Caractérisation of GABAergic neurotransmission within basal ganglia circuit in R6/1 Huntington’s disease mouse model
Zhuowei Du

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Par Zhuowei DU

Characterisation of GABAergic neurotransmission within basal ganglia circuit in R6/1 Huntington’s disease mouse model

Sous la direction de Maurice GARRET

Soutenue le 21 février 2014

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Titre : Characterisation de la neurotransmission GABAergique dans les ganglions de la base chez le modèle murin R6/1 de la maladie de Huntington

Résumé : Nous avons étudié les récepteurs GABA_A dans un modèle de la maladie de Huntington. En combinant des approches biochimiques, moléculaires, électrophysiologiques et de l'imagerie haute résolution, nous avons montré une modification de la neurotransmission GABAergique chez des animaux à des stades pré- et post-symptomatiques. Nos études montrent une diminution de la neurotransmission GABAergique dans le globus pallidus des souris Huntington qui pourrait conduire à une modification des noyaux de sortie des ganglions de la base et de l’activité motrice. L’ensemble de nos résultats permet de définir le rôle de différents types de récepteurs GABA_ A dans le cerveau dans des conditions physiologiques et pathologiques.

Mots clés : récepteurs GABAA, synapses, Striatum, Globus Pallidus, Interneurones.

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Title : Characterisation of GABAergic neurotransmission within basal ganglia circuit in R6/1 Huntington’s disease mouse model

Abstract : We explored GABAergic neurotransmission in a mouse model of Huntington’s disease. Combining molecular, imaging and electrophysiological techniques, we showed changes of GABAergic neurotransmission in presymptomatic and symptomatic R6/1 mice. Our data demonstrated a decreased GABAergic inhibition in the globus pallidus of R6/1 mice, which could result in an alteration of basal ganglia output nuclei and motor activity. Taken together, our results will help to define the contribution of receptor subtypes to inhibitory transmission throughout the brain in physiological and pathophysiological states.

Keywords : GABAA receptors, synapses, Striatum, Globus Pallidus, Interneurones.

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Abbreviation

Ach: acetylcholine

AMPA: $\alpha$-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BACHD: bacterial artificial chromosome HD

BDNF: brain-derived neurotrophic factor

BG: basal ganglia

BIG2: brefeldin A-inhibited GDP/GTP exchange factor 2

CB: cannabinoid

CBP: CREB binding protein

ChAT: choline acetyl transferase

CPu: caudate putamen

CR: calretinin

CREB: cAMP response element-binding protein

DA: dopamine

DARPP-32: dopamine- and cAMP-regulated phosphoprotein of 32 kDa

Drp-1: dynamin-related protein 1

DYN: dynorphin

ENK: enkephalin

EP: entopeduncular nucleus

ER: endoplasmic reticulum

ERAD: ER associated degradation

FSI: fast spiking interneuron

GABA: $\gamma$-aminobutyric acid

GABARAP: $\text{GABA}_A$R associated protein

GAD: glutamic acid decarboxylase

GAT: GABA transporter

GODZ: Golgi-specific DHHC zinc finger protein

GP: globus pallidus

GPCR: G Protein-Coupled Receptors

GPe: GP external segment

GPi: GP internal segment

GRIP: glutamate receptor interacting protein

HAP1: huntingtin-associated protein 1

HD: huntington’s disease
**HEAT**: Huntington, Elongation factor 3, protein phosphatase 2A, and the lipid kinase TOR1

**Hip-1**: Htt-interacting protein-1

**Hippi**: Hip-1 protein interactor

**HTT**: huntingtin gene

**Htt**: huntingtin

**IPSC**: inhibitory postsynaptic current

**IPSC**: large inhibitory postsynaptic current

**IPSP**: inhibitory postsynaptic potential

**KIF5**: kinesin family motor protein 5

**LTD**: long-term depression

**LTP**: long-term potentiation

**MAPK/ERK**: Mitogen-activated protein kinases, also named Extracellular signal-regulated kinases

**mGluR**: Metabotropic glutamate receptors

**MMPs**: Matrix metalloproteinases

**MSK-1**: mitogen- and stress-activated protein kinase-1

**MSN**: medium spiny neuron

**NES**: nuclear export signal

**NL2**: neuroligin2

**NMDA**: N-Methyl-D-aspartate

**nNOS**: neuronal nitric oxide synthase

**NPY**: neuropeptide Y

**NRSF**: neuro-restrictive silencer factor

**NSF**: N-ethylmaleimide-sensitive factor

**PACSIN 1**: PKC and CK2 substrate in neurons 1

**PGC-1α**: peroxisome proliferator-activated receptor gamma coactivator-1 α

**PLIC-1**: protein that links integrin-associated protein with the cytoskeleton-1

**PLTS**: persistent low-threshold spiking interneuron

**polyQ**: polyglutamine

**PPE**: preproenkephalin

**PRIP1/2**: Phospholipase-C-related catalytically inactive proteins 1 and 2

**PSD95**: postsynaptic density protein 95

**PV**: parvalbumin

**REST**: repressor element-1 silencing transcription factor

**ROS**: reactive oxygen species
**SNc**: substantia nigra pars compacta

**SNr**: substantia nigra pars reticulata

**SP**: substance P

**STN**: subthalamic nucleus

**TCA cycle**: tricarboxylic acid cycle

**TH**: tyrosine hydroxylase

**TM**: transmembrane domain

**TOR**: target of rapamycin

**TrkB**: tyrosine kinase receptor

**UPS**: ubiquitin–proteasome system

**VGAT**: vesicular GABA transporter

**VGLUT**: vesicular glutamate transporter

**VMAT**: vesicular monoamine transporter

**VTA**: ventral tegmental area

**YAC**: yeast artificial chromosome
1 Introduction

1-1 GABAergic neurotransmission

\(\gamma\)-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brains, together with glycine which is mainly distributed in the spinal cord, they compose the inhibitory neurotransmission system in the mammalian CNS. In mammalian brains, GABAergic inhibition is essential for controlling excitatory signal transmission, maintaining the excitatory/inhibitory balance of neuronal circuits and filtering input/output information (Smith and Kittler, 2010). Once GABAergic neurons are activated, GABA is released to the inhibitory synaptic cleft from presynaptic compartment and binds to specific transmembrane receptors on the plasma membrane of pre-, post- and extra-synaptic regions. The major effect of GABA is inhibitory in adult brain, but during embryonic or early postnatal development stage it also can be excitatory (Li and Xu, 2008). In mature mammalian brains, the binding of GABA to its receptors results in the influx of Cl\(^-\) and hyperpolarization of the neuron therefore inhibits the generation of action potentials (Goetz et al., 2007). Deficits in GABAergic neurotransmission is implicated in various psychiatric and psychological diseases, including epilepsy, Down syndrome, anxiety disorders, depression, schizophrenia, and autism (Fritschy, 2008, Rudolph and Mohler, 2013). In some neurodegenerative diseases such as HD and PD, the dysfunction of GABAergic neurotransmission also contributes to the motor symptoms.

1-1-1 GABA synthesis, enrichment and degradation

In GABAergic neurons, GABA is synthesized from the excitatory neurotransmitter glutamate using the enzyme glutamate decarboxylase (GAD). There are two isoforms of GAD, GAD65 (also named GAD2) and GAD 67 (also named GAD1), which are named by their molecular weight. The GAD65 is reported to directly interact with the vesicular GABA transporter VGAT (or VIAAT, vesicular inhibitory amino acid transporter), indicating that when glutamate is present at the presynaptic cytosol of GABAergic neurons, it rapidly converts into GABA and enriches in presynaptic vesicles (Jin et al., 2003). There are membrane-bound glutamate transporters EAAT3 (Excitatory amino-acid transporter 3) at presynaptic terminals of inhibitory neurons, which are responsible for taking up
Figure 1-1. Schematic drawing of transmitter release, transport, and synthesis at a GABAergic synaptic terminal. The axonal ending of an inhibitory interneuron (PRE) is drawn on the left, a glial cell (GLIA) on the right. Bottom structure indicates postsynaptic membrane of a target cell (POST), for example, a pyramidal neuron. Transporters are marked by flanking arrows, and synthesizing or degrading enzymes are marked by a centred arrow. Transporters are colour matched to substrates: GABA is shown as blue particles, glutamate in red, and glutamine in green. GS: glutamine synthetase, Mit: mitochondrion, PAG: phosphate-activated glutaminase, SV: synaptic vesicle, and VATPase: vacuolar-type H+-ATPase. For other abbreviations, see the main text. From (Roth and Draguhn, 2012).
glutamate to presynaptic cytosol and serve as GABA synthesis source (Conti et al., 1998a, He et al., 2000) (Fig. 1-1). Recent studies show that glutamine may also serve as an important source for GABA synthesis in immature tissue or during periods of increased synaptic activity (Liang et al., 2006, Brown and Mathews, 2010).

GABA is enriched in presynaptic vesicles of GABAergic neurons by VGAT which is embedded in the vesicular membrane and uses the electrochemical gradient for H⁺ to absorb GABA into small synaptic vesicles (Hsu et al., 1999, Ahnert-Hilger and Jahn, 2011, Roth and Draguhn, 2012). Additionally, chloride gradients between vesicle lumen and presynaptic cytosol may contribute to the vesicular loading of GABA (Ahnert-Hilger and Jahn, 2011, Riazanski et al., 2011, Roth and Draguhn, 2012). It is estimated that the concentration of GABA within vesicles could be as high as 1000 folds comparing to presynaptic cytosol (Edwards, 2007). After release, GABA is cleared and taken by membrane-bound GABA transporters (GATs) into neurons or glial cells. GAT-1 is expressed mainly on neurons while GAT-3 is predominantly observed on glial cells, although the different GAT isoforms are partially overlapping (Minelli et al., 1996, Ribak et al., 1996, Conti et al., 1998b). This uptake also contributes to the modulation of GABAergic neurotransmission (Fig. 1-1).

GABA is finally degraded by GABA transaminase (GABA-T) in the mitochondria of neurons and glial cells. GABA-T induces transamination of GABA and α-ketoglutarate, producing succinic semialdehyde and glutamate (Kugler, 1993). It is estimated that more than 90% of all GABA in the mammalian CNS is degraded in this way and contributes to energy metabolism in the TCA cycle (Roth and Draguhn, 2012). Taking together, the GABA concentration in synaptic vesicles, cytosol and extracellular space is the result of the balance among synthesis, enrichment and degradation. The equilibrium of these mechanisms is important to maintain the physiological role of GABAergic neurotransmission.

1-1-2 GABA receptors

There are mainly two types of GABA receptors which are chloride permeable ligand-gated ion channels (GABA_A receptors) and metabotropic G-protein-coupled GABA_B receptors (GABA_BRs). GABA_A receptors (GABA_ARs) facilitate fast inhibition and play the major role of inhibitory receptors; GABA_BRs mediate a “slow” and “late” inhibition, or function
as auto-receptors that control the typical negative feedback loop of synapses when expressed presynaptically (Isaacson et al., 1993, Misgeld et al., 1995, Scanziani, 2000). Evidence shows that that synaptically released neurotransmitters saturate their receptors (Clements, 1996). Therefore, the strength of GABAergic synapses highly depends on the number of postsynaptic GABA\textsubscript{A}Rs (Otis et al., 1994, Nusser et al., 1997)

1-1-2-1 Structure and assembly of GABA\textsubscript{A}R

GABA\textsubscript{A}Rs are heteropentameric ligand-gated ion channels. So far, totally 19 GABA\textsubscript{A} receptor subunits are identified and grouped into eight subclasses, including six \(\alpha\) (1-6) subunits, three \(\beta\) (1-3) subunits, three \(\gamma\) (1-3) subunits, three \(\rho\) (1-3) subunits and one each of \(\delta\), \(\sigma\), \(\varepsilon\), \(\theta\), \(\pi\) subunit. All of these subunits share a similar structure that includes an extracellular N-terminal domain, four transmembrane domains (TM1-4), and an extended cytoplasmic loop region between TM3 and TM4 that mediates interactions with trafficking and signaling factors (Luscher et al., 2011). A typical GABA\textsubscript{A}R consists of two \(\alpha\) subunits, two \(\beta\) subunits and one \(\gamma\) subunit (can be replaced by other subunits) (Olsen and Sieghart, 2009). Different subunits are distributed differently and have diverse properties, for example the \(\delta\)-containing receptors are usually located extrasynaptically and mediate tonic inhibition; \(\alpha5\)-containing receptors at extrasynaptic sites of pyramidal cells also facilitate \(\gamma\) oscillation and tonic inhibition (Luscher et al., 2011, Rudolph and Mohler, 2013). Different brain regions also have preference to specific subunits, such as in the GP, the levels of \(\alpha1\) and \(\beta2\) are much higher than the striatum while in the striatum, \(\alpha2\) and \(\beta3\) subunits are more expressed (Hortnagl et al., 2013).

1-1-2-2 GABA\textsubscript{A}R anchoring at cell membrane

After synthesis, the GABA\textsubscript{A}Rs are delivered from the endoplasmic reticulum (ER) to the secretory pathway by interaction of \(\alpha\) and \(\beta\) subunits with the protein that links integrin-associated protein with the cytoskeleton-1 (PLIC-1). PLIC-1 may inhibit ubiquitination and ER associated degradation (ERAD) of \(\alpha\) and \(\beta\) subunits, therefore overexpression of it can increase the surface level of GABA\textsubscript{A}Rs (Bedford et al., 2001). In order to deliver GABA\textsubscript{A}Rs to cell membrane, they need to be translocated to Golgi bodies first. The Golgi-specific DHHC zinc finger protein (GODZ) plays an important role and serves to facilitate ER to Golgi translocation of GABA\textsubscript{A}Rs containing \(\gamma2\) subunit. The GABA\textsubscript{A}R \(\gamma2\) subunit is subject to palmitoylation and this modification plays a central role in the surface delivery, clustering and synaptic targeting of GABA\textsubscript{A}Rs.
Figure 1-2. Trafficking of GABAA receptors. The fate of GABA\(_A\)R subunits can be modulated by ubiquitylation and subsequent ER-associated degradation by the proteasome. Ubiquitylated GABA\(_A\)R subunits can also be modulated by their association with PLIC1. PLIC1 facilitates GABA\(_A\)R accumulation at the synapse by preventing the degradation of ubiquitylated GABA\(_A\)Rs. Exit into the Golgi network and subsequent trafficking to the plasma membrane are also facilitated by a number of GABA\(_A\)R-associated proteins. GABARAP associates with the \(\gamma_2\) subunit of GABA\(_A\)Rs and aids in the trafficking of GABA\(_A\)Rs from the Golgi network to the plasma membrane. NSF and BIG2 are also localized to the Golgi network, where they bind to the \(\beta\) subunits of GABA\(_A\)Rs and modulate GABA\(_A\)R trafficking. Palmitoylation of \(\gamma\) subunits occurs in the Golgi apparatus as a result of an association with the palmitoyltransferase GODZ, and is a critical step in the delivery of GABA\(_A\)Rs to the plasma membrane. GRIFs have a role in the trafficking of GABA\(_A\)Rs to the membrane. PRIPs also have essential roles in the trafficking of GABA\(_A\)Rs and in modulating the phosphorylation state of GABA\(_A\)Rs. From (Jacob et al., 2008).
(Keller et al., 2004, Rathenberg et al., 2004, Earnheart et al., 2007). The GODZ is considered as a structurally related palmitoyltransferases and is able to palmitoylate the γ2 subunit. This was shown by reducing the expression of GODZ by shRNA leading to selective loss of GABA_ARs at synapses, as well as decreased whole-cell currents and miniature inhibitory synaptic currents (mIPSCs) (Fang et al., 2006) (Fig. 1-2). Once inside Golgi, GABA_ARs proceed through the trans-Golgi network toward the plasma membrane in association with BIG2 (brefeldin A-inhibited GDP/GTP exchange factor 2) (Shin et al., 2004). BIG2 interacts with the intracellular loop of β subunits and is implicated not only in the exit of GABA_ARs to cell membrane but also in endocytosis of GABA_ARs recycling (Charych et al., 2004, Boal and Stephens, 2010). The GABA_AR associated protein (GABARAP) interacts directly with all γ subunits and is enriched in Golgi and other somatodendritic membrane compartments, and is implicated in GABA_ARs trafficking (Wang et al., 1999, Kittler et al., 2001, Nymann-Andersen et al., 2002) (Fig. 1-2). It is reported that the attachment of phosphatidyl ethanolamine (PE) to the C-terminus of GABARAP is required for dendritic accumulation of GABARAP and for GABARAP-induced cell surface expression of GABA_ARs (Chen and Olsen, 2007, Luscher et al., 2011). Besides the γ subunits, GABARAP interacts with several proteins involved in GABA_ARs trafficking, including Phospholipase-C-related catalytically inactive proteins 1 and 2 (PRIP1/2) which are GABA_AR-associated adaptor proteins for phosphatases and kinases; N-ethylmaleimide-sensitive factor (NSF) which is an ATPase and chaperone of SNARE complexes and important for receptors trafficking; glutamate receptor interacting protein (GRIP) (Kanematsu et al., 2002, Kittler et al., 2004a, Morgan and Burgoyne, 2004, Zhao et al., 2007) (Fig. 1-2). Both PRIP1/2 and NSF directly or indirectly (via GABARAP) interact with β subunits (Kanematsu et al., 2002, Kittler et al., 2004a, Terunuma et al., 2004). PRIP1/2 knockout mice show reduced expression of GABA_ARs as well as decreased association of γ2 subunit-containing GABA_ARs with GABARAP, indicating that PRIP facilitates indirect association of GABARAP with GABA_ARs (Mizokami et al., 2007). NSF and GRIP are involved in activity-induced and Ca^{2+} calmodulin dependent kinase II (CaMK II)-mediated translocation of GABA_ARs to the plasma membrane (Marsden et al., 2007).

1-1-2-3 GABA_AR endocytosis, recycling and degradation

GABA_ARs undergo dynamic exchange between cell surface and intracellular compartments via clathrin- and dynamin- dependent pathways. The endocytosis of
Figure 1-3. Regulation of GABAA receptor endocytosis and post-endocytic sorting. Clathrin-dependent endocytosis is the major internalization mechanism for neuronal GABA₃Rs. The intracellular loops of the GABA₉R β and γ subunits interact with AP2 complex. Once the GABA₉Rs have been endocytosed in clathrin-coated vesicles (CCVs), the vesicles uncoat and fuse with early or sorting endosomes, resulting in the GABA₉Rs being subsequently recycled to the plasma membrane or degraded in lysosomes. HAP1 interacts with the β subunits and promotes receptor recycling to the plasma membrane. From (Jacob et al., 2008).
GABA<sub>A</sub>Rs is regulated by the clathrin adaptor protein AP2 and the phosphorylation state of β and γ subunits (Kittler et al., 2000, Kittler et al., 2005, Kittler et al., 2008). It is reported that the binding affinity of AP2 to β or γ subunits via conserved motifs and the internalization of GABA<sub>A</sub>Rs are negatively regulated by the phosphorylation state (Kittler et al., 2005). Several protein kinases involved in this process, such as PKA, PKC, CaMK II and Akt for β subunits, may regulate the endocytosis of GABA<sub>A</sub>Rs and the strength of GABAergic inhibition (McDonald and Moss, 1994, McDonald et al., 1998, Brandon et al., 2000, Wang et al., 2003) (Fig. 1-3). A dramatic decrease of GABAergic inhibition is observed in animal models of status epilepticus, in which the PKC-mediated phosphorylation of GABA<sub>A</sub>R β subunits is impaired (Terunuma et al., 2008). The β subunits-mediated GABA<sub>A</sub>Rs endocytosis can also be regulated by PRIP1/2 as an adaptor for the serine/threonine-specific phosphatases PP1α and PP2A. The phosphorylation of PRIP in PRIP/PP1α complex or the binding of PRIP to PP2A leads to the activation of the two phosphatases, respectively, and β subunits dephosphorylation-induced internalization (Terunuma et al., 2004, Kanematsu et al., 2006). Comparatively, the γ2 subunit has a different C-terminal specific motif which binds to AP2 via the negative regulation of Fyn and other Src kinase members (Lu et al., 1999, Kittler et al., 2008, Smith et al., 2008, Jurd et al., 2010) (Fig. 1-3).

