST.

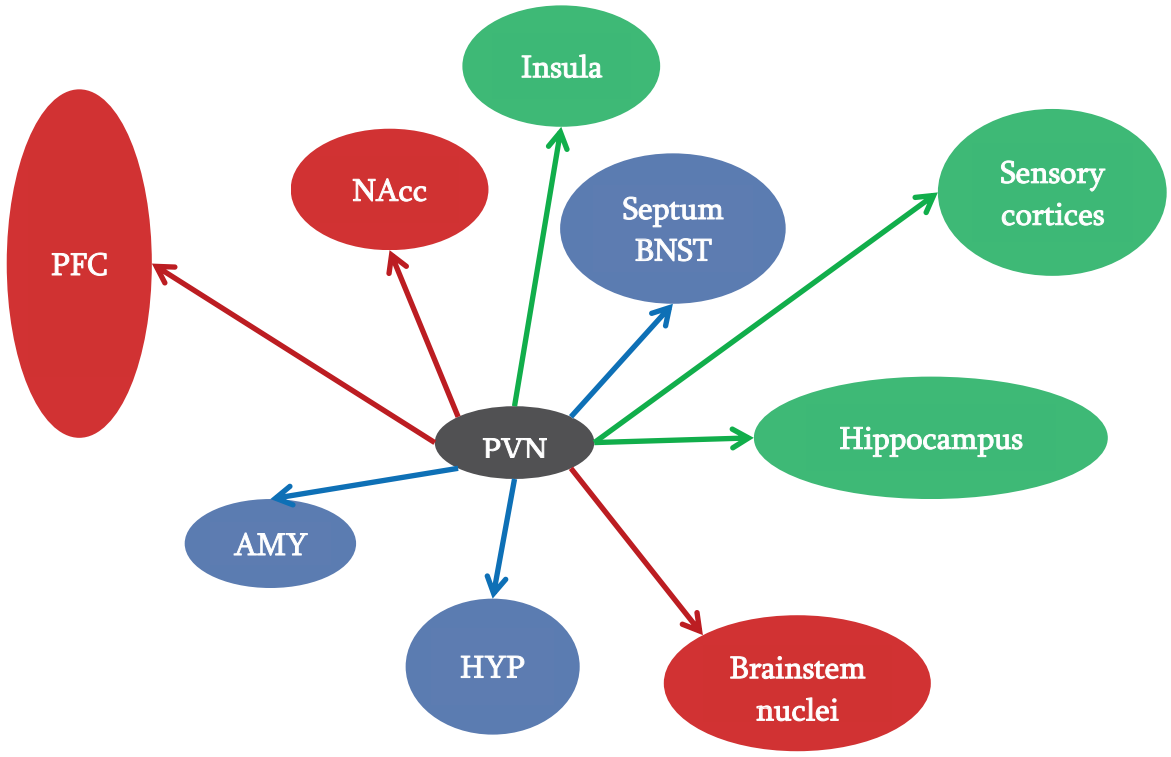

Figure 31. Schematic representation of the three main categories of OT effects: the anxiolytic property of OT is represented by the blue network, the reward modulation by the red one and the perceptual influence by the green pathway. Brainstem nuclei concerned are nucleus of tractus solitary, ventral tegmental area, vagal nucleus, raphe nuclei. NAcc = nucleus accumbens, AMY = amygdala, HYP = hypothalamus, BNST = bed nucleus of the stria terminalis and PVN = paraventricular nucleus. Note that this is a simplification and that some areas are common to several aspects of OT's action, such as the amygdala, that could be involved in perception and anxiety categories of effects. Also, peripheral effects of OT are not represented here.

It should also be noted that some models have included several of these aspects. For instance, Quintana and colleagues have hypothesized that OT effects on social behaviour were due to a dampening of anxiety *via* an action in the brainstem and the periphery, combined to a modulation of social processing (perception and decision making) through amygdala and prefrontal cortex modulation (Quintana et al., 2014). Another recent opinion tried to integrate all three aspects (perception, reward and anxiety) and stated that OT had a direct impact on social reward, increasing both "wanting" and "liking" components while decreasing anxiety in general. This joint effect would produce the modulation of social cognition and thus would increase saliency of social stimuli, but it is unclear how could this occur (Bethlehem et al., 2014).

This leads to the mains problem of theorizing the role of OT in behaviour. For now, the multiplicity of effects and brain areas submitted to OT regulation are hard to gather in a single theory. Thus most of the models are focusing on one of the three aspects, explaining the others by an indirect action. However, we have enough evidence (see part I.2.) to state that OT is exerting, at least, these three actions (perception, reward and anxiety) by acting in the amygdala, prefrontal cortex, insula and nucleus accumbens.

The complexity reaches another level with the fact that some experiments have found effects of OT in non-social context (food, pain, etc.), leading to theories in favour of a general approach / avoidance or anxiety regulation. In my view, it is too soon to establish a model for OT, and there are several reasons for that.

The amount of behavioural effects of OT is probably explained, at least in part, by the poor designs and statistical power of studies in the field (Walum et al., 2015). This is linked to the general reproducibility crisis hitting the neuroscience discipline. But this unfortunately adds to issues specific to OT like intra nasal administration, lack of dose response studies, measurements of plasma OT concentration… Therefore, before one can elaborate a general theory, some time is required to validate or invalidate the supposed effects of OT, especially the ones that cannot be tested in animals, such as trust, cooperation, and other "high level" cognitive skills.

An important factor to consider and that will help to encompass all aspects of OT's action, notably the ones occurring outside the brain (bones and muscles homeostasis, cardiac regulation, etc…) is the evolutionary aspect of OT. We indeed know that this peptide (or its equivalent) has been implicated in social behaviour and feeding in most species in which it was investigated. Therefore, this molecule first developed in organism that did not had a brain, which could explain the multitude of OT effects at the peripheral level. It is to note that a recent theory has focused on this peripheral aspect of OT (Hurlemann and Scheele, 2016; Quattrocki and Friston, 2014). This model suggests that OT modulates self-referential processing notably *via* the insula. However, the reward processing aspect of OT's action does not seem to fit this model. On the other side, it is likely that some functions of OT that were present in basic organisms and animals were lost during evolution. At the anatomical level, some researchers think for instance that OT neurons switched from a volume transmission system to an axonal system (Knobloch and Grinevich, 2014).

Nevertheless, all three main categories of OT effects are highly desirable in the case of ASD. There are many theories about autism, but the major ones are the social motivation deficit (Chevallier et al., 2012), which suggests that patients with autism would suffer from a lack of motivation for social interaction. Thus, OT would be a formidable tool if this

system could be targeted to specifically enhance social reward (both "liking" and "wanting" parts). Another theory would be that ASD are linked to an absence of cognitive empathy (or theory of mind) (Baron-Cohen et al., 1985) and again, OT's action on social perception, learning and processing of information could have positive outcomes in this context. A third well spread theory is the intense world, which states that patients are suffering of an over stimulation of the sensory system (Markram and Markram, 2010). Here as well, increasing saliency of certain (social) stimuli and reducing the importance of others would be crucially needed. Other theories such as the disorder of prediction (which resembles to the theory of mind deficit) have been formulated (Sinha et al., 2014), implying more or less relevance for the OT system. Nevertheless, it can be concluded from the theoretical frameworks presented here about OT and ASD, that the oxytocinergic systems is capable of improving the suspected core social deficits of the pathology and thus encourages further experimental research.

Direction and perspective: The present work opens several possibilities for more experiments. The most straightforward might be to use rodents to investigate the externalization process of the 5-HT_{1A}-R and the internalization of the 5-HT transporter following OT administration, in order to confirm the proposed model (Figure 30).

Another interesting study would be to use other serotonergic radiotracer (especially for the 5-HT 1B receptor) to look at the modulation induced by OT, in both healthy subjects and patients with autism. In the same direction, a crucial step would be to look at the potential heteromerization of OTR with 5-HT receptors. We indeed know the existence of OTR/D2R, so it would not be surprising to find similar structures for OT and 5-HT. To go further in this direction, designing molecules specifically modulating certain types of heteromers could improve the precision of pharmacological therapies by provoking more specific behavioural changes.