The internalized GABA<sub>A</sub>Rs can be either recycled to plasma membrane again or degraded by lysosomes (Arancibia-Carcamo et al., 2009, Twelvetrees et al., 2010). It has been observed that β1-3 subunits are associated with huntingtin-associated protein1 (HAP1) which interacts with huntingtin protein and is involved in neuronal cargo transport. Over expression of HAP1 in cultured neurons interferes with the degradation of internalized GABA<sub>A</sub>Rs by enhancing their cell membrane expression (Kittler et al., 2004b) (Fig. 1-3). A recent study discovered that HAP1 works as an adaptor for the kinesin superfamily member 5 (KIF5) motor protein complex which links to GABA<sub>A</sub>R containing transport vesicles and mediates the delivery of internalized GABA<sub>A</sub>Rs to synapses (Twelvetrees et al., 2010). In addition, the calcium modulating cyclophilin ligand (CAML) is identified to interact with γ2 subunit and may regulate GABA<sub>A</sub>Rs recycling. Deletion of CAML in neurons results in reduced accumulation of GABA<sub>A</sub>Rs at the plasma membrane and at synapses (Yuan et al., 2008). Another recent report detected a Maf1-interacting coiled-coil protein (Macoco) as a novel interacting protein of β3 and γ2 subunits. Overexpression of Macoco increases the surface level of GABA<sub>A</sub>Rs, implicating a role in GABA<sub>A</sub>Rs re-
insertion (Smith et al., 2010). The GABA$_A$Rs that are not involved in recycling will be targeted by ubiquitin system at $\gamma$2 subunits for lysosomal degradation. Additionally in the ER, the ERAD of $\alpha$ subunits and $\beta$ subunits are also regulated by ubiquitin-mediated proteasomal degradation (Arancibia-Carcamo et al., 2009). Disruption of lysosomal functions increases the efficacy of synaptic inhibition (Arancibia-Carcamo et al., 2009).

1-1-2-4 Scaffold proteins, adhesion molecules and GABA$_A$Rs

Scaffold proteins have crucial functions in synapse organization, receptor stabilization and intracellular signaling pathways. At GABAergic synapses, gephyrin which is a 93 KDa polypeptide is considered to play this important role (Sassoe-Pognetto et al., 1999, Sassoe-Pognetto and Fritschy, 2000, Sassoe-Pognetto et al., 2000, Fritschy et al., 2008). Gephyrin interacts with microtubules as well as several regulators of microfilament to form a microtubule and microfilament-associated hexagonal protein lattice that may organize the spatial distribution of receptors and other proteins in the postsynaptic membrane (Mammoto et al., 1998, Ramming et al., 2000, Luscher et al., 2011). Evidence shows that the ability of gephyrin to cluster GABA$_A$Rs is controlled by collybistin which is a guanine nucleotide exchange factor (GEF) that selectively activates the Rho GTPase Cdc42 (Reid et al., 1999, Kins et al., 2000, Fritschy et al., 2008). Although Cdc42 is dispensable for gephyrin and GABA$_A$Rs clustering, collybistin is colocalized with gephyrin at inhibitory synapses and collybistin knockout leads to loss of gephyrin and GABA$_A$Rs clusters in the hippocampus, cortex and basolateral amygdala (Papadopoulos et al., 2007, Reddy-Alla et al., 2010, Saiepour et al., 2010). However, the loss of collybistin may be compensated by other GEFs in in several other brain areas where the clustering of gephyrin and GABA$_A$R is not affected. Most collybistin isoforms generated by alternative splicing contain an SH3 domain which negatively regulates the functions of collybistin (Kins et al., 2000, Harvey et al., 2004). Nevertheless, with the presence of neuroligin2 (NL2), the inhibitory function of SH3 domain in collybistin is abolished therefore collybistin is capable to recruit gephyrin to synaptic compartment and to assemble inhibitory synaptic scaffold (Poulopoulos et al., 2009). Interestingly, gephyrin is not always necessary for the clustering of GABA$_A$Rs, for example a major subset of GABA$_A$Rs comprising $\alpha$1$\beta$$\gamma$2 receptors can accumulate and cluster at synapses independently of gephyrin (Kneussel et al., 2001, Levi et al., 2004, Luscher et al., 2011). Considering the loss of synaptic localization of GABA$_A$Rs composed of $\alpha$2$\beta$$\gamma$2/$\alpha$3$\beta$$\gamma$2 subunits, the scaffolding function of gephyrin at synapses may be receptor subtype specific (Essrich et al., 1998, Kneussel et al., 1999). Recently, the glycogen
synthase kinase 3β (GSK3β) was identified to regulate phosphorylation- and proteolytic cleavage-induced turnover of gephyrin (Tyagarajan et al., 2011). As lithium is a potent GSK3β inhibitor, the lithium mediated enhancement of GABAergic neurotransmission and mood-stabilizing effects for bipolar disorder can be explained by the reduction of gephyrin turnover (Tyagarajan and Fritschy, 2010).

The integrity of synaptic structure which is critically dependent on adhesion complex is fundamental to synaptic function. A typical synaptic adhesion complex is composed of highly interacted presynaptic neurexins with postsynaptic neurexins (Ushkaryov et al., 1992, Ichtchenko et al., 1995, Ichtchenko et al., 1996, Luscher et al., 2011). Interestingly, overexpression of neurexins in neurons induces presynaptic development of GABAergic or glutamatergic synapses, while the increased neurexins may also lead to the formation of GABAergic or glutamatergic postsynaptic compartment (Graf et al., 2004, Chih et al., 2005, Chubykin et al., 2007). At inhibitory synapses including GABAergic synapses, NL2 is the one that selectively located and required for structural and functional synaptic maturation (Graf et al., 2004, Varoqueaux et al., 2004). As what has been described above, addition to forming adhesion complex, NL2 interacts with gephyrin and collybistin, thus contributes to GABA_A R clustering. However, NL2 does not well colocalized with gephyrin and is dispensable for GABA_A R clustering at synapses, indicating some other potential adhesion molecules may contribute to GABAergic synapses (Varoqueaux et al., 2006, Hoon et al., 2009).

It has been reported that the binding of GABA_A Rs to inhibitory scaffold protein is mediated by direct interaction between gephyrin and α2 subunit (Tretter et al., 2008). However the affinity of this interaction is low and cannot strictly confine the GABA_A Rs. As a result, the receptors may travel dynamically on postsynaptic membrane, although it is reported that the clustered synaptic receptors exhibit significantly lower rates of mobility (Jacob et al., 2005). It has been shown that the insertion of GABA_A Rs into plasma membrane occurs predominantly at extrasynaptic regions and they travel to synaptic membranes by lateral diffusion afterwards (Jacob et al., 2005, Thomas et al., 2005). Taken together, the GABAergic inhibition is not only regulated by receptor internalization and recycling, but also lateral diffusion at plasma membrane. Different subunits may have different preference of receptor distribution, such as α4, α5, α6, and δ subunits are mainly localized at extrasynaptic membranes while α2 and α3 subunits are more likely synaptic (Mody, 2001, Egawa and Fukuda, 2013b). It is of interest that GABA_A Rs with low agonist
affinity appear to be clustered at postsynaptic sites, whereas receptors with high affinity are mostly found extrasynaptically (Semyanov et al., 2004, Roth and Draguhn, 2012). Additionally, due to the importance of chloride in GABAergic neurotransmission, the activation of GABA\textsubscript{A}Rs also depends on Cl\textsuperscript{−} equilibrium which is determined chiefly by the relative expression of the Cl\textsuperscript{−} transporters KCC2 and NKCC1 as well as the voltage- and Cl\textsuperscript{−}-sensitive Cl\textsuperscript{−} channel ClC-2 (Andang and Lendahl, 2008, Foldy et al., 2010).

\textbf{1-2 Basal ganglia circuit}

The basal ganglia (BG) are a group of highly interconnected subcortical nuclei which are located in the midbrain, around the thalamus and are strongly connected with the cerebral cortex, thalamus, and other brain areas (Fig. 1-4A and B). The BG are considered to be involved in voluntary movement, memory, and cognitive functions such as procedural learning. Most findings about BG functions were originally obtained from clinical observations and postmortem brain examination of patients with movement disorders, such as Parkinson’s disease and Huntington’s disease. Therefore, the lesions in BG components are also pathological hallmarks in these movement disorders.

Traditionally, the BG are thought to be composed of four groups of nuclei: the striatum, the globus pallidus (GP), the subthalamic nucleus (STN) and the substantia nigra (SN). Among them, the SN can be further divided into two major nuclei, the pars reticula (SNr) and the pars compacta (SNc) (Fig. 1-4A and B). In vertebrates more evolved than rodents, such as primates, the striatum is separated into two parts by internal capsule: the caudate nucleus and the putamen. Thus, the striatum could be named as the caudate putamen (CPu). Similarly, the GP also consists of two parts in more evolved animals: the external segment (GPe) and the internal segment (GPi). The GP in rodents refers to the GPe in higher species while the entopeduncular nucleus (EP) is functionally equivalent to the GPi (Fig. 1-4A and B). The vast majority of neurons in the BG are GABAergic neurons. In the striatum, almost all the neurons are GABAergic, either medium spiny projection neurons (MSNs) or interneurons, with the exception of a small group of giant aspiny cholinergic interneurons. Both segments of the GP (or the GP and the EP in rodents) and the SNr are exclusively made of GABAergic projection neurons. Although the STN contains glutamatergic projection neurons and the SNc is principally composed of dopaminergic
Figure 1-4. Basal ganglia structures in human brain (A) and in mouse brain (B, AChE staining). (A) The human GP is divided into internal (medial) part (GPi) and external (lateral) part (GPe). (B) In rodent, the GP corresponds to the GPe in primate while the entopeduncular nucleus (EP) is functionally similar as GPi. A is from http://syntaptogaming.files.wordpress.com/2012/01/basalganglia_image1.jpg.
Figure 1-5. Organisation of intrinsic connections within the basal ganglia. From (Peter Redgrave (2007), Scholarpedia, 2(6):1825.)
neurons, the GABAergic neurons still represent more than 98% of total neurons in the BG (Tepper et al., 2007).

The BG receive principally glutamatergic inputs from the cerebral cortex and thalamus and the main target of those inputs is the striatum. The cortical and thalamic inputs form about 85% of all the synapses in the striatum, thus although the striatum is composed of fundamentally GABAergic neurons, the majority of synapses within it are glutamatergic. The major recipients of these glutamatergic innervations are the striatal MSNs which account for as much as 95% of total neurons in the striatum and are the principle projection neurons innervating the GP or EP (GPi)/SNr complex. According to the different striatal GABAergic projections, the BG circuit is further divided into mainly two pathways: the direct pathway and the indirect pathway. In the direct pathway, the striatal MSNs project directly to the EP (GPi) and SNr where they make direct synaptic contact with the GABAergic output neurons (Kawaguchi et al., 1990). In the indirect pathway, the striatal GABAergic neurons project to the GP (GPe) to form striatopallidal connections. In turn, the pallidal GABAergic neurons innervate the EP and SNr, or they innervate the glutamatergic neurons in the STN that then project to the output nuclei of the BG (Smith et al., 1998). The outputs of the basal ganglia, which are exclusively inhibitory and GABAergic, are issued largely from the EP and SNr (Fig. 1-5). The targets of the EP and SNr include the thalamus, the tectum and the other segment of SN, the SNc (Celada et al., 1999). The SNc consists of dopaminergic neurons and projects back to the striatum, GP and STN to provide an important feedback control of the BG circuit. The BG output to the thalamus may also control the glutamatergic input to the striatum via the feedback regulation of both the thalamo-striatal and cortico-striatal pathways. In the GP, besides the major GABAergic projections to the STN or output nuclei, a part of neurons project back to the striatum, predominantly to the interneurons, to affect the activation of MSNs and to provide spatiotemporal selection of neurons (Bevan et al., 1998, Koos and Tepper, 1999). In addition to the striatal direct and indirect pathways, the information from the cerebral cortex and thalamus may enter the BG via the STN. The STN receives excitatory inputs from the cortex and the intralaminar thalamic nuclei, and then, send glutamatergic projections directly (or through the GP) to the EP/SNr. The cortico- and thalamo-subthalamic pathways are the fastest routes that can influence the activity of BG output nuclei directly, and have been recognized as the hyperdirect pathways (Nambu, 2004).
The function of the BG loops is not only to relay the information from the cortex or thalamus to the lower midbrain and the brain stem, but also to integrate complex information. Classically, the first fundamental function of the BG architecture is signal selection which decides which functional system or sensory input should be selected to modulate the motor response. This function is achieved by selective removal of tonic inhibitory output on some BG loops while maintaining or increasing it on the others, thus allowing the disinhibited functional system to access to motor system and generate behavioural output (Redgrave et al., 1999). Another important function of the BG loops is associated with reinforcement learning. Although the mechanisms of reinforcement learning are not thoroughly assessed and the models of the BG-mediated reinforcement are still under development, it is widely accepted that the dopaminergic neurotransmission within the BG is critical to ‘reward prediction errors’ and learning process (Montague et al., 1996, Morris et al., 2004). Long-term potentiation (LTP) and long-term depression (LTD) of corticostriatal transmission appears critically dependent on the presence or absence of DA. It is assumed that inputs from the cerebral cortex induce patterns of activity in striatal neurones that are reinforced by phasic DA signalling (Calabresi et al., 2007, Surmeier et al., 2007, Wickens, 2009). Selectivity is achieved by restricting the effects of reinforcement (LTP/ LTD) to specific subsets of recently or concurrently active inputs (Arbuthnott and Wickens, 2007, Redgrave et al., 2011).

1-2-1 The striatum

The striatum is the main input nucleus of the basal ganglia, which receives glutamatergic inputs from the cerebral cortex or thalamus and send inhibitory GABAergic outputs to the GP or EP/SNr. The GABAergic MSNs are the main projection neurons, they account for 95% of total striatal neurons, and are also the main target of the corticostriatal/thalamostriatal projections. Interestingly, each MSN gets only 1 or 2 synapses from a single neuron in the cortex or the thalamus, which means each MSN receives inputs from very large number of cortical or thalamal neurons. (Tepper et al., 2007). The MSNs can be subdivided into two subpopulations depending on different projecting targets. Traditionally, in the direct pathway, the striatonigral MSNs project mainly to the EP and SNr, and express dopamine D_1 receptor, M4 muscarinic acetylcholine receptor (chrm4), dynorphin (DYN), and substance P neuropeptide (SP). The indirect pathway consists of striatopallidal MSNs which mostly project to the GP and co-express
dopamine D_2 receptor, adenosine A_{2A} receptor and enkephalin (ENK) (Gerfen et al., 1990, Graybiel, 1990, Schiffmann and Vanderhaeghen, 1993).

However, there is increasing evidence showing that a subgroup of MSNs coexpress both D_1 and D_2 receptors along with the neuropeptides DYN and ENK (Perreault et al., 2011). Besides the coexpression of D_1 and D_2 receptors, this subgroup of MSNs may also express a novel dopamine D_1-D_2 receptor heteromer complex. The D_1-D_2 coexpressing neurons are widely distributed in the BG, including the striatum, GP, and EP (Perreault et al., 2010). In basal ganglia, the coexpression of D_1 receptor and D_2 receptor has been shown to occur both within neuronal cell bodies and selectively at presynaptic, but not postsynaptic terminals (Wong et al., 1999, Geldwert et al., 2006, Mizuno et al., 2007, Perreault et al., 2010). It is well known that the activation of D_1 or D_2 receptors results in the activation or inhibition, respectively, of cAMP mediated cell signalling pathways (Neves et al., 2002). While the D_1-D_2 heteromeric complex is activated, another phosphorylation pathway, the phospholipase C (PLC) –phosphoinositide (PI) pathway will be activated and result in G_{iso}-PLC-PI induced increase of intracellular calcium release. Consequently, in the striatum, the increased calcium leads to striatal calcium calmodulin kinase IIα (CaMKIIα) phosphorylation and BDNF expression (Lee et al., 2004, Rashid et al., 2007, Hasbi et al., 2009, Ng et al., 2010). A recent study shows that almost all of the D_1-D_2/DYN-ENK coexpressing striatal projection neurons also coexpress GAD67, as well as VGLUT1 and VGLUT2, which implicates that those unique MSNs potentially have both inhibitory and excitatory capabilities. Moreover, it also indicates that the activation of D_1-D_2 heteromers induces a significant reduction in the expression of GABA, relative to glutamate, in the striatum, this may result in a shift between inhibition and excitation (Perreault et al., 2012). Interestingly, a recent report shows that the activation of dopaminergic neurons in the SNc leads to rapid inhibition of MSNs in both direct- and indirect-pathways through vesicular release of GABA. The release of GABA from dopaminergic axons is not dependent on VGAT but relies on the vesicular monoamine transporter, VMAT2 (Tritsch et al., 2012).

Besides the MSNs, there are a small portion of interneurons existing in the striatum. Although the interneurons are not many in number (2-3% of total striatal neurons), they play crucial roles in modulating striatal input and output (Tepper and Bolam, 2004). Biochemically, the striatal interneurons are divided into four different populations: three GABAergic and one cholinergic. The three GABAergic interneuron subtypes can be distinguished neurochemically: one expresses the calcium binding proteins parvalbumin
(PV); one expresses another calcium binding protein calretinin (CR); the last one coexpresses neuropeptide Y (NPY), somatostatin and the neuronal nitric oxide synthase (nNOS) (Rymar et al., 2004).

The PV positive interneurons are the most studied and can be electrophysiologically characterized as fast-spiking interneurons (FSIs) which have the ability to fire at high rate (Kawaguchi, 1993). The FSIs receive glutamatergic excitatory input from the cortex and make strong, dense projections onto MSNs within 300 µm or 500-600 µm depending on different subtypes (Kawaguchi, 1993, Koos and Tepper, 1999). Interestingly, the pattern of corticostriatal projections to FSIs is different from those projections to MSNs: one corticostriatal neuron may form multiple synapses on the same FSI instead of only 1-2 synapses on one MSN (Ramanathan et al., 2002, Tepper et al., 2007). The FSIs are thought to mediate feedforward inhibition in the striatum because they are activated earlier and at lower thresholds than MSNs are (Mallet et al., 2005). One single FSI may form many powerful inhibitory synapses (estimated 135-541 per somata) on MSNs of both direct and indirect pathways to inhibit their firing (Koos and Tepper, 1999, Gittis et al., 2010, Planert et al., 2010). Because the number of FSIs is low in the striatum (0.7% of total striatal neurons), each MSN receives inhibitory synaptic input from very few interneurons, but the inhibitory postsynaptic potentials (IPSPs) produced by FSIs are powerful enough to delay or suppress the firing of MSNs (Koos and Tepper, 1999). Comparatively, the feedback inhibition mediated by axon collaterals of the MSNs is weak, although they are more numerous, they do not produce strong effects at the soma (Tunstall et al., 2002). Together, it suggests that feedforward inhibition may have a major role in adjusting the excitability of MSNs and controlling the action potential generation. Feedback inhibitory synapses may interact with voltage sensitive conductance in the dendrite to alter the electrotonic structure of the spiny neuron (Koos et al., 2004, Wilson, 2007). Besides the corticostriatal input, the FSIs are also reported to receive GABAergic projections from local MSNs and the PV-containing neurons in the GP, and a cholinergic input from the cholinergic interneurons within the striatum (Chang and Kita, 1992, Bolam et al., 2000). They are also reported to have dopamine D5 receptors, thus can be activated by D1-like-receptor agonist (Centonze et al., 2003a). All of these may implicate a complex modulation of GABAergic inhibition in the striatum and interactions among different interneurons.

The NPY interneurons have the least dense axonal arborisation of all striatal interneurons and are classified into the persistent and low-threshold spike (PLTS) neurons. Single action
potentials in NPY cells produce large inhibitory postsynaptic currents (IPSCs) in MSNs and the decay kinetics is 10 fold longer than those induced by FSIs (Ibanez-Sandoval et al., 2011). Those NPY interneurons receive both cholinergic input from striatal cholinergic interneurons and dopaminergic input from the SNc, which suggests an intrinsic regulation of NPY cells (Tepper and Bolam, 2004, English et al., 2012). In addition, the NPY interneurons are also the source of several neuromodulators, such as somatostatin, NPY and nitric oxide (NO). They may regulate corticostriatal synaptic plasticity or striatal output, thus further investigation is required to clarify their true functions (Centonze et al., 2003b, Gittis and Kreitzer, 2012). The calretinin positive interneurons are relatively less studied and there are controversies about the morphological and electrophysiological profiles of those neurons. It may be due to the existence of different subtypes of calretinin-containing neurons (Tepper et al., 2010). Another population of GABAergic interneurons in rodents is the one that express tyrosine hydroxylase (TH). The TH positive interneurons receive excitatory inputs from the cortex and thalamus, as well as inhibitory input from striatal MSNs, and exert a modulation on MSNs. Interestingly these TH positive cells produce both GABA and dopamine, therefore implicating a potential novel dopaminergic regulation within the striatum (Ibanez-Sandoval et al., 2010, Tepper et al., 2010).

The cholinergic interneurons are a group of large aspiny neurons expressing choline acetyltransferase (ChAT) and are the main source of acetylcholine within the striatum (Suzuki et al., 2001). They receive excitatory glutamatergic projections from the cerebral cortex and thalamus, the inhibitory GABAergic afferent from local MSNs and dopaminergic input from the SNc. Their intense axon arborisation forms synaptic contacts with virtually all striatal MSNs as well as a part of GABAergic interneurons (Koos and Tepper, 2002, Tepper and Bolam, 2004, Ghiglieri et al., 2012). The cholinergic interneurons typically fire slowly and regularly, with action potentials of long duration as well as lengthy and slow spike after hyperpolarization (Tepper and Bolam, 2004). It is reported that cholinergic interneurons are important in the integration of DA, adenosine, and endocannabinoid signalling systems (Tozzi et al., 2011). There are also evidences showing that they are involved in corticostriatal synaptic plasticity and are important in reward-based learning or motivated behaviour (Calabresi et al., 2000, Morris et al., 2004, Calabresi et al., 2007).