In this line, a lot of scientists are now looking for alternative ways to stimulate the oxytocinergic system, since the limitations of intra nasal OT. For instance, new bivalent OT agonists (capable to activate OTR homodimers) seem to produce stronger effects with smaller molar concentration (Busnelli et al., 2016). Others have been looking at the melanocortin receptor, which is known to trigger OT release (Modi et al., 2015) and a lot of agonists to OT (carbetocin, and many laboratory developed molecules) crossing the blood brain barrier and resisting to degradation. Some of them are partial agonists (activating only OTR coupled to a particular G protein) which could lead to more specific effects.

In addition to all these molecules, some attention should be dedicated to the vasopressinergic system, as it is involved in social behaviour as well (Albers, 2012; Caldwell et al., 2008) and may provide an interesting pathway to modulate social behaviours (especially in males since vasopressin in male voles seems to have a major importance in social behaviours).

Thus, although the field is facing the deception of the weak effects of OT (at least not strong enough to become an approved pharmacotherapy), many new paths to explore have been recently open.

In parallel, more fundamental questions are raised by our work. Indeed, we have found an interaction of OT with 5-HT in the amygdala, insula and OFC, but it remains unclear what is the role of this pathway (as opposed to the action of OT on GABAergic interneurons). Thus, another way to pursue the research presented here would be to investigate the effects of combined OT 5-HT manipulation, e.g., blocking 5-HT_{1A}-R and administering OT to see the behavioural consequence. Ideally, these could be done in non-human primates, allowing local injections and recording of electrophysiological activity, as well as tasks testing the various aspects of OT's role (perception, reward, anxiety).

To terminate, the ethical aspect of modulating social behaviour should be evoked (Wudarczyk et al., 2013). Indeed, while in the case of autism or other really impairing social disorders, the use of pharmacotherapy to improve their behaviour is easily understandable, the presence of these molecules on the market bring some more difficult questions. Despite its feeble effects, OT is already sold by some unscrupulous companies. However, the consequence on health and social aspect of people taking it should be negligible, thus it remains "only" a financial scam. However, the situation would be completely different in the case of effective drugs enhancing or degrading social behaviour. Especially since the effects of OT stimulation will not be easily detectable as it is a natural hormone and behavioural modulation should be subtle in healthy subjects. If we disregard the case of people drugging others, as this is obviously condemnable, it should be questioned to what extent society should let people modulate their own social behaviours? The consequences of a population with no shyness are hard to imagine but should not be considered lightly as it might produce some instability. The life mode we adopted is in part based on social norms regulating most of daily social interactions. Therefore, introducing molecules modulating social behaviour may have an impact when looking at this level.

V. References

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VI. Annexes

VI.1. Blood microsampling from the ear capillary in non-human primates

These article was made in collaboraton with Sebastien Ballesta. The aim was to develop a blood sampling technique suitable for animal undergoing head restained behavioural tasks and possibly electrophysiological recordings.

Lab Anim OnlineFirst, published on May 12, 2015 as doi:10.1177/0023677215586911

Short Report

Laboratory Animals
0(0) 1–4

DOI: 10.1177/0023677215586911
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Blood microsampling from the ear capillary in non-human primates

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Abstract

Blood sampling from awake non-human primate (NHP) is classically performed under constraint in the cephalic or saphenous vein. It is a challenging, potentially harmful and stressful procedure which may lead to biased results and raises ethical concerns. Laboratory NHPs undergo a head-restrained procedure allowing for a safer procedure to collect blood from NHPs ears. Using regular capillary blood collection devices 500µL of blood can be easily withdrawn per puncture point which is sufficient to perform most of the usual modern biological assays. This procedure has been validated by measuring total proteins, cortisol and vasopressin concentrations from concomitant blood samples taken from the saphenous vein and the ear capillary vessels of macaques (n=16). We observed strong correlations between the blood concentrations of total proteins, cortisol and vasopressin (respectively: r=0.72, r=0.63, r=0.83, all p-values<0.01) taken from the saphenous vein and from the ear capillary. There were no significant differences between blood concentrations taken from the saphenous vein and the ear capillary. Our alternative to classical blood collection procedure is harmless and can be routinely performed, therefore can improve scientific results whilst increasing animal welfare in accordance to the 3R principles.