All in one, the functions of striatum are complex. The striatal nuclei receive, integrate and process the information from the cerebral cortex and thalamus under the modulation of
striatal microcircuits, and then send outputs via different pathways. The striatum is involved in smooth and conscious movement but also in cognitive behaviours such as reward-based learning. However, due to the complexity of striatal organization, many details of striatal microcircuit and the roles of striatum inside the BG macrocircuit are still missing.

1-2-2 The globus pallidus

The globus pallidus (GP, GPe in primate) is an important component of the BG. It locates medially to the striatum and laterally to the internal capsule. As what has been discussed above, the GP is playing an essential role in the indirect pathway of the BG macrocircuit. It receives a large number of GABAergic afferent fibres from the striatum. However, not all the striatal GABAergic inputs are from the indirect pathway, because MSNs involved in the direct pathway also project axonal collaterals to the GP. (Kawaguchi et al., 1990, Parent et al., 1995, Wu et al., 2000). The estimated number of boutons belonging to the direct-pathway collateral axons is approximately half of that belonging to the striatal indirect-pathway axons (Kawaguchi et al., 1990). The GP also receives glutamatergic projections from the STN and some dopaminergic inputs from the SNc. It is also reported that the GP may receive sparse afferents from the intralaminar thalamic nuclei, the peduncloptine tegmentum, the EP (GPi) and the cerebral cortex (Fink-Jensen and Mikkelsen, 1991, Kita, 1994, Naito and Kita, 1994b, Yasukawa et al., 2004). The efferent of GP is also complex. It virtually projects to all the basal ganglial components including the striatum (GABAergic interneurons and MSNs), the STN, the EP/SNr, as well as the GP itself (axon collaterals) (Kita, 2007, Jaeger and Kita, 2011).

The GP is an integrative hub coordinating neuronal activity across the BG, it participates in multiple feedback and feed-forward loops. The GP is almost exclusively composed of GABAergic projection neurons. In a rat study, it is reported that the GP neurons can be differentiated by three calcium binding proteins: parvalbumin (PV), calbindin D-28k (CB) and calretinin (CR) (Cooper and Stanford, 2002). The PV positive neurons represent about 66% of total pallidal neuronal population and distribute throughout the GP with the highest density in the lateral part (Kita and Kitai, 1991, Cooper and Stanford, 2002). The CB containing neurons constitute approximately 2% of total GP neurons and can be observed throughout the GP in a complementary pattern to PV cells (Cooper and Stanford, 2002). The CR neurons are very sparse (less than 1%) and are not labelled by striatal colloidal
gold particles, thus they may represent a subpopulation of pallidal interneurons. No co-expression of calcium binding proteins is observed in the GP. Due to the fact that approximately 30% of GP neurons are not labelled by any of the three calcium binding proteins, it seems that every GP neuron appears to express either one single type of calcium binding protein or none at all (Cooper and Stanford, 2002). Some other studies also suggest that the neuronal population in the GP can be neurobiochemically divided into two groups: the PV positive cells (about 60%) and the neuropeptide precursor preproenkephalin (PPE) mRNA containing neurons (about 40%) (Kita, 1994, Hoover and Marshall, 1999, 2002). Recently, new electrophysiological studies support that the GP neurons can be grouped into two subpopulations according to their neuronal firing patterns: a major group of GP neurons (about 75%) preferentially discharge during the inactive component of the cortical slow (1Hz) oscillation when most cortical, striatal and STN neurons are quiescent, thus named as GP-TI neurons; another population of GP neurons (more than 20%) are likely discharge during the active component and called GP-TA neurons (Mallet et al., 2008). The GP-TI neurons are considered as the prototypic GP neurons which target the downstream BG nuclei including the STN, EP and SNr. Most GP-TI neurons express PV while none of them express PPE. Besides the long-range axon collaterals that project to the BG downstream nuclei, the GP-TI neurons give rise to extensive local collaterals and some of them even have collaterals modestly innervate the striatal GABAergic interneurons. The GP-TA neurons are exclusively PPE positive and only innervate the striatum with the special GABAergic/enkephalinergic projections. Their targets include three major striatal interneurons (PV, NOS/NPY, ChAT) as well as MSNs. They also emit local axon collaterals although these collaterals are relatively restricted and the number of boutons is much smaller than GP-TI cells. None of GP-TA neurons projects to the BG downstream nuclei. Considering the technical limits and the fact that about 40% GP neurons are PPE positive and some GP-TA neurons also contain PV, the real number of GP-TA cells may be under estimated while the percentage of GP-TI neurons is over estimated (Mallet et al., 2012). The GP-TA neuronal projections may be an important source of striatal enkephalin, thus play a role in regulation of MSNs firing (Blomeley and Bracci, 2011). In addition, the PV positive and negative (PPE positive) GP neurons are positively and negatively modulated by dopaminergic transmission, respectively. This may implicate a novel dopaminergic regulation in the BG macrocircuit and a dichotomous role of GP in dopaminergic dysfunction such as PD (Hoover and Marshall, 2002).
The GP is controlled by multiple projections, different circuits and various neurotransmitters, thus its function is under integrated regulations. As what has been discussed above, there are mainly two types of GABAergic inhibitory inputs in the GP, which are from the striatum (striatopallidal input) and from local collaterals of neighbouring pallidal neurons (pallidopallidal input). The striatopallidal synapses are usually distributed at distal dendritic compartments and express relatively higher GABA$_\text{A}$ $\alpha_2$ subunits; comparatively, the pallidopallidal synapses are more somatic and proximal, containing more GABA$_\text{A}$ $\alpha_3$ subunits (Gross et al., 2011). As the most abundant GABA$_\text{A}$ $\alpha$ subunit, the expression of $\alpha_1$ subunits is highly presented at both somatic and dendritic locations. Electrophysiologically, the IPSC of striatopallidal input shows significantly slower rise time, decay time and half-width than the pallidopallidal collaterals. Compared to pallidopallidal synapses, striatopallidal synapses have a greater paired-pulse ratio, indicative of a lower probability of release (Sims et al., 2008). Those observations may be the results of the different synaptic subcellular distribution and synaptic components of the two GABAergic inputs. Along with GABA$_\text{A}$ receptors, evidences show that two types of GABA$_\text{B}$ receptors, GABA$_\text{B}$-R1 and GABA$_\text{B}$-R2 are also widely distributed in the GP. The GABA$_\text{B}$-Rs are mainly localized on extrasynaptic membranes and some on presynaptic membranes of GABAergic and glutamatergic boutons (Smith et al., 2000, Chen et al., 2004, Charara et al., 2005). A high level of GABA is required to active GABA$_\text{B}$-Rs. It seems that activation of both striatal axons and local pallidal neurons is necessary to evoke GABA$_\text{B}$-R-mediated responses (Kita et al., 2006). The activated GABA$_\text{B}$-Rs then may regulate the spontaneous firing activity of GP as well as a slow IPSP in the GP after burst stimulation in the striatum (Kaneda and Kita, 2005, Kita et al., 2006). The presynaptic GABA$_\text{B}$-Rs also play the role of auto-receptors, thus when they are activated, the release of GABA in the GP is reduced (Kita, 2007). The GP receives glutamatergic inputs from the STN to modulate the local GABAergic neurotransmission. Evidence shows that both striatalpallidal and local axon collateral terminals have group III mGluRs (Bradley et al., 1999). Activation of these receptors can presynaptically reduces both GABAergic and glutamatergic transmission in the GP (Matsui and Kita, 2003). The group I mGluRs are also observed in the GP but their function is not clearly understood (Paquet and Smith, 2003, Mitrano and Smith, 2007). As a part of the indirect pathway and the target of striatal projections, the GP express adenosine A$_{2A}$ receptors, dopamine D$_2$ receptors as well as the cannabinoid CB1 receptors at the striatopallidal terminals. Those receptors control the GABAergic and glutamatergic tone in the GP and are involved in motor dysfunctions like
In addition, the GP receives abundant serotoninergic (5-HT) innervation from the dorsal raphe (Charara and Parent, 1994). The main effect of the 5-HT projections is to suppress the glutamatergic excitation (through 5-HT\textsubscript{1A}-R) and GABAergic inhibition (through 5-HT\textsubscript{1B}-R) (Kita et al., 2007, Hashimoto and Kita, 2008, Rav-Acha et al., 2008).

1-3 Huntington’s disease

Huntington’s disease (HD) is an inherited autosomal dominant neurodegenerative disease and is characterized by motor dysfunctions, emotional and cognitive deficits. It was comprehensively and accurately described in 1872 by George Huntington who became internationally recognized soon after. Although he was not the first one who described this disorder, the disease was still named after him since then (Bates, 2005). Huntington’s disease is characterized by motor, emotional, and cognitive disturbances. Most people with HD develop typical motor symptoms include jerky involuntary movements, muscle tone dysregulation (hypotonia or hypertonia), swaying gait and late stage dysphagia which greatly increases the risk of aspiration (Stewart, 1988). Cognitive and behavioral changes, such as impulsive behavior, substance abuse, apathy, sexual promiscuity and depression may occur years before movement disorders begin (Cangemi and Miller, 1998). HD patients also show weight loss and cachexia. Cachexia, together with aspiration complications, are the most common causes of death (Martin, 1984). Due to knowledge and technique restrictions, the genetic mechanism of HD had been undiscovered for more than one century until about 20 years ago. In 1993, with new gene-mapping and genomics technologies, a consortium of scientists discovered that HD was associated with an unstable expanded CAG trinucleotide repeat in the huntingtin gene (HTT) on chromosome 4. They reported that in normal condition, the exon-1 of HTT gene contained less than 35 CAG repeats at the 5’ end, whereas HD patients had more than 40 trinucleotides (group, 1993). Individuals with 36-40 CAGs may develop a slow pathological progression with onset at late life or even without significant symptoms (Andrew et al., 1993, Brandt et al., 1996). The expanded CAG trinucleotides lead to an elongated polyglutamine (polyQ) stretch at the N-terminus of the huntingtin protein (Fig. 1-6). The mutated huntingtin with long polyQ stretch can self-associate to β-sheet structures and gradually precipitate in
Figure 1-6. Diagram of regions of the Htt protein. Full-length htt is composed of ~3144 amino acids, with a molecular weight of 350kDa. The N-terminal 17 amino acid (N17) region is highly conserved and contains multiple post translational modifications, such as phosphorylation and ubiquitination, which could strongly affect the pathogenesis. Following the N17 region are the polyglutamine (polyQ) and polyproline (polyP) regions. The function of polyproline is still largely unclear. Studies have suggested a hot spot of proteolytic cleavage exists between amino acids 400 and 600. Putative proteases include caspases 2, 3, 6, calpain, and matrix metalloproteinase 10. Up to 16 HEAT repeats with unknown functions have been identified in Htt. Multiple regions of phosphorylation have been identified. The C-terminus contains a leucine-rich nuclear export signal (NES), which may target full-length Htt to cytoplasm. Another leucine-rich NES could exist in the N17 region. From (Zheng and Diamond, 2012).
neurons (Perutz et al., 1994). Interestingly, the longer polyQ stretch is, the earlier symptoms occur and the faster the disease progresses. The discovery of the genetic basis of HD is a milestone in HD research history. Based on it, various transgenic animal models have been generated and many therapeutic methods are under development.

1-3-1 The structure of huntingtin

In humans, the huntingtin gene is expressed ubiquitously in all tissues with the highest levels in the central nervous system and testes (DiFiglia et al., 1995). It encodes a protein of approximately 350 kDa, which is named as huntingtin (Htt), with several subdomains including the polyQ tract at the N-terminus (Fig. 1-6). The polyQ tract is a potential membrane association signal, but in vivo deletion of polyQ only slightly influences animal’s energy metabolism, learning and memory. It seems that polyQ is not essential for Htt’s basic functions. However, in cell cultures, the ablation of polyQ shows elevated ATP level and early senescence (Clabough and Zeitlin, 2006). Besides the polyQ repeats, Htt also contains 16 clustered HEAT (Huntington, Elongation factor 3, protein phosphatase 2A, and the lipid kinase TOR1) repeats (in several groups) which may be involved in intracellular transport (Andrade and Bork, 1995). In addition, there is one conserved NES (nuclear export signal) in the C-terminus and probably another NES in the N17 (N-terminus 17 amino acid) region, which are important for nuclear transport (Xia et al., 2003, Cornett et al., 2005) (Fig. 1-6). The physiological function of Htt has not been fully described but it is clearly crucial to embryonic development, neuronal maturation and cell survival.

1-3-2 Wild-type huntingtin is protective and essential for neuron survival

In an Htt gene knockout mouse model, the homozygous knockout mice showed embryonic lethality as a result of severe apoptosis, while the heterozygous mice displayed increased motor activity and strong cognitive deficits due to increased neuronal necrosis in the globus pallidus (GP) and the subthalamic nucleus (STN) of the basal ganglia (Nasir et al., 1995). Thus, inactivating Htt gene postnatally can lead to dystrophic axons and neuronal apoptosis, which indicates an anti-apoptotic role that Htt may play in the nervous system (Dragatsis et al., 2000). Additionally, many transgenic (i.e. R6/2) or brain injury (ischemia, traumatic brain injury, and spinal cord injury) models, can lead to the progressive depletion of full length Htt while overexpression of wild-type Htt can significantly protect neurons
against apoptosis both in vivo and in vitro (Rigamonti et al., 2000, Zhang et al., 2003, Leavitt et al., 2006). Taken together, these data suggest neuron-protective functions of the full-length wild-type Htt. In a knock-in HD mouse model (Hdh140Q), the animal’s lifespan is extended by overexpression of full-length Htt lacking its polyQ stretch, which can significantly reduce mutant Htt aggregation via activation of the autophagy process and the clearance of truncated N-terminal Htt aggregates (Zheng et al., 2010). The mechanism of neuronal protective effect of Htt may be due to the blockage of caspase 9 and 3 which are activated in apoptotic process, as well as the interruption of pro-apoptotic Bcl-2 family members (Bik and Bak) (Rigamonti et al., 2000, Rigamonti et al., 2001). It also seems that the existence of wild-type Htt may up-regulate the expression of BDNF (brain-derived neurotrophic factor) which is an important neurotropic factor involved in neuron growth, differentiation and survival, via suppression of the BDNF transcriptional repressor complex, REST (repressor element-1 silencing transcription factor)/NRSF (neuro-restrictive silencer factor). However, it is not clear whether the loss of BDNF contributes to HD symptoms (Zuccato et al., 2001). Another possible mechanism may be due to the reduced binding affinity between mutant Htt and Hip-1 (Htt-interacting protein 1). Free Hip-1 binds to Hippi (Hip-1 protein interactor) to form a pro-apoptotic complex which recruits procaspase-8 and triggers a cell-death pathway. As a result, the binding of wild-type Htt and Hip-1 is neuronal protective by preventing neurons from initiating the apoptotic process (Gervais et al., 2002). Overall, the wild-type Htt is physiologically protective and important for neuron survival while HD pathology is the results of both loss of WT Htt protection and gain of mutant Htt toxicity. However, the functions, the interactions and the protective mechanisms of Htt are not yet fully deciphered.

1-3-3 Mutant huntingtin in HD pathogenesis

1-3-3-1 Proteolytic cleavage of mutant huntingtin

Htt is vulnerable to a number of proteases, including caspases, calpains and some Matrix metalloproteinases (MMPs). The proteolytic cleavage of mutant Htt results in different N-terminal fragments containing the polyQ stretch which induces even more cytotoxicity and aggregation than full length mutant Htt. The presence of truncated N-terminal fragments can be detectable in both HD mice and human patients before the loss of neurons in the striatum (Davies et al., 1997, DiFiglia et al., 1997, Wellington et al., 2002) (Fig. 1-7). The cleavage efficiency of caspases increases with the length of polyQ tract which may explain
Figure 1-7. Representative intracellular events in neurons expressing mutant HTT. In HD, processing of mutant HTT by caspases, calpains and MMPs facilitates the formation of intracellular aggregates, which are mainly degraded by autophagy. Failure in the clearance of HTT proteolytic fragments eventually results in excessive cytosolic Ca$^{2+}$ concentration and organelle dysfunctions. From (Bano et al., 2011).
the correlation between the toxic effects and the number of CAG repeats in HD (Goldberg et al., 1996). Blocking Htt cleavage pharmacologically or genetically can reduce the toxicity and neuropathology in HD, which indicate that the caspases-dependent proteolysis may be an important step in HD pathogenesis (Graham et al., 2006). Htt is also a substrate of calpains which are a group of calcium-dependent proteases. In HD, the level of glutamate release is elevated from afferent neurons, and this leads to enhanced NMDA receptor activity and increased intracellular calcium. The calpains are then activated to cleave the mutant Htt into a series of toxic N-terminal fragments containing polyQ stretches which promote the NMDA receptor-mediated excitotoxicity (Gafni and Ellerby, 2002, Gladding et al., 2012). Another study combining western blot and RNAi shows that a group of MMPs are involved in mutant Htt proteolysis. Reducing the activity of MMP can suppress neuronal dysfunction and cell death in both HD mouse (Hdh^{111Q/111Q}) striatal cell culture and a Drosophila model of HD (Miller et al., 2010b). In summary, these findings indicate that proteolytic cleavage of mutant Htt is a key step in neurodegenerative process of HD and inhibition of the activity of those proteases may be a potential therapeutic approach to treat or at least to remit the HD symptoms (Fig. 1-7).

1-3-3-2 Aggregation of mutant Htt
The intracellular aggregates of mutant Htt, which are also named as inclusion bodies, are closely correlated with HD symptoms. They are a pathological hallmark of HD and can be found in neurons before significant neuronal death (Wellington et al., 2002). Many conformational types of aggregates have been described, including fibrils which are long ordered polymers, oligomers which may range from 2 to hundreds monomers and amorphous aggregates. In vitro, the wild-type Htt N-terminal fragment peptide forms a compact coil that resists aggregation, while the mutant Htt fragment induces a more extended conformation with an exposed polyQ which is apt to aggregate into globular oligomers. The newly formed oligomer also contains polyQ structure that can further interact with other monomers or oligomers, and form amyloid-like aggregates (Thakur et al., 2009). The expanded polyQ Htt is aggregation-prone both in vitro and in vivo, and the aggregation of Htt fragments highly depends on the length of polyQ and the size of N-terminal fragments (Hackam et al., 1998, Li and Li, 1998, Martindale et al., 1998, Legleiter et al., 2010).
The role of mutant Htt aggregation in the pathogenesis of HD remains controversial and confusing. On one side, because Htt is involved in intracellular transport, receptor endocytosis and synaptic vesicle recycling, the mutation of Htt may alter the release of neurotransmitters and synaptic transmission. The aggregation process of inclusion bodies may interact with other proteins, such as transcription factors and proteasomes, thus, trigger toxic effects (Arrasate and Finkbeiner, 2012). The intracellular aggregates of mutant Htt could also influence autophagy system and mitochondrial function (Bano et al., 2011) (Fig. 1-7). On the other side, the connection between inclusions and cell loss is not clear. For example, in human HD patients, the mutant Htt aggregates are denser in the cerebral cortex than in the striatum, while the cell loss is severe in the striatum but very low in the cerebral cortex (Gutekunst et al., 1999). Even in the striatum, the inclusions are found predominantly in interneurons compared to the more vulnerable medium spiny neurons (Kuemmerle et al., 1999). In a YAC120 mouse model, the frequent and widespread inclusions can be found but without evidence of neuronal dysfunction and neurodegeneration. Those findings indicate that the mutant Htt inclusions are not pathological in vivo and the inclusion bodies could be dissociated from neuronal death (Slow et al., 2005). In fact, instead of inclusion bodies, the more soluble forms of mutant Htt, such as oligomers, are linked with neuronal death in neuron culture. The amount of soluble Htt governs the survival of neurons and is correlated to the risk of cell death. Comparatively, the inclusion body formation reduces the level of toxic soluble mutant Htt and prolongs the survival of neurons. Thus, inclusion bodies may be beneficial coping response to the HD pathogenesis (Arrasate et al., 2004, Miller et al., 2010a). However, in another study, after a yeast-based high-throughput screening assay, a small compound named C2-8 is found to be capable to reduce the mutant Htt aggregation both in cultural slices and in Drosophila (Zhang et al., 2005). This compound can penetrate the blood-brain barrier and significantly improve the motor performance and reduce neuronal atrophy in vivo (R6/2 mouse model). Interestingly, instead of reducing the number of inclusions, the effect of this compound is mainly to decrease the aggregate volume, which means the inclusions are much smaller after treatment (35% reduction). Although this C2-8 improves the motor performance and delays the motor impairment, it has no effect on survival (Chopra et al., 2007). All of these indicate that the principle of this compound may be that it changes the steps of aggregate formation and make the mutant Htt more prone to aggregate into more compacted aggregate inclusions. Thus, this effect reduces the soluble toxic mutant Htt and saves the neurons. But at later stage, this effect is weakened by high
amount of soluble Htt, so the toxicity will finally lead to the death of the animal. However, this hypothesis still needs to be tested.

1-3-3-3 Mutant Htt and transcriptional dysfunction

A number of transcriptional factors and cofactors are reported to interact with mutant Htt, including LANP, PQBP-1, N-CoR, ARA24, CBP, p53, mSin3A, TAFII130, ETO/MTG8, p160/GRIP1, Sp1, C-terminal binding protein (CtBP), CA150, SC35 and MLF1 (Okazawa, 2003, Moumne et al., 2013). The binding of mutant Htt and the aggregation process may sequester those factors, leading to transcriptional dysfunction. For example, CBP (CREB binding protein) is a coactivator for CREB (cAMP response element-binding protein) mediated gene transcription which plays an important role in cell survival. So CBP is a major mediator of survival signals in matured neurons. In HD, CBP is found to bind to mutant Htt via its own polyQ stretch (15 in mouse, 18 in human), thus is sequestered from the nucleus and relocated to mutant Htt inclusions. This may be one reason for neuron atrophy (Nucifora et al., 2001). Another example is the REST/NRSF transcription complex which has been mentioned above. The REST/NRSF binds the NRSE region within the BDNF promoter to inhibit the gene expression. Wild-type Htt binds REST/NRSF and prevents its binding to BDNF promoter, while mutant Htt has low affinity to this complex resulting in the down-regulation of BDNF expression (Zuccato et al., 2001, Zuccato et al., 2003). The down-regulated BDNF together with the reduction of its receptor, tyrosine kinase receptor (TrkB), lead to the decreased TrkB-mediated Ras/MAPK/ERK1/2 signaling which is associated with increased cell sensitivity to oxidative damage and cell loss (Gines et al., 2010). The activity of another MARK downstream kinase, mitogen- and stress-activated protein kinase-1 (MSK-1), is also impaired, which leads to defective H3 phosphorylation and deficient c-Fos induction (Roze et al., 2008). This may result in reduced cell survival. Another study shows that the expression of a transcriptional coactivator, PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1 α) which regulates mitochondrial biogenesis and respiration, is reduced in both mouse and human HD brains. The mutant Htt can associate with the transcriptional promoter of PGC-1α and affect the CREB/TAF4-dependent transcriptional pathway (Cui et al., 2006). The reduction of PGC-1α may change the mitochondrial function and energy metabolism in HD.
Taken together, mutant Htt can alter the levels of transcriptional products (Moumne et al., 2013). Whether HD symptoms are truly a result of multiple genetic dysfunctions remains unclear, but it is certain that the genetic dysfunctions caused by mutant Htt contribute greatly in HD pathogenesis.

1-3-3-4 Mutant Htt and clearance pathways: autophagy and UPS

Autophagy is a fundamental cellular catabolic process, which is involved in cell survival, differentiation, development, and homeostasis. It starts with the formation of a double membrane vesicle, named autophagosome, which is made by the expansion of an isolation membrane (phagophore) to enclose the target cytoplasmic materials. The autophagosome with sequestered cargo content is then fused with a lysosome to form an autolysosome where all the captured cytoplasmic materials as well as the inner membrane are degraded. Autophagy occurs at low level in normal condition to maintain cellular homeostasis but can be activated to respond to cell stress (Levine and Kroemer, 2008). As autophagy is one of the major protein degradation pathways, its dysfunction is tightly related to various protein-aggregation-associated neurodegenerative diseases, including HD. The dysregulation of autophagy can cause metabolic stress and is one of the main reasons for cell death in HD (Sarkar and Rubinsztein, 2008).

In autophagy process, one of the most important regulators is the target of rapamycin (TOR), which is an inhibitory complex regulated by class I phosphoinositol 3-kinase (PI3K), the serine/threonine kinase Akt and the 50-AMP-activated protein kinase (AMPK) (Kroemer et al., 2010). Inhibition of the TOR complex leads to activation of the downstream TOR kinases, and then the formation of autophagosome. In HD, the activity of autophagy is increased, as suggested by: (i) more abundant huntingtin-enriched cytoplasmic vacuoles in cells expressing mutant Htt (Kegel et al., 2000); (ii) excessive endosomal–lysosomal-like organelles and vesicles in HD brains (Sapp et al., 1997); (iii) increased numbers of autophagosomes have been found in lymphoblasts of HD patients (Nagata et al., 2004). Interestingly, it seems that the increased activity of autophagy is due to the sequestration of TOR by mutant Htt aggregates. This is the result of self-destruction of Htt aggregates. Therefore, compounds that can inhibit TOR, such as rapamycin, lead to increased clearance of aggregates and reduced cell toxicity in cell and animal models (Ravikumar et al., 2003, Ravikumar et al., 2004). Besides this, drugs that suppress the increase of intracellular calcium ions may also enhance the autophagy activity indirectly by
decreasing the activity of calpains, hence preventing the degradation of kinases involved in TOR signaling, like Beclin-1 and Atg related proteins (Yousefi et al., 2006, Russo et al., 2011). It is also reported that the inhibition of inositol monophosphatase (IMPase) may also trigger autophagy by depletion of free inositol, thus lower the level of 1, 4, 5-trisphosphate (IP3). This autophagy induction is independent of mammalian TOR because the IP3 levels had no effect on the autophagy-inducing property of mammalian TOR inhibition (Sarkar et al., 2005). However, although the autophagy system seems to be activated and the assembly of autophagosomes is at a high level, the degradation efficiency of mutant Htt is very low in HD. This may due to the sequestration of autophagic cargo by Htt aggregates or the failure of recognition of autophagic targeting signals, such as p62 which is a polyubiquitin-binding protein and is also involved in autophagic cargo recognition (Martinez-Vicente et al., 2010). As a result, other than enhancing autophagy, improving the cargo targeting and the autophagic efficiency may be a better therapeutic strategy to improve HD symptoms. But at this moment, the mechanisms of autophagy targeting are still not fully understood.

The ubiquitin–proteasome system (UPS) is the major pathway that controls the degradation of proteins in eukaryotic organisms. This pathway is initiated by the attachment of ubiquitin to target protein (ubiquitination) and followed by proteasome-induced unfolding, translocation and proteolysis. It has been reported that both ubiquitin modifications and proteasome subunits can be found in Htt aggregates which means the mutant Htt is targeted by UPS for degradation. However, the folding of mutant Htt and the sequestration of UPS components into inclusions may change activity of UPS (Davies et al., 1997, DiFiglia et al., 1997). In addition, during the UPS-induced proteolysis, the prolonged polyQ site may be trapped in the proteasome being not efficiently degraded and blocking the entry of other substrates into the catalytic zone (Venkatraman et al., 2004, Imarisio et al., 2008). The alteration of UPS mediated degradation in HD is also suggested by the facts that: proteasome inhibitors increase the level of inclusions while proteasome enhancers, like chaperones, reduce the Htt aggregates (Carmichael et al., 2000). Moreover, evidence has shown that UPS and autophagy are closely connected with each other by several proteins which are associated with ubiquitination, like p62 and HDAC6 (histone deacetylase 6, a cytoplasmic deacetylase interacting with ubiquitin) (Seigneurin-Berny et al., 2001, Hook et al., 2002). The mechanisms of ubiquitin in clearance pathway in HD may be much more complex than what has been described. Further study in ubiquitin system is necessary to
understand the principles of cellular protein catabolism and that may not only be beneficial to HD but also various other types of diseases.

1-3-3-5 Mutant Htt and mitochondrial deficiency

Energy metabolic defect has been well described in brain tissues of HD patients and animal models, as well as cultured HD striatal neurons (Browne and Beal, 2004). It is not surprising that energetic metabolism affects the normal functions of neurons, or even leads to neuronal cell death, because neurons require high energy to restore membrane potential after frequent impulses, to generate dendrite outgrowth and maintain synaptic connections, to control membrane receptors and their internalization, to control the trafficking and recycling of various vesicles, and also require energy to release and re-uptake diverse neurotransmitters.

Mitochondria are the most important energy metabolic organelles and also the key sensor of cell damage. Mitochondrial morphology and bioenergetics status are found to be affected in HD. In HD striatal neuron cultures, mitochondrial fragmentation and cristae alterations have been characterized. This may be due to the increased activity of phosphatase calcineurin which dephosphorylates the mitochondrial fission GTPase dynamin-related protein 1 (Drp1) to elevate its activity. The mutant Htt may also directly interact with Drp1 and change its structure and function (Costa et al., 2010, Song et al., 2011). The up-regulated activity of Drp1 impairs mitochondrial fission-fusion balance, thus triggers the mitochondrial fragmentation and cristae remodeling which decreases cristae surface area and volume. In addition, the decreased activity of several important enzymes, including respiratory chain complexes I, II, III, and IV, which are involved in energy metabolism, have also been reported, indicating an impaired energy generating in HD (Zheng and Diamond, 2012).

Besides energy metabolism, mitochondria have also been known to have an important role in buffering cytoplasmic calcium levels in order to respond to neuronal activity. They take up calcium actively from the cytosol by the power of mitochondrial proton gradient that drives the calcium accumulation. Moderate increase in mitochondrial calcium concentration can produce an adjustment of ATP production to meet the energy demand of neuronal activity. But in pathological conditions, calcium overload may result in extensive mitochondrial depolarization which leads to the opening of the mitochondrial permeability transition (MPT) pore and increased susceptibility to calcium transient (Nicholls, 2009).
HD, the depolarized mitochondria lose the function of calcium buffering, together with the increased calcium influx from over-activated NMDA receptors, make the striatal neurons which are high energy demanding, particularly vulnerable to apoptosis. Due to the energy producing role the mitochondria play in cells, they are also one of the most important sources of reactive oxygen species (ROS) which may cause oxidative stress. The ROS are chemically reactive molecules containing oxygen, mainly including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), and reactive nitrogen species (like NO). They can cause lipids peroxidation, protein degradation and nucleic acid damage. In HD, an increased lipofuscin, a product of unsaturated fatty acid peroxidation, broken DNA fragments and the accumulation of 8-hydroxy-2’-deoxyguanosine (OH$^8$dG) which is a marker of ROS are found to be increased in brains (Browne et al., 1999, Hersch et al., 2006). However, the origin and the role of ROS in HD have not been defined clearly. But it has been reported that aconitase, which is an enzyme in mitochondrial TCA cycle containing iron-sulfur cluster, is impaired in HD patients and mouse model (Tabrizi et al., 1999, Tabrizi et al., 2000).

Altogether, there is a clear link between mitochondrial dysfunction and HD pathology. But it is not yet sure that whether it is a cause, epiphenomenon or coping effect in the neurodegenerative process. Further studies in this organelle may lead to new therapeutic strategies to HD.

1-3-4 Altered neurotransmission in HD

According to investigations of neurotransmission and plasticity in HD mouse models, the abnormal synaptic transmission occurs long before the onset of classical disease indicators, like motor dysfunctions. Some recent human studies also suggest that early cognitive deficits occur years prior to cell death or predicted clinical diagnosis in HD carriers, probably due to synaptic and cellular dysfunction (Paulsen et al., 2008, Schippling et al., 2009, Orth et al., 2010). The altered neurotransmission which underlies the early symptoms of HD may progress and trigger cell death in the later stage of this disease. Although the function of Htt is still not clearly assessed, it is believed to have a role in vesicular trafficking, exocytosis and endocytosis. Some studies show that mutant Htt interacts with a number of pre- and postsynaptic proteins involved in vesicle transport, receptors internalization and synaptic structure maintenance (Li et al., 2003, Borrell-Pages et al., 2006, Rong et al., 2006). These interactions with mutant Htt may alter the synaptic
transmission with the loss-of-function of wild-type Htt combining the gain-of-function of mutant Htt.

Progressive loss of neuronal complexin II is shown in cells expressing mutant Htt, in R6/2 transgenic mice and in human HD patients (Morton and Edwardson, 2001, Morton et al., 2001). Complexin II is involved in neurotransmitter release by interacting with the soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor (SNARE) complex, which regulates membrane fusion between the synaptic vesicle and the presynaptic plasma membrane. The protein and mRNA levels of rabphilin 3A, which is a protein involved in priming and docking of vesicles to the plasma membrane, are both progressively reduced in R6/1 transgenic mouse model of HD along with the onset of motor deficits and behavioral disturbances (Smith et al., 2005). The PKC and CK2 substrate in neurons 1 (PACSIN 1) is a neurospecific phosphoprotein which plays an important role in synaptic vesicle and receptor recycling, thus is involved in endocytosis. This protein interacts with Htt in a polyQ-length-dependant pattern, therefore the interaction is enhanced with mutant Htt and it may lead to sequestration of PACSIN 1. In brains from HD patients, the expression level of PACSIN 1 seems normal but it is relocated from nerve terminals to neuronal cell bodies. This re-localization may impair the endocytosis in HD (Modregger et al., 2002). The huntingtin-associated protein 1 (HAP1) is another protein interacting with Htt and have a role in neurotransmission. In normal condition, HAP1 interacts with p150Glued of dynactin, and then dynactin binds dynein. The interaction of dynactin and dynein is necessary for vesicular transport along microtubules. But in HD, the binding affinity between HAP1 and mutant Htt is increased by the expanded polyQ. This may result in the depletion of HAP1 from its functional HAP1/dynactin/dynein complex and lead to impaired vesicular transport along microtubules. Importantly, the attenuated BDNF transport may be due to this mechanism (Gill et al., 1991, Li et al., 1995, Gauthier et al., 2004). Abnormal phosphorylation in HD can also affect neurotransmission. Synapsin I is one of the major phosphoproteins regulating neurotransmitter release by attaching the vesicles to actin and microtubule cytoskeletalles. Its function highly depends on site-specific phosphorylation and dephosphorylation. In R6/2 HD mice, the phosphorylation of synapsin I is altered and reduced affinity to actin filament is observed. This may be due to the decreased expression level of calcineurin-B involved in dephosphorylation process in neurons (Lievens et al., 2002).
Although the molecular mechanisms underlying impaired neurotransmission in HD are still largely unknown, after years of research, there are more and more evidence showing that the changes in different neurotransmission pathways in HD are important in the disease development, especially in early stage. Because the neurotransmission dysfunctions usually occur before the onset of classic symptoms, they may be the trigger for HD pathogenic progress.

1-3-4-1 Glutamatergic neurotransmission in HD

Glutamate is the predominant excitatory neurotransmitter in mammalian central nervous system which is crucial for a wide range of physiological processes, including neuron development, communication and integrative brain function. It exerts its actions by binding with its receptors which are mainly divided into two groups, ionotropic receptors and metabotropic receptors. Ionotropic glutamate receptors are ligand-gated cation channels that mediate the fast excitatory neurotransmission. There are three types of ionotropic glutamate receptors which are N-methyl-D-aspartate receptor (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors. Metabotropic glutamate receptors (mGluR) belong to the subfamily C of G Protein-Coupled Receptors (GPCRs) and can be further divided into 3 subgroups depending on sequence homology and G protein coupling specificity. Metabotropic glutamate receptors may modulate synaptic transmission by regulating protein synthesis through second messenger systems, thus play a slow but prolonged change in synaptic connections (Olney, 1994, Ribeiro et al., 2011).

In HD, besides the mutant Htt inclusions, another hall-mark of the disease is the selective loss of striatal medium spiny neurons (MSNs). Because the striatal MSNs receive extensive excitatory glutamatergic innervations from the cerebral cortex (cortico-striatal pathway) and the thalamus (thalamo-striatal pathway), the changes of glutamatergic neurotransmission resulting in excitotoxic neuronal damage have been described as a key event in HD pathogenesis. The excitotoxicity hypothesis is based on the facts that (1) the glutamate release in the striatum is increased when the glial glutamate transporter (GLT-1) which is responsible for glutamate clearance, is down regulated in R6 HD model (Lievens et al., 2001, Nicniocaill et al., 2001), (2) the mRNA and protein levels of mGluR1, 2 and 3 are all decreased. The mGluR2 is reported to locate presynaptically and control the release of glutamate (Cha et al., 1998, Cartmell and Schoepp, 2000). The decrease of mGluR2
Figure 1-8. Synaptic and Extrasynaptic Receptors in HD. The left side shows synaptic and extrasynaptic NMDA receptors functioning in a balanced manner in the normal condition. On the right in HD there is an increase in extrasynaptic NMDA receptor function due to increase in either the number of receptors and/or an increase in extracellular glutamate that shifts the functional balance toward the extrasynaptic receptors enhancing the proapoptotic pathway. From (Levine et al., 2010).
leads to an increased glutamate secretion. Together with impaired clearance system, the glutamatergic neurotransmission is significantly increased in HD, which progressively induces excitotoxicity and MSNs loss in the striatum.

However, the mechanisms that link to the postsynaptic receptor responses to the increased glutamatergic input are still not clear. In presymptomatic HD mice, it has been shown that an increased glutamatergic neurotransmission in the striatum contributes to striatal neuronal stress. But this increased sensitivity mainly relies on NMDA receptors because neither spines density nor AMPA receptors mediated current frequency and amplitude is changed (Cepeda et al., 2003, Milnerwood and Raymond, 2007, Cummings et al., 2010, Milnerwood et al., 2010) and cell swelling is not differentially affected by non-NMDA receptor activation between control and symptomatic HD mice (Levine et al., 1999). The increased NMDA receptor mediated current is linked with cell coexpressing NR1/NR2B subunits but not NR1/NR2A subunits in HD mice (Chen et al., 1999). Moreover, increasing the expression level of NR2B subunit in brains from HD mice may exacerbate disease phenotype and pathology (Heng et al., 2009). Collectively, these data suggest that glutamate vulnerability may highly depend on the receptor subunit composition and the interaction between mutant Htt and NR2B may contribute to this selective neuronal dysfunction and degeneration (Chen et al., 1999). Besides the NR2B mediated toxicity, some other studies suggest that the location of NMDA receptors may be important for their roles in cell survival (Levine et al., 2010). In general, activation of synaptic NMDA receptors generates pro-survival signaling including BDNF transcription, whereas activation of NMDA receptors at peri- and extrasynaptic sites is believed to be neurotoxic and may induce cell death (Milnerwood and Raymond, 2010) (Fig. 1-8). Thus, the balance of activity or the numbers of each type NMDA receptor at each location can sway the outcome between cell survival and cell death (Levine et al., 2010). According to this hypothesis, a study shows increased extrasynaptic NMDA receptor induced currents and signaling in the YAC128 HD mice. Because the extrasynaptic NMDA receptors require glutamate spillover and higher activity for their activation compared to synaptic receptors, low dose of NMDAR competitive antagonist (memantine) can selectively silent them. The blockade of extrasynaptic NMDARs leads to increased CREB activity and enhanced performance of HD mice (Milnerwood et al., 2010). In the same study, evidence indicates that the elevated extrasynaptic NMDAR current is associated with an increased number of NR2B subunit-containing extrasynaptic NMDARs. Increased extrasynaptic signaling,
which is due to about 50% elevation of NR2B-containing NMDARs at peri- and/or extrasynaptic sites, is specifically associated with cognitive deficits, motor impairments and neurodegeneration in older HD mice (Milnerwood et al., 2010). Another study shows that the activation of synaptic NMDAR can induce Htt inclusions while the extrasynaptic signaling reduces inclusion formation. The reduction of inclusion makes cells more vulnerable by impairing the PGC-1α cascade (see above) and increasing the level of the small binding protein Rhes which has been shown to sumoylate and disaggregate mutant Htt (Okamoto et al., 2009). Interestingly, as described above, the mutant Htt can directly alter the expression of PGC-1α by associating with its promoter. Together with the role of NMDAR in HD, both the expression level and the function of PGC-1α are significantly impaired by mutant Htt.

The mechanisms of the altered NMDAR distribution and enhanced NR2B subunit mediated signaling in HD have yet to be studied thoroughly. But the interaction between mutant Htt and synaptic proteins may contribute to the alteration of NMDAR. The postsynaptic density protein 95 (PSD95) is a member of the membrane-associated guanylate kinase (MAGUK) scaffold family which exclusively characterizes excitatory synapses. PSD95 binds NMDA receptors directly via NR2 subunits and AMPA receptors indirectly via their auxiliary TARP (Transmembrane AMPA regulatory protein) subunits to form clusters in the postsynaptic membrane and linking receptors to cytoskeletal and signaling proteins (Newpher and Ehlers, 2008). PSD95 also interacts directly with Htt and this interaction is significantly impaired with mutant Htt (Harjes and Wanker, 2003), leading to enhanced NR2B-PSD95 binding and extrasynaptic NMDAR localization in HD mice (Milnerwood et al., 2010). Reducing NR2B-PSD95 binding leads to decreased mutant Htt toxicity in vitro is also shown (Fan et al., 2009). The NMDAR trafficking may also be regulated by post-translational modification, such as palmitoylation which is a process to transfer the palmitate to proteins via chemical modification of cysteine residues. One of the Htt-interacting proteins (HIP14) is a palmitoyl transferase (Huang et al., 2004). The misregulated NMDAR localization may be due to altered palmitoylation that increases binding between PSD95 and NR2B (Fan et al., 2009). In addition, it is reported that the NMDAR surface expression may be regulated by phosphatase, like striatally enriched tyrosine phosphatase (STEP). STEP seems to be involved in excitotoxicity through extrasynaptic NMDARs (Xu et al., 2009).
Other neurotransmission pathways may also influence the glutamatergic neurotransmission in HD. For example, it is well established that dopamine receptors are widely distributed in the striatum and their activity regulates glutamatergic neurotransmission via either modulation of glutamate release (presynaptic) or glutamate receptor function (postsynaptic) (Cummings et al., 2006). This will be discussed later. In addition, the total expression levels of glutamate receptors are also important in glutamate signaling and excitotoxicity. In symptomatic R6/2 HD mice, both decreased NMDAR and AMPAR expression is observed in the striatum and later in the cortex (Cha et al., 1998, Cha et al., 1999). This corresponds to the studies in human HD that show selective reductions of NMDA and AMPA receptors in patients in both caudate and frontal cortex (Young et al., 1988, Wagster et al., 1994). These findings may relate to the failure of cortico-striatal pathway and motor dysfunctions in HD. However, the levels of glutamate receptors change across HD progression is still controversial, because other studies suggest the expression of glutamate receptors may not be changed in either HD mice (Jarabek et al., 2004) or human patients (Dure et al., 1991).