Keywords: *Blood sampling; non-human primates; head-restrained; refinement*

Introduction

When it comes to non-human primates (NHP), researchers face ethical concerns which sometimes require a shift of experimental strategy (Abbott, 2014). Classical methods of blood sampling in non-human primates usually imply challenging and stressful procedures which increase the risk of injury for both the animals and the experimenters. In addition, extensive handling of the primates by the experimenter requires habituation. Such trust relationship might be altered by this potentially harmful sampling procedure, making behavioural training harder and longer. In addition, the behavioural and physiological consequences of stress can induce potential scientific misinterpretation (reviewed in (Reinhardt, 2003)).

Despite a recent growth of interest in micro sampling methods in rodents (Nilsson et al., 2013), no comparable alternative blood sampling method has been reported for non-human primates. We propose here an original micro sampling method from the ear capillary of head-restrained NHPs. Since the blood contains biomarkers that provide insights into brain functioning (Filiou and Turck, 2011), the need for a convenient and ethical blood sampling procedure for NHP used in neurosciences is thus important. To validate our sampling method, we assessed the concentrations of plasma total proteins and two hormones (cortisol and vasopressin) in samples taken simultaneously from the ear capillary and the saphenous vein. Our hypothesis is that blood collected from the ear capillary will give similar information to blood collected from the saphenous vein.

Methods

Animals

This study was approved by our local animal experimentation ethics committee (CELYNE) and used experimental procedures complying with the recommendations of the local authorities on Animal Care (Direction Départementale des Services Vétérinaires, Lyon, France) and the European Community standards for the care and use of laboratory animals. All animals were individually or socially housed at the Centre de Neuroscience Cognitive in Bron, France. Subjects were 16 macaques (14 males, 9 mulatta and 7 fascicularis, mean age=6.1, sd=2.9, mean weight=7.4 kg, sd=1.7).

Blood sampling procedures

We took advantage of a veterinary control procedure that required anesthetizing the animals (ketamine 10mg/kg) in the morning to collect concomitant blood samples from the ear capillary and the saphenous vein.

Blood collection from the saphenous vein was performed using EDTA tubes and a 23G needle.

We describe in Figure 1 the ear capillary blood sampling procedure on vigilant animals. Once the subjects had had their head fixed using a classic head restraint system, we first familiarized them with ear manipulation. Prior to the puncture, the ear was cleaned and shaved in order to avoid blood contaminations. The ideal locations to collect blood are around the lobule vein and at the extremity of the ear (see Figure 1A). However, it is also possible to collect blood in other areas of the external ear. Using a micro puncture system (Safety-Lancet super, blade of 1.5mm, Sarstedt), a small puncture was made at the selected location which was adapted to each monkey (Figure 1B). Immediately after the puncture, a drop of blood appeared and started to ooze out (Figure 1C). The first drop of blood was systematically discarded. Blood was collected using a Microvette® 300 (Sarstedt), with the tip inclined at 45° downwards to the drop of blood to insure optimal collection (Figure 1D). If the blood flow stopped before the desired volume was collected, massaging around the puncture site was performed to stimulate blood flow. Depending on the puncture site, 100 µL to more than 500µL of blood can be withdrawn from each puncture point. Once enough blood was collected, a one-minute compression of the puncture site was performed to allow proper coagulation. The whole process requires only one experimenter.