**1-3-4-2 GABAergic neurotransmission in HD**

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian central nervous system. It performs inhibitory action through binding GABA to two classes of GABA receptors: GABA<sub>A</sub> receptors which are ligand-gated ion channels and GABA<sub>B</sub> receptors which are metabotropic G-protein coupled receptors. Importantly, the majority of neurons in the basal ganglia which is considered to control smooth movement are GABAergic. Especially two components of the basal ganglia, the striatum and the globus pallidus (GP), are composed of almost exclusively GABAergic neurons. Therefore, GABAergic neurotransmission plays an important role in movement control and its dysfunction will lead to movement disorders, such as HD. How GABAergic neurotransmission changes in HD and the mechanisms underlying these changes have yet to be fully investigated.

Based on postmortem human HD studies, it is well established that the atrophy of the caudate nucleus and putamen (the striatum) is described as one of the most evident hallmarks. And the GABAergic projection neurons (MSNs) which represent more than 90% of striatal neurons are particularly vulnerable in HD (Graveland et al., 1985). In a human brain autoradiography binding study, several receptors expressed in the basal ganglia
including GABA_A receptors underwent a disease-stage-dependent alteration. The GABA_A receptors binding decreased gradually at early and middle stages, and totally disappeared in the striatum at advanced stage. In the GPe (the GP in rodents) GABA_A receptors are significantly increased in HD at all disease grades, which may suggest compensation to the loss of striatal GABA signal (Glass et al., 2000). In an immunohistochemistry study, the expression levels of GABA_A receptor α1 subunit, γ2 subunit and gephrin (the major scaffolding protein at inhibitory synapses) were measured in human HD brains. Immunoreactivity of both α1 and γ2 subunits are increased in both GPe and GPi (entopeduncular nucleus, EP) in HD while gephrin is not altered (Thompson-Vest et al., 2003). However, due to the small sample size used in this study, the results may be questionable. Another study on the alterations of GABA_A and GABA_B receptors in human HD shows the upregulation of both GABA_A receptor subunits (α1, β2, β3 and γ2) and GABA_B receptor (R1) in the GP. The increased receptor immunoreactivity is not only in punctate but also diffused distributed throughout all dendrites and neuron soma which indicates an abnormal distribution of receptors in HD and possibly increased extrasynaptic receptors (Allen et al., 2009).

The changes of GABAergic neurotransmission in animal HD models are less studied than glutamatergic neurotransmission. It has been shown that in the somatosensory cortex, the inhibitory postsynaptic currents (IPSCs) are initially increased in frequency and subsequently decreased at the time when overt behavioral phenotype appears in R6/2 mice (Cummings et al., 2009). In the striatum, the frequency of inhibitory GABA receptor-mediated synaptic events is increased in the R6/2, YAC128 transgenic, and CAG140 knock-in models of HD at symptomatic age (Cepeda et al., 2007, Cummings, 2007). A recent study in R6/2 and BACHD (97Q) mice shows that the increased GABA synaptic activity in the striatum infringes principally the indirect pathway MSNs and could dampen the striatal output to the GP at symptomatic age. In the same time, the connectivity between striatal MSNs via axon collaterals from MSNs themselves is significantly reduced, but bidirectional connectivity occurs in HD but not in wild-type mice. Two types of interneurons, the parvalbumin (PV) expressing fast spiking interneurons (FSIs) and persistent low-threshold spiking interneurons (PLTSs) are also investigated and enhanced FSI activity is observed. Although there is no significant connectivity change, the frequency of spontaneous action potentials of PLTS increases which may suggest increased inhibition from PLTS as well (Cepeda et al., 2013). Taken together, the
increased GABAergic neurotransmission within the striatum seems to be a compensation for the dysregulated glutamate release: the increased glutamatergic activity and reduced GABAergic inhibition in cortical neurons result in increased corticostriatal input which leads to excessive glutamate release and excitotoxicity in the striatum; the striatal interneuron induced GABA synaptic activity is enhanced to prevent the glutamate induced neuronal damage, thus protecting MSNs from excitotoxicity.

In HD, the mutant Htt may also directly alter the trafficking of GABA\textsubscript{A} receptors. The number of GABA\textsubscript{A} receptors expressed on the surface membrane and at synaptic sites is a critical determinant of inhibitory synapse strength (Jacob et al., 2008). In addition, GABA\textsubscript{A} receptors need the function of the kinesin family motor protein 5 (KIF5) to traffic to synapses. The adaptor linking the GABA\textsubscript{A} receptors to KIF5 has been demonstrated as HAP1 (see above). Therefore, the formation of receptors-HAP1-KIF5 motor complex determines the transport of GABA\textsubscript{A} receptors along microtubules in dendrites and the recycling of receptors back to synapses after internalization. The binding affinity of mutant Htt and HAP1 is increased along with the length of expanded polyQ, thus depletes the cytoplasmic HAP1. As a result, the formation of motor complex is likely to be disrupted and the GABA\textsubscript{A} receptors synaptic delivery is diminished. Consequently, the mutant Htt may lead to insufficient synaptic inhibition and elevated neuronal excitability in HD (Twelvetrees et al., 2010, Yuen et al., 2012). The presynaptic compartment of GABA synapses can also be changed in HD and account for the abnormal GABAergic neurotransmission. An \textit{in situ} hybridization study in brains from R6/2 transgenic mice shows that the mRNA expression levels of glutamic acid decarboxylase (GAD) 67, which is an enzyme responsible for GABA synthesis, are normal in the striatum but decreased in the frontal cortex, parietal cortex, GP, entopeduncular nucleus and substantia nigra pars reticulate (Gourfinkel-An et al., 2003). Because GAD67 represents the basal GABA synthesis ability, it can be used as an index of GABA activity. Those changes indicate the disconnection of each basal ganglia components and altered corticostriatal pathway. Another GAD form, GAD65, is reported to localize mainly in nerve terminals and forms a protein complex with the heat-shock protein 70, the vesicular GABA transporter (VGAT) and cysteine string protein (CSP). This protein complex provides a structural basis for a functional coupling model between GABA synthesis and transport into synaptic vesicles (Jin et al., 2003). Therefore, GAD65 is also a good indicator for GABAergic synaptic transmission. Palmitoylation is a critical post-translational modification that controls the
function of GAD65 by regulating its trafficking from the Golgi membrane to presynaptic terminals (Kanaani et al., 2004, Kanaani et al., 2008). The palmitoylation of GAD65 is mediated by Hip14 (huntingtin interacting protein 14, see above) which also interacts with other neuronal proteins, such as 25 kDa synaptosome-associated protein (SNAP 25), PSD-95, synaptotagmin I and CSP (Huang et al., 2004). With the existence of mutant Htt, the expression of HIP14 is disrupted and the GAD65 clusters are significantly reduced. In a HD cellular model study, it is shown that subcellular localization of GAD65 is altered when mutant Htt is present, and overexpression of Hip14 can rescue the palmitoylation of GAD65 and improve GAD65 trafficking (Rush et al., 2012).

1-3-4-3 Dopaminergic neurotransmission in HD

In the brain, dopamine (DA) is classified as a member of catecholamine family and acts as a neuromodulator. It is involved in numerous functions, including motor control, cognition, emotion, food intake, reward-motivated behavior and addiction. The dopaminergic neurons are relatively few in number. They are mainly distributed in the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA), the hypothalamus and a few other regions in the brain, and different DA systems control different neural functions (Bjorklund and Dunnett, 2007). For example, the nigrostriatal DA pathway originating from the SNc and projecting to the striatum controls smooth movement; the mesocortical DA pathway which arises from the VTA, projects to cingulate, entorhinal and prefrontal cortices, is important in cognition and schizophrenia; the mesolimbic DA pathway which arises from the VTA and projects to the limbic striatum (a part of the striatum) and olfactory tubercle, is involved in reward, addiction and depression (Andre et al., 2010). The DA receptors are a group of GPCRs and can be classified into two subgroups according to their different G-protein activity. The D₁-like receptors, including D₁ and D5 subunits, are coupled to Gsα which activates adenylate cyclase and increase the level of cyclic adenosine monophosphate (cAMP), thus enhance the downstream pathway. To the contrary, the D₂-like receptors consist of D₂, D₃ and D₄ subunits and are coupled to Giα which inhibits adenylate cyclase and the cAMP pathway (Neves et al., 2002). Among those subunits, the D₁ and D₂ are predominant. Therefore, the dopaminergic neurotransmission depends largely on the activity of these two subunits.

All striatal MSNs, which represent more than 90% of the total neurons in the striatum, receive dopaminergic projection from the SNc. The MSNs can be further divided into
mainly two groups depending on their own projection pathways. The direct pathway consists of MSNs expressing D₁ receptors, substance P and dynorphin, and it projects to the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus (GPi, in rodent entopeduncular nucleus EP) or the SNc. The MSNs in the indirect pathway are expressing D₂ receptors, adenosine A₂A receptors and encephalin, and they project to the GPe (GP) which further projects to the GPi (EP) and SNr or the subthalamic nucleus (STN) (Gerfen et al., 1990, Schiffmann and Vanderhaeghen, 1993, Steiner and Gerfen, 1999). Reports show that, compared to the direct pathway, the indirect pathway MSNs projecting to the GP are more excitable and thus could be more vulnerable to abnormal glutamate release or receptor dysfunction (Cepeda et al., 2008). One of the main functions of DA receptors is to modulate the response of MSNs to excitatory glutamate input from the cortex. Generally speaking, activation of D₁ receptors increases striatal NMDA and AMPA current while activation of D₂ receptors decreases them (Levine et al., 1996). The D₁ receptor mediated enhanced glutamate response may be due to different pathways, such as cAMP formation, NMDA receptor subunit phosphorylation and activation of voltage-gated Ca²⁺ channels (Blank et al., 1997, Dunah and Standaert, 2001, Liu et al., 2004). There are also a number of D₂ receptors which are located presynaptically on corticostriatal terminals suggesting that DA can modulate glutamate release to the striatum directly by activating D₂ receptors, and thus control the excitatory input (Bamford et al., 2004). In addition, D₂ receptors can also be found on nigrostriatal DA neuron terminals to function as auto-receptors and regulate DA release (Andre et al., 2010).

In HD patients, it is hypothesized that the chorea in early HD may be induced by increased DA signaling and the late stage akinesia may be the result of decreased DA level (Bird, 1980, Stahl et al., 1986). There is one study in HD patients showing increased levels of DA as well as its major metabolite DOPAC in the cerebrospinal fluid (Garrett and Soares-da-Silva, 1992). Additionally, antagonists of DA receptors and DA-depleting agents reduce chorea, and that L-DOPA exacerbates chorea in HD (Andre et al., 2010). The biphasic change of DA levels may be the cause of biphasic movement symptoms of early and late HD. A study of positron emission tomography (PET) for D₁ and D₂ ligands shows that the density of D₁ and D₂ receptors are reduced even in asymptomatic patients and the loss of receptors progresses by 3-5% each year (Andrews et al., 1999). The loss of DA receptors in asymptomatic or early HD may correlate with early cognitive deficits in patients, such as attention, executive function, learning and memory (Backman and Farde, 2001). The
decrease of DA receptors also correlated with the duration of the disease and the decrease of GABA content which reflects a loss of neurons in the striatum (Ginovart et al., 1997). Additionally, postmortem analyses show decreased striatal binding of DA transporter (DAT) which regulates DA reuptake as well as down-regulated level of vesicular monoamine transporter type-2 (VMAT-2). These may suggest a loss of nigrostriatal projections in the late HD stage (Backman et al., 1997, Ginovart et al., 1997, Suzuki et al., 2001).

Progressive reduction of DA levels as well as decreased D\textsubscript{1} and D\textsubscript{2} receptor binding have been found in both R6/2 and YAC128 HD mice (Chen et al., 2013). It has been shown that both DAT level and long-term potentiation (LTP) induced by D1-receptor are reduced in the striatum of R6/2 mice (Kung et al., 2007, Stack et al., 2007). These indicate that the DA transmission may be altered in HD and loss of nigrostriatal inputs in the striatum may contribute to the symptoms. Accordingly, selective down-regulation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) has been observed in presymptomatic R6/2 HD mice. DARPP-32 is not only involved in dopaminergic neurotransmission, as an important substrate of cAMP, it participates in other neuronal transmitter signalling pathways like glutamate and serotonin. Its disruption may contribute to the dysfunctions of NMDA, AMPA and DA receptors in HD (Bibb et al., 2000). There is evidence that the DA neurons could be regulated by frontal cortical glutamatergic neurons via a direct cortico-nigral projection (Naito and Kita, 1994a). The direct link between DA and glutamate neurons may explain the biphasic pattern of both DA and glutamate levels in the striatum of HD. The main function of DA in the brain seems to act as a filter to regulate the active inputs and enhance the signal-to-noise ratio. Therefore, excessively high or low level of DA both induces neuropathology (Chen et al., 2013). A recent modelling work suggests that inflexibility rather than inconsistency is more relevant to explain changes during aging and neurodegeneration (Hong and Rebec, 2012). In HD, the impairment in DA and glutamate neurotransmission is likely to be attributed to a reduction in signal-filter and noise-control capacity. As disease develops, the regulative capability is progressively decreasing, which will finally lead to neuronal dysfunction, increased noise and inflexibility in the brain (Chen et al., 2013). Earlier studies show that the MSNs in the indirect pathway (D\textsubscript{2} positive) are more excitable and thus more sensitive to glutamate release, thus may be more vulnerable in some neurodegenerative diseases (Kreitzer and Malenka, 2007, Cepeda et al., 2008). However, a recent study in HD mouse
models (YAC128 and BACHD) shows that the direct pathway MSNs receive more excitatory input at early stage while the indirect pathway MSNs are not affected. At symptomatic stage, both of the pathways receive less excitatory input compared to wild-types while inhibitory transmission is increased in D2 MSNs (Andre et al., 2011). These findings may be due to the fact that the YAC128 and BACHD mouse models display mild phenotypes and do not recapitulate a shorter lifespan as in human HD. It is also possible that the earlier hypothesis should be reconsidered. As the neuronal activities in the striatum is a complex balance in GABA, glutamate, and dopamine transmission in addition to interactions among two MSN pathways and interneurons functions, the truth behind neuropathology in HD still needs to be investigated.

**1-3-4-4 Other neuronal signals changes in HD**

The cannabinoid system is an important modulatory signalling system that controls through the activation of cannabinoid receptors a number of biological functions inside or outside nervous system. Cannabinoid receptors are GPCRs and one of the most abundant GPCRs in human brain. Two types of cannabinoid receptors have been identified, the CB1 receptors which are mainly distributed in the central nervous system, and the CB2 receptors which are found predominantly in the immune system. Besides these two major cannabinoid receptors, several other types of receptors related to cannabinoid signals have been discovered. These include the transient receptor potential vanilloid type 1 (TRPV1) cation channel and the GTP binding protein-coupled receptor GPR55 (Sagredo et al., 2012).

Several evidences show that the activation of cannabinoid system in the CNS is neuroprotective: (i) CB1 receptors are located presynaptically on glutamatergic terminals and the activation of those receptors can reduce excitotoxicity; (ii) the activation of CB1 may close the voltage-sensitive calcium channels which are activated in response to NMDA receptor over-activation (Demuth and Molleman, 2006); (iii) by activation of CB2 receptors on glial cells, the release of cytotoxic factors including inflammatory cytokines is reduced and the production of prosurvival molecules, such as anti-inflammatory cytokines and neurotrophic factors, is increased (Sagredo et al., 2007). In HD patients and HD mice, CB1 receptor binding, CB1 expression and endocannabinoids levels have been found reduced (Allen et al., 2009, Dowie et al., 2009). The early dysfunction of cannabinoid system may lead to loss of protection of MSNs and make them susceptible to neurotoxicity and cell death. However, the effects of CB1 receptor agonists tested in different mouse models are not clear. More precisely, the deletion of CB1 receptors in R6/2 mice shows
exacerbated motor and neurological impairments and these phenotypes are eased by chronic treatment of CB1 receptor agonist (THC, Δ(9)-tetrahydrocannabinol) (Blazquez et al., 2011). Importantly, CB1 receptors are also highly expressed presynaptically on striatal MSNs. In our lab, a study shows that systemically chronic administration of a CB1 agonist (WIN 55,212) is able to stop the appearance of motor deficits and to enhance the number of huntingtin striatal inclusions (see Pietropaolo et al. supplementary information). It seems that the activation of CB1 receptors may induce the down-regulation of both glutamatergic and GABAergic neurotransmissions, although, differently. CB1R agonist induces greater reduction of glutamatergic neurotransmission and smaller diminution of GABAergic neurotransmission in R6/1 as compared to WT mice (Chiodi et al., 2012). This indicates a shifted balance between CB1 receptor-regulated GABA and glutamate transmissions, resulting in decreased neuroactivity which may be beneficial to HD neuropathology. A recent study shows that the down-regulation of CB1 receptors in the striatum in HD is selective. In both HD mouse models and HD patients, the decreased CB1 expression is restricted to MSNs in indirect pathway and interneurons expressing neuropeptide Y (NPY)/neuronal nitric oxide synthase (nNOS). The NPY interneurons in HD mice also display a diffused mutant Htt expression in the soma (Horne et al., 2013). These findings reveal the selective vulnerability of indirect pathway MSNs and NPY interneurons in HD. An HD cellular model shows that the treatment of cannabinoids can increase the expression level of CB1 receptors as well as the expression of BDNF-2 and PGC-1α, thus improve some molecular abnormalities, such as ATP deficit and spontaneous GABA release (Laprairie et al., 2013). Furthermore, a recent work using CB2 knockout BACHD mice demonstrates that the functions of CB2 receptors are also important in HD. The deletion of CB2 receptors accelerates the disease development and severity while treatment with CB2 agonist extends the life span and suppresses disease phenotype (Bouchard et al., 2012).

In the striatum, besides the dominant GABAergic neurons (MSNs and interneurons), there are a small number of large aspiny cholinergic interneurons which are the main source of acetylcholine (Ach) within the striatum. They receive glutamatergic inputs from the cortex and thalamus, and contact MSNs by their highly arborized axons (Izzo and Bolam, 1988). In post-mortem HD striatum, the decreased activity of choline acetyl transferase (ChAT) which is involved in the synthesis of Ach, and the reduced protein expression of vesicular Ach transporter (VACHt) have been observed, indicating a dysfunction of cholinergic
interneurons and impaired cholinergic neurotransmission in general (Suzuki et al., 2001, Massouh et al., 2008). In order to restore the cholinergic tone in human HD patients, a few trials to inhibit the activity of acetylcholinesterase which is the enzyme that degrades Ach, have been tested but the results are not clear (Ghiglieri et al., 2012).

Adenosine A$_{2A}$ receptors, a type of stimulatory GPCRs activated by adenosine, are highly expressed in the basal ganglia especially on striatopallidal MSNs and cholinergic interneurons (Rosin et al., 1998). It has been reported that a transient increase in A$_{2A}$ receptor density in the striatum of presymptomatic R6/2 HD mice was followed by dramatic reductions in later stages, suggesting that A$_{2A}$ may be involved in HD pathogenesis including the early excitotoxic stage (Tarditi et al., 2006, Domenici et al., 2007). The physiological roles of A$_{2A}$ receptors depend highly on their synaptic localization. In the striatum, A$_{2A}$ receptors could be located presynaptically on the corticostriatal glutamatergic terminals, or postsynaptically on striatal neurons and dendrites. Activation of presynaptic A$_{2A}$ receptors may facilitate glutamate release and exacerbate the excitotoxicity in HD, thus the blockade of them could be beneficial to the disease. However, the postsynaptic A$_{2A}$ receptors play important roles on GABAergic neurotransmission, neurotrophin functions (like BDNF) and interactions with other neurotransmissions (CB1, D$_{2}$, mGlu5) so their inhibition may induce striatal deficits (Tebano et al., 2010, Orru et al., 2011). So far, the results of A$_{2A}$ receptor manipulation in HD are conflicting, but it is clear that A$_{2A}$ receptor knockout significantly worsen motor performance, molecular neuropathology and survival of a HD mouse model (N171-82Q) (Mievis et al., 2011). Additionally, two recent reports from a same laboratory show that neither A$_{2A}$ receptor agonist nor antagonist treatment is effective on NMDA-induced toxicity in R6/2 HD mice, but both of them modify the expression of NMDA receptor subunits (Ferrante et al., 2010, Martire et al., 2010). Besides neurons, A$_{2A}$ receptors are also expressed on striatal glial cells and may be important in the modulation of response to cell injury and inflammation (Brambilla et al., 2003). Adenosine A$_{2A}$ could be a potential drug target in HD therapy, but due to its wide-spread distribution and complex functions, more detailed mechanisms as well as more target-specific drugs still need to be revealed.

1-3-5 Animal models of HD

Beyond the limited studies in post-mortem human HD brains, many animal models have been generated to imitate the pathological factors of human HD and to be used for the
pathogenic mechanism research. Before the identification of HTT gene, HD animal models were produced according to the excitotoxic hypothesis: the over-activated glutamatergic neurotransmission in the striatum leads to striatal lesion and the HD pathology. So the glutamate analogs, such as quinolinic acid or NMDA, were injected into the striatum to mimic the early excitotoxicity in the disease (Beal et al., 1986). There are another type of animal models which is generated based on the mitochondrial dysfunctions observed in HD. 3-nitro-propionic acid (3NP) is a mitochondrial toxin which blocks succinate oxidation. Systemic injection of 3NP into rat can replicates some aspects of HD, including chorea, dystonia, some cognitive deficits, and striatal degeneration (Brouillet et al., 1999). Although these chemical animal models cannot reproduce the pathological mechanisms induced by mutant HTT gene, they are still used to study neuroprotection and neuroregeneration in HD.

Mutant mouse models of HD have been generated after the discovery of the CAG-expanded HTT gene in human patients. Those models are powerful tools because they imitate not only the behavioural symptoms or striatal lesion in HD, but also mutant Htt intra-neuronal inclusions, altered protein interactions, abnormal neurotransmissions and changed lifespan. They thus are very helpful to study molecular mechanisms and potential therapies. There are mainly three groups of mouse models available: transgenic mouse models with human HTT gene fragment, transgenic mouse models with full-length human HTT and human HTT knock-in models (Fig. 1-9). The first transgenic R6 mice lines were generated by randomly inserting a 1.9 kb fragment containing about 1 kb human Htt promoter and exon 1 of the mutant human HTT gene into mouse genome (Mangiarini et al., 1996). Depending on the number of CAG repeats in the inserted human HTT gene, the R6 mice are further divided into R6/1 (about 113-116 CAG) and R6/2 (about 144-150 CAG) lines. The R6 mice develop progressive neuropathological phenotypes, such as reduced brain and striatal volume, shortened lifespan, body weight loss, extensive intra-neuronal inclusions, altered behaviour and cognition, aberrant synaptic plasticity and impaired neurotransmissions (Mangiarini et al., 1996, Murphy et al., 2000, Milnerwood et al., 2006, Cayzac et al., 2011). Due to the different number of CAG repeats, the two R6 lines have some differences: the motor symptom onset of R6/1 is around 18 weeks while that of R6/2 is about 6 weeks; the lifespan of R6/1 can be up to 40 weeks, R6/2 is only 13 weeks at most; the intra-neuronal inclusions can be observed in R6/1 after 9 weeks but in R6/2 it is
<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Transgenic or Knockin</th>
<th>Gene Characteristics</th>
<th>Promoter</th>
<th>Repeat Length</th>
<th>Motor Symptom Onset</th>
<th>Lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6/2</td>
<td>Transgenic fragment</td>
<td>Exon 1 of human HTT gene</td>
<td>~150</td>
<td>10 weeks</td>
<td>10-13 wks</td>
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<tr>
<td>R6/1</td>
<td>Transgenic fragment</td>
<td>Exon 1 of human HTT gene</td>
<td>116</td>
<td>18 weeks</td>
<td>32-40 wks</td>
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<tr>
<td>N171-82Q</td>
<td>Transgenic fragment</td>
<td>First 171 AA of human HTT (exons 1, 2, part of 3)</td>
<td>82</td>
<td>3 months</td>
<td>16-22 wks</td>
<td></td>
</tr>
<tr>
<td>Tg100</td>
<td>Transgenic fragment</td>
<td>First ~3 kb of human HTT cDNA</td>
<td>100</td>
<td>3 months</td>
<td>Normal</td>
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<tr>
<td>HD94</td>
<td>Transgenic fragment</td>
<td>Chimeric human/mouse HTT exon 1</td>
<td>94</td>
<td>4-8 weeks</td>
<td>Normal</td>
<td></td>
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<tr>
<td>YAC72</td>
<td>Transgenic full-length</td>
<td>Full length human HTT gene</td>
<td>Human HTT</td>
<td>16 months</td>
<td>Normal</td>
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<tr>
<td>YAC128</td>
<td>Transgenic full-length</td>
<td>Full length human HTT gene</td>
<td>Human HTT</td>
<td>6 months</td>
<td>Normal</td>
<td></td>
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<tr>
<td>BACHD</td>
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<td>Full length human HTT gene (floxed exon 1)</td>
<td>Human HTT</td>
<td>2 months</td>
<td>Normal</td>
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<td>HdhQ72, Q80</td>
<td>Knockin</td>
<td>Endogenous murine Htt gene, expanded CAG inserted</td>
<td>Mouse Htt</td>
<td>12 months</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>HdhQ111</td>
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<td>Endogenous murine Htt gene, chimeric human/mouse exon 1</td>
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<td>24 months (gait)</td>
<td>Normal</td>
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<td>HdhQ94</td>
<td>Knockin</td>
<td>Endogenous murine Htt gene, chimeric human/mouse exon 1</td>
<td>Mouse Htt</td>
<td>2 months (rearing)</td>
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<tr>
<td>HdhQ140</td>
<td>Knockin</td>
<td>Endogenous murine Htt gene, chimeric human/mouse exon 1</td>
<td>Mouse Htt</td>
<td>4 months</td>
<td>Normal</td>
<td></td>
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<tr>
<td>HdhQ150</td>
<td>Knockin</td>
<td>Endogenous murine Htt gene, expanded CAG inserted</td>
<td>Mouse Htt</td>
<td>100 weeks</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1-9. Commonly Used Mouse Models of HD.** From (Crook and Housman, 2011).
before 4 weeks. Therefore, the R6/2 line is a more severe model than the R6/1 line, and like in human patients, the symptoms onset is related to CAG repeats length.

Another transgenic model is the N171-82Q mouse line which is created by expressing a cDNA encoding an N-terminal fragment (171 amino acids) of Htt with 82 polyQ under the control of the mouse prion promoter (Schilling et al., 1999). This mouse line also exhibits most of the HD symptoms, such as behavioural abnormalities, intra-neuronal inclusions, brain atrophy and progressive weight loss. The symptoms onset of N171-82Q mice is at about 12 weeks and they have a lifespan between 16 and 24 weeks.

The yeast artificial chromosome (YAC) transgenic HD mice are created by inserting full-length human HTT gene with expanded CAG repeats (46, 72 or 128) (Hodgson et al., 1999). These YAC transgenic mice are relatively mild HD models, because they have a normal lifespan, no body weight loss and they even do not show the inclusion bodies, one of the HD hallmarks. But they still present HD phenotypes at old age and are good models for early stage HD research. For example, the YAC128 mice exhibit hyperkinesia as early as 12 weeks and other behaviour deficits from 24 weeks, striatal and cortical atrophy at 48 weeks. The YAC128 mice even exhibit a small reduction of neuron number in the striatum after 48 weeks, which is not clearly observed in the other transgenic models.

Another full-length transgenic HD mouse line is generated by using bacterial artificial chromosome (BACHD) with entire human HTT containing 97 CAG repeats (Gray et al., 2008). Like the YAC models, the BACHD mouse line is also a mild HD model. These mice have normal lifespan, exhibit progressive motor deficits starting as early as 2 months but symptoms become robust by 6 months of age, show neuronal synaptic dysfunction at 6 months, and selective neurodegeneration in both striatum and cortex after 48 weeks. This model has also been used for various mutations, for example, to define the effect of specific N-terminal mutations in the Htt gene (Gu et al., 2009, Mishra et al., 2012).

In transgenic mouse models, human HTT gene is inserted randomly into the genome and it may interfere with the activity of other genes. Additionally, the overexpression of human HTT may induce artificial effects. In order to solve these disadvantages, the knock-in HD models have been generated by using the exon 1 of mutant human HTT gene to replace the normal mouse HTT gene exon 1 (HdhQ), or by inserting more CAG repeats directly into endogenous HTT gene (Hdh(CAG)). In these mouse models, the mutated HTT is placed in the appropriate position within mouse genome and is regulated by the endogenous mouse
gene expression system (Menalled, 2005). However, those mouse models do not show overt phenotypes until very old age. For example, the Hdh(CAG)150 mice (150 CAG repeats) exhibit neuronal inclusions only after 9 months of age and the abnormal behaviours (including claspning) occur at 2 years; the HdhQ140 (140 CAG repeats) mouse line shows relatively early behavioural abnormalities from 8-16 weeks and loss of striatal neurons from 2 years (Hickey et al., 2008). Those knock-in models may be very important to study the early functional deficits or more subtle HD phenotypes, like early metabolic changes, neurotransmission dysfunctions or network alterations. Therefore these KI mice can be useful to study how to delay the onset of HD symptoms.

Besides the mouse models, some other animal models have also been created. For example, a transgenic rat HD model has been generated by expressing a truncated HTT gene fragment (1.9 Kb) carrying 51 CAG repeats under the control of the endogenous rat Htt promoter (von Horsten et al., 2003). This rat model is characterized by adult-onset progressive motor phenotypes (40-50 weeks), formation of intracellular inclusions (72 weeks), shortened lifespan (98 weeks) and body weight loss. Because the anatomy, physiology, function, and circuitry of human brains are much more complex than rodent brains, the use of larger animals which are closer to humans could be better to mimic the pathological features, especially the cognitive behaviours observed in human patients. Recently, a transgenic HD rhesus monkey model has been developed by expressing exon1 human mutant HTT with 84Q and the green fluorescent protein under the control of the human ubiquitin promoter. These monkeys show key HD features, including dystonia, chorea, seizure and abundant cellular Htt aggregates (Yang et al., 2008). Due to the close genetic and evolutionary connections between non-human primates and human beings, the rhesus monkey model could be the most powerful model for HD study: they can display the cognitive changes in human HD, which cannot be mimicked by rodents; they can show the detailed brain alterations by functional in vivo imaging; the pathological dysfunctions in the brain are able to be described more accurately. However, the use of monkeys in HD studies is restricted by the relatively long lifespan, low breeding, and complicated housing or experiment processes.
1-4 Scientific research rationale

As described above, the GABAergic neurotransmission which is widely distributed in the CNS is the major inhibitory neurotransmission in the brain. Two important components of the basal ganglia system, Caudate Putamen (CPu) and Globus Pallidus (GP), are almost exclusively made of GABAergic neurons. For the reason that basal ganglia circuit plays an important role in control of movement, the abnormal GABAergic neurotransmission in CPu and GP may induce or be the consequence of movement deficits, such as those observed in HD. This disease is caused by a mutation in the huntingtin gene leading to dysfunctions of MSNs or even cell death in the CPu.

In my thesis project, I have started a new project, using molecular methods, state-of-the-art imaging techniques combined with stereology and electrophysiology to investigate the change of GABAergic neurotransmission during HD development. A R6/1 HD mouse model is used in order to assess the disease progression in presymptomatic (2 months) and symptomatic (6 months) ages. The aim of this study is to develop a strategy to identify and appraise the GABAergic synaptic connections in the CPu and GP.

Firstly, we verified our R6/1 mouse model by measuring their body weight and brain weight. We found a significant reduction of both body weight and brain weight at symptomatic age as previously showed, but not at presymptomatic age. With acetylcholinesterase (AChE) staining, we also observed both CPu and GP atrophy, as well as decreased AChE activity at 6 months. By using high performance liquid chromatography, we showed that the level of several neuromodulators is altered in R6/1 mice even at 2 months, suggesting abnormal neuromodulation in this model. In order to assess GABAergic neurotransmission, we used Western blotting to investigate the expression levels of some proteins which are involved in GABAergic neurotransmission. Those protein markers included presynaptic markers, GABA_A receptor subunits, and anchor or scaffold proteins. Our results showed alterations of those markers in the GP and/or CPu. For example, in R6/1 mice, α1 subunit was significantly increased in the CPu of symptomatic mice and decreased in the GP at both presymptomatic and symptomatic ages; α5 and δ subunits were dramatically increased and decreased, respectively, in the CPu at 6 months; gephyrin, NL2 and GAD67 expression were all decreased in the GP at both ages while the reduction of GAD65 was only observed at symptomatic stage. These
results indicated a developmental dysfunction of GABAergic neurotransmission in basal ganglia circuit of R6/1 mouse model. In the CPu, we used fluorescent immunohistochemistry to show that α1 subunit is expressed in PV neurons as well in a group of unidentified neurons of WT mice. In R6/1 mice, at 6 months, this specific α1 expression is dramatically decreased. These results in the CPu suggest a decreased inhibition of specific striatal interneurons, which might lead to elevated activity of these interneurons and enhanced inhibition of striatal MSNs. In the GP, we show a decrease of both sIPSCs (spontaneous inhibitory post-synaptic currents) and mIPSCs (miniature inhibitory post-synaptic currents) suggesting GABAergic dysfunctions developed before the onset of motor symptoms (2 months). Then we combined confocal microscopy, mosaic scanning and stereology to study the alteration of GABAergic neurotransmission at synaptic level. We found a reduction of total number of GABAergic synapses in the GP of R6/1 model at 6 months and an alteration of GABAergic synapses on PV positive neurons. All the data implicated a weakened GABAergic inhibition in the GP of R6/1 mice which could result in the disinhibition of basal ganglia output nuclei and decreased motor activity.
2 Materials and methods

2-1 Animals

Male R6/1 transgenic mice and the age-matched male wild type littermates were used in the experiments. They were from the crossbreeding of male R6/1 (C57/B6 background) and female C57/B6 mice. This R6/1 line expresses exon 1 of human huntingtin gene with an expanded 116-126 CAG trinucleotide repeats. All animals were genotyped by PCR with DNA extracted from tail specimens. The animals were housed with 12-12 hours’ light-dark circle (light on at 8 a.m.) and unlimited food/water access.

2-2 Frequently used solutions

Standard artificial cerebra-spinal fluid (ACSF) contains 126 mM sodium chloride (NaCl), 2.5 mM potassium chloride (KCl), 1.25 mM sodium phosphate monobasic monohydrate (NaH$_2$PO$_4$·H$_2$O), 2 mM calcium chloride dehydrate (CaCl$_2$·2H$_2$O), 2 mM magnesium sulfate hepta-hydrate (MgSO$_4$·7H$_2$O), 10 mM D-Glucose and 26 mM sodium bicarbonate (NaHCO$_3$). The solutions should be freshly prepared before experiment.

Sucrose ACSF contains 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$·H$_2$O, 5 mM CaCl$_2$·2H$_2$O, 10 mM MgSO$_4$·7H$_2$O, 10 mM D-Glucose, 26 mM NaHCO$_3$ and 0.23 mM sucrose (78.72 g/L). The solutions should be freshly prepared before experiment.

Phosphate buffered saline (PBS) contains 150 mM NaCl (8.78g/L), 2 mM KH$_2$PO$_4$ (0.272 g/L) and 8 mMNa$_2$HPO$_4$ (1.424 g/L). Adjust pH to 7.4.

Solutions for acetylcholinesterase staining (AChE). Stock solution (acetate buffer): 0.2M sodium acetate (NaAC) in distilled water (27.2g in 1L), adjust pH to 5.9. Solution A: 1.875g Glycine, 1.25g Copper sulfate pentahydrate (CuSO$_4$·5H$_2$O) in 50mL distilled water. Pre-incubation solution: 10mL solution A diluted in 90mL Stock solution. Incubation solution: 100mg Acetyl thiocholine iodide dissolved in 100mL Pre-incubation solution.
Cryoprotector for reservation of brain slices: 30% ethylene glycol and 30% glycerol in normal PBS solution. High concentrated PBS (5 times) and distilled water are needed in the preparation.

Urethane for anaesthesia. Make solution of 100 g/L; Use it 0.1 mL per 10g animal body weight. Mounting medium for fluorescent immunohistochemistry. This home-made mounting medium is composed of 75% glycerol, 25% Coon’s buffer and 0.1% paraphenylene diamide. The Coon’s buffer is made of 0.01 M sodium diethylbarbiturate and 0.1 M NaCl. Adjust the pH to 7.4. Paraphenylene diamide is the anti-bleaching reagent.

### 2-3 Animal weight

Animals were taken between 2 p.m. and 4 p.m. After recording the body weight, the animal was left in isoflurane anesthesia system for 5 minutes (3% isoflurane). Then the animal was decapitated and the brain was taken. After removing the spinal cord to medulla oblongata and the olfactory bulb, the brain was kept in a tube with 4% paraformaldehyde (PFA, PBS solution). The weight of the brain could be measured by weighing the difference of the tubes.

### 2-4 Acetylcholinesterase staining

(All of the mentioned solutions see 2-2 solutions for Acetylcholinesterase staining)

The brains from animal weighing were kept in 4% PFA for at least 24 hours and washed in PBS overnight before use. A Leica VT1000s vibratome was used for slicing. The cerebellum was totally removed in order to stick the brain more firmly on the sectioning stage. The vibratome speed was set at 8 and the amplitude was 5. 60 µm coronal sections were collected from the beginning to the end of the dorsal striatum. A small cut in the cortex of the left hemisphere was generated to distinguish the side. All of the slices were kept in 24-well cultural plate with PBS. The slices were washed in stock solution (see above) for 5 minutes then were incubated in pre-incubation solution (see above) for 15 minutes. Afterwards, the slices were incubated in incubation solution for 4 hours in room temperature with constant shaking. Following 5 minutes wash in stock solution, the slices were then immersed in 1% ammonium sulfate (dilute in stock solution, 500 µL per well) for at least 2 minutes until the brown color was stable. Then, the ammonium sulfate
solution was removed and all of the slices were washed in stock solution for 3 times, 5 minutes each time. Because of the strong irritative smell of ammonium sulfate, all the steps concerning this solution were performed in a fume hood. Then, all of the slices were placed on gelatin coated glass slides in good order and dried overnight in a fume hood. The next day, the slides were dehydrated with 50%, 70%, 95%, 100% ethanol and 100% Xylene, then mounted in a mounting medium (coverquick, Labonord, France).

The acquisition and volume estimation were performed with a Leica microscope (Laborlux,) and Mercator software (Pro V6.22, Explora Nova). As illustrated in Figure 1-5B, the Caudate Putamen (CPu, or Striatum) and the Globus Pallidus (GP) were distinguished by the brown stains. The strong brown semilunar part is the Striatum and the relatively weak elliptical part at the ventral Striatum is the GP. One of every two slices was used for acquisition and calculation. The volume calculation was carried by Mercator software. The gap between each slice was set at 120 µm.

2-5 Brain tissue preparation for immunohistochemistry

2-5-1 Preparation of 16 µm sections on glass slides.  

2-5-1-1 Tissue preparation.

The tissue was prepared with the protocol used to prepare acute slices for patch-clamping. In order to keep the neurons alive as much as possible, all the process should be done on ice before the fixation with 4% PFA (made of PBS). First, freshly made sucrose ACSF (see solutions) was kept in -80°C for 40 minutes to make it ice-liquid mixture. All the surgery tools, the chamber of Leica VT1000s vibratome and the standard ACSF (for temporarily storing the slices, see solutions) were cooled by ice beforehand. The iced sucrose ACSF and standard ACSF were also saturated by carbogen bubbling. The next, animals were anaesthetized by 3% isoflurane for 5 minutes and then further anaesthetized by intraperitoneal injection of urethane (see solutions). After transcardial perfusion with cold sucrose ACSF, the animal was decapitated and brain was cut into 350 µm sagittal slices by vibratome (speed at 5, frequency at 9). The reservoir was filled with cold sucrose ACSF with constant carbogen bubbling. The slices were kept in carbogen saturated standard ACSF. When all of the slices of interest were collected, they were moved to 24-well culture plate and fixed by 4% PFA for 30 minutes. The fixation time was decided and optimized from a previous immunohistochemistry study (Schneider Gasser et al., 2006).

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Following wash in PBS for 3 times (10 minutes each time), the slices were stored in 30% sucrose (PBS solution) in fridge (4 °C) overnight for cryoprotection. The next day, all slices were mounted on glass slides and frozen with freezing gel (Freeze gel, Labonord, France) in -43 °C isopentane. The slices could be stored in -80 °C freezer for later use.