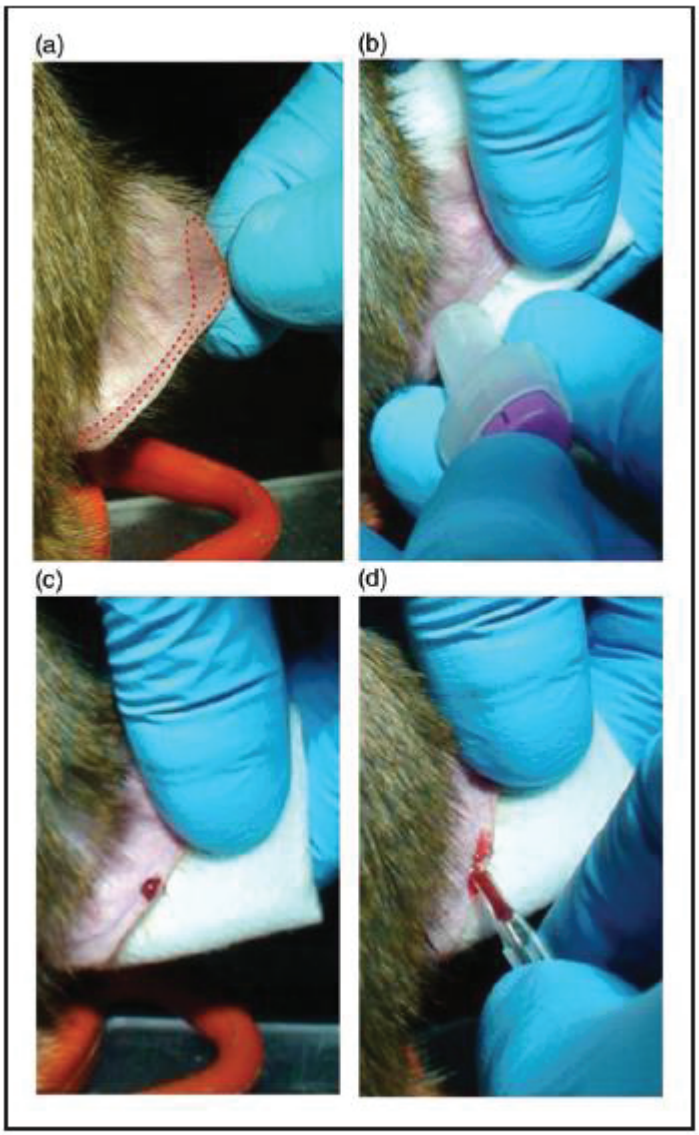

Figure 1. Ear capillary blood sampling procedure. (a) Optimal puncture site is delimited by a dashed line. (b) Puncture performed using a Safety-Lancet. (c) A successful puncture. (d) Blood collection using a Microvette®.

Assays

All blood samples were immediately centrifuged at 4°C for 10 minutes at 2000xg to separate and extract plasma. Plasma was stored at -80°C until assay. Total protein concentration was assessed with a Lowry protein colorimetric assay (Bio-Rad), cortisol and vasopressin levels with a commercially prepared enzyme immunoassay kits (respectively: Life Science Inc. and Enzo Life Science). Due to technical issues, only twelve concomitant

samples could be assessed for vasopressin. Intra-assay coefficients were all <10%. Excepting 3 samples for cortisol, none of the samples fell below assay sensitivity.

Results

All p-values are corrected for multiple comparisons (Bonferroni). No significant differences were found between venous and capillary concentrations of protein, vasopressin and cortisol (Wilcoxon signed rank test, all p-values>0.1).

Concentrations of capillary proteins were correlated with concentrations of venous proteins (r=0.73, p=0.008; Figure 2A). To correct for non-Gaussian distribution, cortisol and vasopressin concentrations values were log transformed, we found correlations between venous concentrations and capillary concentrations (respectively: r=0.84, p<0.01and r=0.65, p=0.021, Figure 2B and C).