2-4-1-2 Cryostat re-slicing

In order to balance the temperature, the 350 µm frozen slices (-80 °C) were left in cryostat (Leica CM3000, set at -18 °C) for at least 30 minutes before cutting. At the end of this step, the tissue was pasted to the cutting platform with freezing gel. The platform was adjusted and the thickness was set at 16 µm. 2% gelatin coated slides were used for collecting those thin slices. The tissue-attached slides were thoroughly dried on heating plate (60 °C) to improve the tissue attachment and then stored at -80°C until immunohistochemical labeling.

2-5-2 Preparation of 100 µm free-floating slices.

The brains were removed from animals as described in section 2-5-1 “Preparation of 16 µm sections on glass slides”. The speed and frequency of vibratome were set at 3.5 and 9, respectively. The thickness of slices was set at 100 µm. When a single brain was finished, the slices were transferred to 24-wells culture plate with 2 to 3 slices each well. The slices were fixed with 4% PFA for 30 minutes, and then washed 10 minutes, 3 times in PBS. Afterwards, the brain slices were stored in cryoprotector (see solutions, §2-2) in -20 °C. The slices could be reserved in such condition for years. Each time before use, wash the slices with PBS twice and 0.3% Triton X-100 (in PBS) twice, 10 minutes each time.

2-6 Immunohistochemistry

2-6-1 Standard fluorescent immunohistochemistry on slides.

Slides were taken from -80 °C freezer and left in room temperature until dry. Then they were rehydrated and washed 3 times in PBS, 10 minutes each time. After incubation in blocking solution for one hour, the slides were incubated overnight in primary antibodies (diluted in blocking solution) at room temperature. The blocking solution contained 4-8% serum of the species in which the secondary antibodies were raised and 0.3% Triton X-100 in PBS. The next day, the primary antibodies were discarded and the slides were washed in
PBS 3 times, 10 minutes each time before incubating in blocking solution containing secondary antibodies conjugated to fluorescent probes. In order to avoid the light bleaching of the secondary antibodies, from this step the incubator should be covered as much as possible. After one hour’s incubation in secondary antibodies, the slides were washed in PBS as before and then mounted by home-made mounting medium (see solutions-mountain medium). The slides were left in the dark for one hour for liquid exchanging, then the excessive mounting medium was removed and the slides were sealed by transparent nail varnish. When the nail varnish was dry, the slides were ready for use.

All the primary and secondary antibodies used in the experiments are listed:

Primary antibodies

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<td>Company</td>
<td>Raised species</td>
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<td>--------------------</td>
<td>----------------</td>
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**Secondary antibodies**

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</tr>
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<td>rat</td>
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</tbody>
</table>

2-6-2 Fluorescent immunohistochemistry on free-floating slices.

Slices from Cyroprotector (see Solutions) were taken and washed as described above. Then the slices were incubated in blocking solution (500 µL per well for 24-well plate) for one hour at room temperature. When the incubation was finished, the blocking solution was replaced by diluted primary antibodies (diluted in blocking solution), 300 µL per well in 24-well culture plate. The slices were left in room temperature for one hour with constant shaking, and then were stored overnight at 4 °C. The next day, the primary antibodies were
removed and the slices were washed 3 times, 10 minutes each time in PBS. Then slices were incubated in secondary antibodies (diluted by blocking solution) for 1 hour with constant shaking and aluminum foil wrap. When the incubation was finished, the slices were washed in PBS again as described before. Next, the slices were transferred onto 2% gelatin coated slides and excessive solution was removed as much as possible with absorbent paper. Slides were then mounted with mounting medium and sealed by nail varnish.

2-7 Stereology in synapse quantification

The brains were cut into 350 µm sagittal slices as described before in §2-6-1 “Preparation of 16 µm sections on glass slides-Tissue preparation”. For one animal, all slices containing the GP were collected. In order to distinguish the two different hemispheres, a cut was made in the frontal cortex of the right hemisphere. The slices from the same hemisphere (usually 4-5 slices) were put in order and marked during the freezing process. All of those serial slices were stored in -80 °C freezer for re-slicing. The re-slicing step was performed as described in “Preparation of 16 µm slide-attaching sections- Cryostat re-slicing”. One set of serial 350 µm slices were cut into 16 µm sections on gelatin coated slides, one section each slide. If one section was lost (folded or destroyed) the slide with that section would be marked and replaced by the adjacent one in later immunohistochemistry. All the sections were put in order, numbered and stored in -80 °C. There were usually 60-80 sections for one hemisphere.

One slide every 10 were chosen for triple-channel-fluorescent immunohistochemistry. The presynaptic protein VGAT, postsynaptic protein α1 subunit, and neuron specific protein parvalbumin were revealed with different fluorescent probes. The microscope was a Leica SP8 WLL2 on an upright stand DM6000 (Leica Microsystems, Mannheim, Germany), using objective HCX Plan Apo CS2 63X oil NA 1.40. The confocal microscope was equipped with a white light laser 2 (WLL2) with freely tuneable excitation from 470 to 670 nm (1 nm steps) and a diode laser at 405 nm. The scanning was done using either a conventional scanner (10Hz to 1800 Hz) or a resonant scanner (8000Hz). The microscope was composed of 2 internal PMT, 2 internal hybrid detectors and 1 external PMT for transmission. For the quantification of synapses in the GP, the mosaic scanning mode was
Figure 2-1. Illustration of Imaris software. (A) The pre- and postsynaptic compartments are calculated by the number of spots on the two channels. The diameter of spots are set at 0.5 µm. (B) The filter of “quality” are fixed (such as 15%) during the quantification to normalize the difference among sections. (C) The pre- and postsynaptic spots are colocalized with a distance less than 0.5 µm.
chosen. The image stacks were made of 5 images spaced 0.29 µm in depth. To increase acquisition speed and sensitivity, the resonant scanning mode was used with two HyDs (hybrid detection system) and the 488 nm and 647 nm fluorescent probes were scanned the same time as there is no over-lap with these two channels. The stitched mosaic images were deconvolved with “Huygens professional” (Scientific Volume Imaging, The Netherlands) and analyzed with “Imaris” (Bitplane scientific software, Switzerland). During the analysis, “Spots detection” was used with “Estimated Diameter” at 0.5 µm (estimated diameter of synaptic compartment, Fig. 2-1A). In order to normalize different slides and make comparison between wild-type and R6/1 mice, in “Filters” step a fixed percentage of quality filter (such as 15%) was applied to all of the images (Fig. 2-1B). When both of the pre- and post-synaptic “spots” were calculated, the function of “colocalize spots” was applied to quantify the number of colocalized particle pairs. The distance of particles from two different channels was set at less than 0.5 µm (estimated distance between pre- and post- synaptic regions) from the centers (Fig. 2-1C). These colocalized particle pairs were considered as synapses. Afterwards, all mosaic stacks from the same brain were used to calculate the average density of synapses by the equation:  
\[
D = \frac{\sum N_i}{d \times \sum S_i}
\]
\(D\) is the average density; \(N_i\) is the number of synapses from each mosaic stack; \(d\) is the depth of each stack; \(S_i\) is the area of the GP on each mosaic stack.

2-8 Western blot

2-8-1 Tissue dissection and protein extraction

350 µm acute slices were prepared as described in §2-6-1 “Preparation of 16 µm sections on glass slides- Tissue preparation” (before the fixation). All the slices of interest were kept in ice-cooled standard ACSF with constant carbogen bubbling. Then the slices were taken one by one to dissect out the interesting regions (GP, CPu, thalamus, hippocampus, motor cortex) under a Nikon SMZ800 brightfield microscope with SCHOTT KL1500 LCD illuminator. For each set of experiment, 3 to 4 animals were used for dissection. The dissected proteins were kept in ice-cooled Eppendorf tubes. All of the Eppendorf tubes were weighed beforehand. When the dissection was finished, the amount of tissue in each tube could be calculated by measuring the weight changes of the tubes. The RIPA buffer (Sigma-Aldrich, R0278) with protease inhibitor cocktail (Roche Diagnostics, 11836153001) was used for protein extraction at a ratio of 20 µL per milligram of tissue. The brain tissues
were disrupted and homogenized by a sonicator (Ultrasons, Annemasse, France) with ice bath (keep the temperature at about 4 °C). The homogenates were centrifuged (1-15k, Sigma) at 13000 rpm for 30 minutes at 4 °C. The supernatants were collected as the total cell lysates.

2-8-2 Protein concentration assay

The assay was performed with RC DC™ protein assay kit (BIO-RAD). The protein ladder was made as shown in the following table:

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<th>blank</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Sample dilution 1:5</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.3</td>
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The solution A’ is a mixture made of 50 µL solution S and 2.5 mL solution A. It needs to be prepared freshly and be used within 10 minutes. After being prepared, the protein ladders, as well as the samples were left in darkness for 15 minutes to allow the color reaction to proceed sufficiently. The light absorption was measured by a spectrophotometer (Thermo Spectronic Biomate 3). The standard linear equation of protein concentration was calculated with protein ladders. The protein concentration of 5 times diluted samples were then calculated with the standard equation. According to the calculation, all samples were divided into aliquot parts containing 15 µg of proteins per tube. All aliquots were frozen and stored at -20 °C.

2-8-3 Solutions for western blot

Electrophoresis solution was made of 3 g/L Trizma base (25 mM), 15 g/L glycine (200 mM), 1 g/L SDS (1%) and distilled water to 1 liter.
Transfer-membrane solution was made of 3 g/L Trizma base (Tris, 25 mM), 15 g/L glycine (200 mM), 0.2 L methanol (20%) and distilled water to 1 liter.

4 times loading buffer was made of 40% glycerol, 8% SDS, 0.5 M Tris-HCl, 0.2% Bromophenol and distilled water. Before use, mercaptoethanol was added to the loading buffer at a ratio of 1:5.

Tris-Buffered Saline (TBS-T) was made by 20 mM Tris, 500 mM NaCl, 0.1% Tween20 and distilled water. The pH was adjusted to 7.5.

Resolving gel (10%) was made of 8.1 mL deionized water, 6.7 mL 30% acrylamide, 5 mL 1.5 M Tris (pH 8.8), 200 µL 10% SDS, 100 µL 10% ammonium persulfate and 25 µL TEMED. The total volume was 20 mL.

Stacking gel (4%) was made of 7.6 mL deionized water, 1.6 mL acrylamide, 2.2 mL stacking gel buffer (62.5 mL 1M Tris, 5 mL 10% SDS, deionized water to 100 mL, pH 6.8), 50 µL 10% ammonium persulfate and 20 µL TEMED. The total volume was about 11.5 mL.

2-8-4 Samples preparation

6 µL of loading buffer was added to each sample aliquot and electrophoresis solution was added to a final volume of 20 µL. The samples were then shaken by vortex and centrifuged.

2-8-5 Electrophoresis and Western blot

The gels were made by using a BIO-RAD electrophoresis system. Mini gels (1.5 mm space) with 15 wells were made. After loading the samples, the system voltage was set at 120 V for 120 minutes (depending on the molecular weight of the protein of interest). When the electrophoresis was ended, the gels were taken to assemble “transfer-membrane-sandwich” with PVDF membrane, chromatography paper and thin sponges. The current was set constantly at 300 mA for 90 minutes. The transfer-membrane was taken in ice-cooled transfer-membrane solution with stirring. Afterward, the membranes were taken and left in ambient environment for dry. Then, they were rehydrated and washed in TBS-T. The membranes were blocked in 5% skimmed milk (TBS-T solution) for 1 hour with constant shake. When this was done, the milk solution was replaced by primary antibodies (diluted with TBS-T) and left in 4 °C overnight with constant slow shake. The next day, the
antibody solutions were removed and the membranes were washed in TBS-T 5 times, 10 minutes each time. And then, the peroxide conjugated secondary antibody solution (made by blocking solution) was used to incubate the membranes for 1 hour. After 3 washes in TBS-T (10 minutes each wash), the membranes were immersed in ECL substrate (chemiluminescent substrate system, KPL) for 1 minute then locked in autoradiography cassette (Fisher Biotech). In the darkroom, the detection was performed with high performance autoradiography film (Amersham Hyper film, GE Healthcare) and Kodak developer & fixer. The films were scanned by EPSON V600 photo scanner and the images were analyzed with ImageJ (Rasband, W.S., National Institutes of Health, Bethesda, Maryland, USA).

The list of antibodies used in Western blot:

Primary antibodies

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<th>Antibody</th>
<th>Company</th>
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GAD67 | Synaptic Systems | Rabbit | 1:2500
---|---|---|---
GAD65 | Synaptic Systems | Rabbit | 1:4000
Vgat | Synaptic Systems | Mouse | 1:1500
VMaT2 | Synaptic Systems | Rabbit | 1:1000
GAPDH | Abcam 6C5 | Mouse | 1:100,000

Secondary antibodies:

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<td>Rabbit</td>
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### 2-9 Electrophysiology

#### 2-9-1 Slice preparation

Twenty four mice were used for the electrophysiological recordings. Experiments were carried out by Marine Chazalon and Jérôme Baufreton. Animals were anaesthetized with ketamine/xylazine and perfused transcardially with ice-cold modified artificial cerebrospinal fluid (ACSF), equilibrated with 95% O₂–5% CO₂, containing (in mM): 230 sucrose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 10 MgSO₄, and 10 glucose. The brain was removed, submerged in ice-cold modified ACSF and sectioned into 350 μm thick slices with a vibrating blade microtome (VT1200S; Leica Microsystems, Germany) in the parasagittal plane. Slices containing GP were then stored in ACSF equilibrated with
95% O₂–5% CO₂, and containing (in mM): 126 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 2 MgSO₄ and 10 glucose.

2-9-2 Whole-cell voltage-clamp recordings

Single slices were transferred to a recording chamber, which was perfused continuously with modified ACSF heated to 32–34° C, equilibrated with 95% O₂–5% CO₂, and containing (in mM): 126 NaCl, 26 NaHCO₃, 3 KCl, 1.25 NaH₂PO₄, 1.6 CaCl₂, 1.5 MgSO₄ and 10 glucose. GP neurons were visualized with infrared gradient contrast video microscopy (Eclipse workstation; Nikon, Japan) and a ×63 water-immersion objective (E600FN, Nikon, Japan). Somatic patch clamp recordings were made using pipettes (impedance, 3–7 MΩ) prepared from borosilicate glass capillaries (G150–4; Warner Instruments, Hamden, CT, USA) with a micropipette puller (P-97; Sutter Instruments, Novato, CA, USA) and were filled with CsCl-based internal solution (in mM): 135 CsCl, 3.6 NaCl, 1 MgCl₂, 10 Hepes, 0.1 EGTA, 0.4 Na₂GTP, and 2 Mg₁.₅ATP, 2 QX-314 (pH 7.2, 294 mosmol l⁻¹) for the recording of miniatures and spontaneous IPSCs in voltage clamp mode.

Data were recorded using a Multiclamp 700B amplifier controlled by Clampex 9.0 (Molecular Devices, Sunnyvale, CA, USA). Signals were digitized at 20 kHz and low-pass filtered at 6 kHz, respectively. Junction potential of CsCl-based internal solution filled electrodes was of 4 mV and was not corrected. Miniatures and spontaneous IPSCs were recorded at a holding potential of -60 mV. Series resistance was monitored by a step of -5 mV every 50 s. Data were discarded when the series resistance increased by >20%. Control (25.4 ± 1.10 MΩ, n= 61) and R6/1 (25.6 ± 2.53 MΩ, n= 61) groups of recordings had similar values for series resistances (P > 0.05; Mann-Whitney U-test). Recordings were performed in the presence of 1 µM (2S) -3-[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl)(phenylmethyl)phosphinic acid (CGP55845; Tocris Bioscience, Bristol, UK), 50 µM d-(−)-2-amino-5-phosphonopentanoic acid (APV; Ascent Scientific, Cambridge, UK), 20 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX; Ascent Scientific) to block GABA_B, NMDA and AMPA/kainate receptors, respectively. Miniatures IPSCs recordings were performed in presence of 0.5µM of tetrodotoxin (TTX, Ascent Scientific). In some recordings, 20 µM 4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid hydrobromide (GABAzine/SR-95531) was applied to confirm that the remaining synaptic
events were indeed sensitive to a selective GABA<sub>A</sub> receptor antagonist. Recordings and analysis of the data were done blind of the genotype of the animals.

2-9-3 Statistics

Composite cumulative distributions of mIPSCs and sIPSCs were compared using Kolmogorov-Smirnov (K-S) test and population data using Mann-Whitney U (M-WU) test.

2-10 High performance liquid chromatography (HPLC)

2-10-1 Tissue processing for histological verification and post-mortem analysis

Before the sacrifice, animals were kept in the vivarium for six weeks without any testing. Mice were gently taken outside the vivarium to be immediately decapitated (within a few seconds) in a quiet room next door. Brains were removed rapidly, frozen using liquid nitrogen and stored until dissection at -80°C. Dissection of brain areas was performed on a frozen microtome. Bilateral punches of discrete regions were selected using a magnifying glass from frontal brain sections (250 μm) with stainless steel cannulae of 500 or 800 μm inner diameter and pooled. Samples were stored in pre-weighed, small eppendorf tubes (0.6 ml volume) and stored at -80°C. The day of the biochemical analysis, after weighing the eppendorf, tissues were homogenized in 50 μl of 0.1N HClO<sub>4</sub>, sonicated, and centrifuged at 13,000 rpm for 30 min at 4°C. Aliquots (20 μl) of the supernatants were injected into the HPLC system without dilution in the mobile phase.

2-10-2 Chromatographic analysis

Tissue concentrations of monoamines and their metabolites were measured by a sensitive HPLC-ECD system. Samples were kept at 4°C using an automated autosampler (Shimadzu, SIL-20A, Paris, France) and injected into the HPLC column (Hypersyl C18, 150 X 4.6 mm, 5 μm; C.I.L.-Cluzeau, Sainte-Foy-La-Grande, France) protected by a Brownlee–Newgard precolumn (RP-8, 15 X 3.2 mm, 7 μm; C.I.L.-Cluzeau). The mobile phase, delivered at 1.2 ml/min flow rate using a HPLC pump (LC20-AD, Shimadzu, France) was as follows (in mM): 60 NaH2PO<sub>4</sub> , 0.1 disodium EDTA, and 2 octane sulfonic acid plus 7% methanol,
adjusted to pH 3.9 with orthophosphoric acid and filtered through a 0.22 mm Millipore filter. Detection of NA, DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) was performed with an amperometric cell Ag/AgCl (VT-03) coupled to a programmable detector (Decade II Antec, AlphaMos, Toulouse, France). The potential of the electrode was set at +500 mV. Output signals were recorded on a computer (Beckman, system GOLD). Under these conditions, the sensitivity for NA, DA, 5-HT, DOPAC and 5-HIAA was 5, 2, 15, 12 and 10 pg/20μl, respectively, with a signal/noise ratio of 3:1. The time of elution for a sample was 30 minutes and standard solutions containing all the compounds of interest at known concentrations have been regularly injected into the system.

2-10-3 Data analysis

Tissue concentrations of monoamines and metabolites, expressed in ng/mg of tissue weight, and ratio between their metabolites and neurotransmitter (DOPAC/DA and 5-HIAA/5-HT) are presented in as the mean ± SEM of values. Aberrant data were discarded on the basis of the value outside the range of the average mean ± two standard deviations. We used this strict criterion with the risk of excluding relevant values, because of the size of our samples. Inter-individual variability in monoamine tissue content was assessed by using the Tukey’s boxes showing median, 25th and 75th percentiles, 10th and 90th percentiles and outliers (5th and 95th percentiles).
3 Results

3-1 Validation of R6/1 mouse model and phenotypic alterations

R6/1 mice develop progressive HD phenotypes, including reduced brain and striatal volume as well as body weight loss (Mangiarini et al., 1996). To validate our R6/1 mice line, we evaluated these parameters on mice available in our laboratory at the ages of 2 months (asymptomatic) and 6 months (symptomatic). We found that both the body weight and brain weight of R6/1 mice were significantly reduced at symptomatic age by 38% (WT: 33.99 ± 1.26g, R6/1: 21.34 ± 0.555g) and 25% (WT: 0.447 ± 0.006g, R6/1: 0.336 ± 0.043g), respectively (Fig. 3-1A). With acetylcholinesterase (AChE) staining (Fig. 3-1B), we found that in R6/1 mice at symptomatic age, the volumes of CPu (Caudate Putamen) and GP (globus pallidus) were also decreased by 30% (WT: 10.50 ± 0.220 mm$^3$, R6/1: 7.30 ± 0.355 mm$^3$) and 10% (WT: 0.908 ± 0.025 mm$^3$, R6/1: 0.816 ± 0.027 mm$^3$) (Fig. 3-1C). Additionally, by measuring the AChE staining, we found that there was a dramatic reduction (36 ± 1.9%) in 6-month-old R6/1 mice, suggesting a decrease of AChE activity and an impairment of cholinergic neurotransmission (Fig. 3-1D) in line with previous findings (Suzuki et al., 2001, Massouh et al., 2008).

3-2 Neurochemical markers along the nigrostriatal tract in wild-type and R6/1 mice.