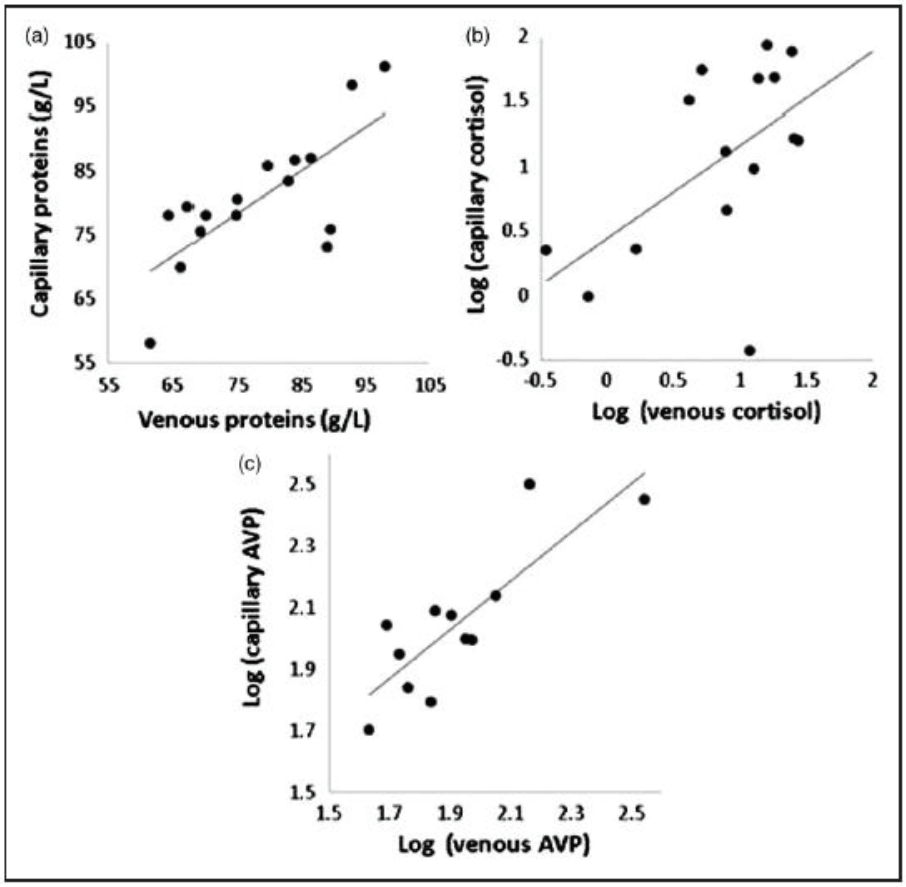

Figure 2. Correlations between venous and capillary concentrations. (a) Total proteins. (b) Cortisol. (c) Vasopressin. AVP = vasopressin.

Discussion

The present study proposes ear capillary blood sampling as a suitable and harmless way to collect blood from a head-restrained NHP.

Our physiological analysis validates this method as a reliable technique to measure blood concentration meaningful biomarkers such as cortisol or vasopressin. Indeed, all measured biomarkers strongly correlated between ear capillary samples and venous samples. Moreover, there were no significant differences between blood concentrations taken from the saphenous vein and the ear capillary.

This blood sampling technique requires minimal animal handling and is unlikely to produce any significant discomfort. Indeed, even though no controlled objective measures of pain were performed, we did not notice any changes in facial mimicry or general activity of the macaque whilst being sampled. Importantly, the ear lobe puncture has been shown to be less painful than classical arterial puncture in humans (Dar et al., 1995). In addition, our sampling method was performed in the animal facility for more than one year and we did not notice any infections of the puncture site. Additionally, as neurosciences experiments may require water restrictions, we propose that such sampling methods should also be routinely used to assess the hydration states of the animals (Yamada et al., 2010).

The main limit of our procedure is the lower amount of blood that can be collected compared to classical venipuncture. However, recent assay procedures require smaller volumes (usually around 200 µL of plasma), therefore, depending on the number of molecules measured, one puncture should generally provide enough blood for further biological analysis.

In conclusion, ear capillary blood sampling is a good alternative when behavioural training is precluded. It can represent a routine procedure in neurosciences as it provides unaltered data, increases the well-being and safety of both animals and experimenters and requires only one experimenter to be performed.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.