Neurons within the CPu are controlled by glutamate and GABA. The plasticity of these excitatory/inhibitory inputs is modulated by monoamines including dopamine (DA). We assessed the level of dopamine, noradrenalin, serotonin and metabolites in the tissue from several brain domains from R6/1 and WT littermates at 2- 4- and 6-months of age. Figure 3-2 illustrates the outcome of nigrostriatal neurochemical markers at different ages of R6/1 and wild mice. In the CPu, tissue DA and DOPAC contents were stable from 2 to 6 months’ old in WT mice. Tissue DA and DOPAC were significantly lower at 2-months in R6/1 mice and a progressive decrease was observed for both markers in R6/1 mice. The decrease reached more than 60% at 6 months of age compared to WT littermates. DA
turnover (DOPAC/DA) was similar in WT and R6/1 at 2-months (respectively: 0.19 ± 0.03 and 0.17 ± 0.05; Student’s t-test). The turnover progressively decreased in WT and increased in R6/1 mice. The turnover was significantly lower at 6 months compared to R6/1 mice (0.14 ± 0.22 versus 0.26 ± 0.03; p<0.001, Student’s t-test). In the SN, a decrease of both markers in R6/1 mice was also noticed compared to WT. The decrease was less pronounced compared to the CPu at 6 months (50%). DA turnover was similar (approximately 0.5) in both mice whatever the age considered (data not shown).

The NA contents in WT mice at 2-months of age corresponded to 297 ± 50 pg/mg in the SN and 77 ± 9.5 pg/mg in the CPu. These levels were not significantly different in R6/1 whatever the age considered (ns, Student’s t-test, data not shown).

We found small differences regarding the tissue content for 5-HT markers (supplementary Fig. S1). The tissue content in the SN was lower for 5-HT and 5-HIAA in R6/1 mice (146 ± 21 and 78 ± 12 pg/mg respectively) compared to WT (281 ± 54 and 164 ± 36 pg/mg) at 4-months (p<0.05, Student’s t-test). The turnover was similar whatever the age (data not shown). In the CPu, we found a lower content of 5-HIAA in R6/1 at 6 months (p<0.05, Student’s t-test). While no modification was observed between R6/1 and WT regarding 5-HT content, a significant decrease in 5-HT turnover was noticed at 2 (0.77 ± 0.04 in WT versus 0.51 ± 0.1 in R6/1; p<0.05, Student’s t-test) and 4 (0.79 ± 0.05 in WT versus 0.39 ± 0.07 in R6/1; p<0.01) months (Fig. S1).

3-3 protein expression in the CPu and other brain regions

3-3-1 Western blots in the CPu and other brain regions

In order to evaluate GABAergic neurotransmission during HD progression, we quantified expression levels of several indicators essential for GABAergic synaptic functions using Western blot on protein extracted from brain structures at asymptomatic age (2 months) and symptomatic age (6 months). Besides the CPu, we also analyzed brain extract from thalamus, hippocampus and motor cortex, which are potentially affected in HD. Note that our data are representative of at least three or more often four independent Western blot
analyses. Moreover, each individual analysis was performed with pooled brain structures from at least 3 mice of the same age and same genotype. We therefore believe that our data represent an average of protein expression in the different mice avoiding individual variability.

Analyzed proteins were divided in three groups: proteins found in i) presynaptic or ii) postsynaptic compartments and iii) proteins involved in scaffolding or anchorage. Figure 3-3A shows expression level of GABA\(_A\)R \(\alpha_1\) subunit in the CPu with a trending increase at 2 months and a statistically significant increase to \(173.5 \pm 24.8\%\) at 6 months. On the contrary, expression level of \(\alpha_1\) subunit decreased significantly to \(73.5 \pm 5.1\%\) in the thalamus, to \(63.5 \pm 10.1\%\) in the hippocampus, and to \(66.4 \pm 11.9\%\) in the cortex at 6 months. GABA\(_A\)Rs present in synapses are associated with \(\gamma_2\) subunits. Expression of this subunit (Fig. 3-3B) is significantly increased in the CPu to \(125.5 \pm 8.9\%\) while no significant changes were measured in the thalamus, hippocampus, or cortex. Extrasynaptic receptors are mainly associated with GABA\(_A\)Rs containing either \(\alpha_5\) or \(\delta\) subunits. Figure 3-3C shows a trending or significant increase of \(\alpha_5\) at 2- and 6 months, \(127.0 \pm 21.9\%\) and \(185.8 \pm 17.6\%\) respectively, in the CPu. In the hippocampus, no change was observed between R6/1 and WT. Contrary to \(\alpha_5\), the expression of \(\delta\) subunit was decreased to \(65.4 \pm 1.6\%\) in the CPu at 6 months (Fig. 3-3D) Note that the \(\delta\)-subunit used in this study was validated on protein extracts from a \(\delta\)-KO mouse (Supplementary Fig. S2). GABA\(_A\)Rs are pentameric complexes that contain \(\beta_1\)-3 as mandatory subunits. Their expression may therefore reflect expression level of GABA\(_A\)Rs as a whole. Then, we analyzed \(\beta_2\) and \(\beta_3\) subunits that are respectively moderately or highly expressed in the CPu (Boyes and Bolam, 2007, Hortnagl et al., 2013). \(\beta_2\) displayed significant or trending increase at 2 or 6 month (Fig. 3-3E, \(143.6 \pm 16.3\%\) or \(124.8 \pm 16.1\%\), respectively), no change was measured for \(\beta_3\) (Fig. 3-3F). Taken together, these data suggested a change in receptor subtype expression in the CPu of R6/1 mice rather a change in the number of receptors.

Because GABAergic neurotransmission is also regulated by presynaptic mechanisms, expression level of proteins involved in GABA synthesis or release were measured. A significant decrease of the vesicular transporter was seen in extracts from the cortex (Fig. 3-4A, \(74.2 \pm 9.0\%\)) while no significant changes were measured in CPu, thalamus or hippocampus. An antibody recognizing both enzymes responsible for GABA synthesis (GAD 65 and GAD 67) revealed a significant decrease in the hippocampus and thalamus of 6-month-old R6/1 (72.3 \(\pm\) 7.5 \% and 35.8 \(\pm\) 2.4 \%, respectively, Fig. 3-bB) while there
was no difference in the CPu or cortex. These analyses were also performed in the CPu of 2- and 6-month old mice by using antibodies specific for GAD 67 (Fig. 3-4C) or GAD 65 (Fig. 3-4D): a decrease of GAD 67 level to 76.9 ± 3.5 % was measured at 2 months. No significant changes were seen at 6 months or for GAD 65 expression level at 2- or 6-months. Because a GABA release controlled by VMAT2-containing vesicles has been reported (Tritsch et al., 2012), we measured the expression level of this vesicular transporter (Fig. 3-4E); no significant changes were seen in the CPu or hippocampus. Gephyrin is directly associated with synaptic GABA$_A$Rs and neuroligin 2 is an adhesion molecule, associated with inhibitory synapses, interacting with gephyrin (Fritschy et al., 2008, Poulopoulos et al., 2009). Gephyrin expression was significantly decreased in the thalamus, hippocampus, and motor cortex from symptomatic R6/1 mice (63.2 ± 4.9 %, 56.0 ± 5.9 %, and 61.2 ± 4.0 %, respectively; Fig. 3-5A). No significant changes were seen in the CPu at 2- or 6-months in R6/1 mice. The expression level of NL2 was significantly decreased in the motor cortex of R6/1 mice at symptomatic age (54.9 ± 6.2 %, Fig 3-5B) while no significant changes were measured in the CPu, thalamus, or hippocampus.

3-3-2 Immunohistochemistry in the CPu

When GABA$_A$R $\alpha_1$ subunit was labeled on mouse brain sections, in addition to the expected labeling all over the neuropil in the CPu, we noticed that some cell bodies were decorated by antibodies (Fig. 3-6) in agreement with previous findings (Pirker et al., 2000). In order to identify these cells, multiple labeling was performed (Fig. 3-6A-E). Some of these cells are parvalbumin interneurons where an $\alpha_1$ labeling is present on cell body membrane and proximal dendrites (Fig. 3-6A). No colocalization with calretinin or somatostatin dense-core vesicles labeling were detected (Fig. 3-6B). These cells are not cholinergic neurons as they are not labeled by choline acetyltransferase (ChAT) antibodies (Fig. 3-6C). In addition to the $\alpha_1$ labeling on PV interneurons we also found a stronger labeling on NeuN positive but PV negative cells (Fig. 3-6D). These neurons expressing a high level of $\alpha_1$ are DARPP-32 negative showing that they are not MSNs (Fig. 3-6E). These unidentified neurons are characterized by a large dendritic tree displaying varicosities (Fig. 3-6F). The expression of $\alpha_1$ on PV and unidentified neurons were analyzed in the CPu of brain sections from 2- and 6-month-old WT and R6/1 mice (Fig. 3-7). While $\alpha_1$ labeling is clearly detected on PV neurons on WT and R6/1 littermates at 2 months (Fig. 3-7A, C), $\alpha_1$ labeling was absent from R6/1 mice at 6 months (Fig 3-7,
compare B and D). For quantification, PV cells and α1 subunit were labeled with visible-wavelength emission (Alexa Fluor 488) and far-red-emitting dye not visible by eye (Alexa Fluor 647), respectively (Fig 3-7A-D). PV cells were identified by eye and imaged on confocal microscope, and then α1 labeling was evaluated on all PV positive cells. These analyses clearly show a dramatic decrease of α1 subunit expression in PV positive interneurons in the CPu of R6/1 mice at 6 months after the onset of the disease (Fig. 3-7E). For the unidentified cells expressing a high level of α1 in the CPu, α1 labeling was performed with visible-wavelength emission dye (Fig 3-7 F, G) and the number of neurons expressed in each section were counted (Fig 3-7C). The mean number of neurons was dramatically decreased in the CPu of R6/1 brain sections both at 2- and 6-months (Fig 3-7G).

### 3-4 Data from the GP

#### 3-4-1 Western blots in the GP

As described above in the study on CPu we investigated GABAergic neurotransmission in the GP during HD progression. We firstly tested the protein expression levels of several GABA_A receptor subunits which are highly expressed in the GP, including α1, β2 and γ2 subunits. The data showed that both the α1 and β2 subunits were significantly decreased in the GP of 2- (α1 has a reduction to 67.6±9.2%, β2 is reduced to 70.9±10.4%) and 6-month-old (α1 and β2 are reduced to 59.7±5.8% and 49.6±8.4%, respectively) R6/1 mice while the γ2 did not change at 6 months (88.1±9.7%) (Fig. 3-8A-C). Next, we measured the expression of the GABA synthetase and vesicular transporters in asymptomatic (2-month-old) and symptomatic (6-month-old) R6/1 mice. Our data showed a significant decrease of GAD to 51.8±7.4% (GAD67 and GAD65 isoforms, Fig. 3-8D) in the GP of symptomatic R6/1 mice. With two specific antibodies against GAD 67 and GAD 65, respectively, we showed that the expression of GAD67 was largely reduced in the GP of both 2- (reduced to 43.3±5.7%) and 6-month-old (reduced to 65.8±7.7%) R6/1 mice while the level of GAD65 was significantly decreased only in the GP of symptomatic R6/1 mice (reduced to 47.9±4.3%) but not presymptomatic mice (81.5±8.6%) (Fig. 3-8E, F). The immunoreactivity of Vgat showed a significant reduction in the GP of asymptomatic R6/1 mice to 63.9±6.2% and a trending increase at symptomatic stage (135.4±20%) (Fig. 3-8G).
We also measured the alterations of inhibitory scaffold protein gephyrin and an adhesion protein NL2 which is predominantly expressed in inhibitory synapses. Our results revealed that the expression of both the two inhibitory synaptic markers was significantly decreased in the GP of asymptomatic (NL2 and gephyrin have reductions to 72.5±9.6% and 63.8±1.7%, respectively) and symptomatic R6/1 mice (NL2 is decreased to 60.3±7.0% and gephyrin is decreased to 71.4±10.0%) (Fig. 3-8H, I), suggesting that an alteration of GABAergic neurotransmission occurred before the appearance of motor symptoms.

3-4-2 Electrophysiology in the GP

GABA$_A$ receptor-mediated synaptic transmission was recorded in GP neurons and isolated by bath application of APV (50µM) and DNQX (20µM) and CGP55845 (1µM) to block ionotropic glutamate receptors and GABA$_B$ receptors, respectively. Electrophysiological recordings of GP neurons were performed in whole cell configuration, voltage clamp mode at a holding potential of -60mV. GABA$_A$ receptor-mediated synaptic currents were inward when recorded with CsCl-filled electrodes.

In order to determine if there was an alteration in GABA$_A$ receptor-mediated synaptic transmission in an aged-dependent manner, we recorded GABAergic synaptic transmission in asymptomatic (2 month-old) and symptomatic (6 month-old) and R6/1 animals and aged-matched WT mice. First we investigated action potential-dependent GABAergic synaptic transmission by recording spontaneous post-synaptic currents (sIPSCs). We found a significant decrease in the frequency and amplitude of spontaneous IPSCs in R6/1 mice compared to WT animals at 2 months and 6 months (Fig. 3-9). Cumulative distributions of sIPSCs inter events intervals were significantly shifted to the right (Fig. 3-9C, I; p<0.01, K-S test) suggesting a reduction in the frequency of sIPSCs and cumulative distributions of amplitude were significantly shifted to the left meaning a reduction of the sIPSCs amplitude. The reduction in frequency and amplitude of sIPSC in R6/1 mice aged of 2 and 6 months was also observed on population data (Fig. 2-9E-F, K-L) p<0.05, M-W Utest). These results show an alteration of GABAergic synaptic transmission in GP of R6/1 mice and suggest that this alteration is present before the appearance of motors deficits and involved both the pre and post synaptic elements.

In order to discriminate whether the alteration of GABAergic transmission was dependent of neuronal activity, we investigate quantal GABAergic transmission. We recorded
miniatures post-synaptic currents (mIPSCs) in WT and R6/1 animals at 2 and 6 months in presence of tetrodotoxin (TTX) which blocks voltage-dependent sodium channels (Na_v). Under these recordings conditions, we found a significant decrease in the frequency but not the amplitude of mIPSCs in R6/1 compared to WT animals at 2 months (Fig. 3-10A, B) and 6 months (Fig. 3-10G, H), respectively. Cumulative distribution plots showed a significant increase in mIPSCs inter event intervals (Fig. 3-10C; p < 0.05; K-S test)) but no change in amplitude ((Fig. 3-10D,G)) in R6/1 mice (red traces) compared to WT mice (black traces) at 2 and 6 months. The reduction in frequency but not in the amplitude of mIPSC in R6/1 mice aged of 2 months and 6 months was also observed at the population level (Fig. 3-10E, F, K, L). These results suggest that the alteration of GABAergic transmission in the GP is predominantly due to a defect in presynaptic release of GABA.

Taken together, these results suggest that the alteration of GABAergic synaptic transmission of the striato-pallidal indirect pathway precedes the appearance of motors deficiencies and is maintained over time in the R6/1 mouse model of Huntington’s disease.

3-4-3 Immunohistochemistry in the GP

Neurotransmission relies on several factors including the number of synapses and connectivity pattern (Brickley and Mody, 2012). In order to appraise synapses in the GP we are developing a strategy that combines multiple labeling, high resolution imaging, and image analyses. The microscope is a Leica SP8 using objective 63X. The confocal microscope is equipped with a white light laser freely tuneable excitation from 470 to 670 nm (1 nm steps). We used a resonant scanner (8000Hz) and 2 internal hybrid detectors to increase acquisition speed and sensitivity enabling mosaic scanning and stack acquisitions. In addition, the image stacks are made of 5 images spaced 0.29 µm in depth. The 488 nm and 647 nm fluorescent probes are scanned the same time as there is no overlap with these two channels. The stitched mosaic images are deconvolved (Fig. 3-11). These images allow the detection of individual synapses as well as the identification of different cell types as PV cells (Fig 3-11A1, A2) or dendrites (Fig 3-11A3). We used these images to quantify the number of PV neurons in the whole GP. This analysis showed that 6-month old R6/1 mice and WT littermates have the same number of PV neurons in the GP (Fig. 3-11B).
Our high resolution mosaic images were also used to quantify the number of VGAT/α1 synapses in WT and R6/1 mice. These analyses are currently done in collaboration with the private company “Explora Nova” using by developing and adding a plug-in to their software “MorphoStrider”. We also use the software “Imaris” (Fig. 3-12A-C) available at the “Bordaux Imaging Center” facility. These analyses can also be performed by using the “ImageJ” software (Fig. 3-12E). Analyses rely on the identification of VGAT- and α1-labeling as pre- and post-synaptic objects, respectively. Labelling is considered as synapses when the centres of pre- and post-synaptic objects are within a distance between 0 and 500 nm. This quantification shows a decrease in the density of synapses in the GP of 6-month old R6/1 mice (Fig. 3-12 D).
**Figures for results**

**Figure 3-1. Phenotypic alteration of 6-month-old R6/1 mice.** A, B body (WT n=12, R6/1 n=8) and brain (WT n=11, R6/1 n=8) weight. Respectively. C, Expression of acetylcholinesterase showing the striatum (CPu) and globus pallidus (GP) in mouse brains of 6-month-old WT and R6/1 littermates D, E. This labeling was used to measure the striatum volume (WT n=6 brains, R6/1 n=7) (D) as well as the expression level of the enzyme(n=3 brains) (E). ± SEM. **p < 0.01; ***p < 0.001.
Figure 3-2. Dopamine and DOPAC content (pg/mg) in the striatum and substantia nigra of 2-, 4-, and 6-month-old WT and R6/1. The content of Dopamine (DA) and metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) extracellular levels were measured by HPLC and expressed in pg/mg ± SEM, n=4, each age and each genotype. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3-3. Western blotting analysis of brain extracts from 2 month- or 6 month-old WT and R6/1 mice with GABA<sub>A</sub>R subunit antibodies. Membrane preparations extracted from striatum (CPu), thalamus (Tha), hippocampus (Hip), and cortex (Cort) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted. A, Representative Western blot out of four shows α1 subunit immunoreactivity (top panel). The blot was also probed for GAPDH as loading control (bottom panel). The bar graphs represent the means of four independent experiments and show α1 subunit expression after normalization to the corresponding expression of GAPDH; ratio measurement in wild type (WT) was set at 100% compared to ratio in R6/1 mice. Experiments show a trending increase in expression level in the striatum of 2-month-old R6/1 mice (n=4) and a significant increase at 6 months (n=4) while significant decreases was measured in the thalamus, hippocampus, and cortex of 6-month-old R6/1 mice. B, In the striatum of 6-month-old R6/1 mice, a significant increase of γ2 subunit expression (n=3) was measured while no change in the thalamus or trending increase in the hippocampus or cortex were measured. C, A trending increase of α5 subunit expression in the striatum of 2-month-old R6/1 mice (n=4) and a significant increase at 6 months (n=4) was measured while no significant change in the hippocampus was detected. D, In the striatum, a trending decrease of δ subunit expression at 2 months (n=4) and a significant decrease at 6 months (n=4) were measured. E, In the striatum, a significant increase of β2 subunit expression at 2 months (n=4) and a trending increase at 6 months (n=4) was measured. F, β3 subunit expression did not change in the striatum at 2 and 6 months. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3-4. Western blotting analysis of brain extracts from 2 month- or 6 month-old WT and R6/1 mice with pre-synaptic marker antibodies. Membrane preparations extracted from striatum (CPu), thalamus (Tha), hippocampus (Hip), and cortex (Cort) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted. **A**, Representative Western blot out of four shows vesicular transporter Vgat immunoreactivity (top panel). The blot was also probed for GAPDH as loading control (bottom panel). The bar graphs represent the means of three independent experiments and show Vgat expression after normalization to the corresponding expression of GAPDH; ratio measurement in wild type (WT) was set at 100% compared to ratio in R6/1 mice. In 6-month-old R6/1 mice, experiments show a trending decrease in expression level in the hippocampus and a significant decrease in the cortex while no significant change in the striatum and thalamus was detected. **B**, labeling with an antibody recognizing both GABA synthesizing enzymes GAD 65 and 67 showed a significant decrease in the thalamus and hippocampus of 6-month-old R6/1 mice (n=3) while no significant change in the striatum and cortex was measured. **C**, In the striatum of 2-month-old R6/1 mice (n=4), a significant decrease of GAD 67 expression was measured while expression at 6 month (n=4) did not change. **D**, the expression of GAD 65 did not change in the striatum of 2- and 6-month-old R6/1 mice (n=4). **E**, the expression of the vesicular transporter of monoamines, VMAT2, did not change in the striatum of 2- and 6-month-old R6/1 mice (n=4). *p< 0.05; ***p<0.001.
Figure 3-5. Western blotting analysis of brain extracts from 2 month- or 6 month-old WT and R6/1 mice with gephyrin and neuroligin 2 antibodies. Membrane preparations extracted from striatum (CPu), thalamus (Tha), hippocampus (Hip), and cortex (Cort) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted. A, Representative Western blot out of four shows anchoring protein gephyrin immunoreactivity (top panel). The blot was also probed for GAPDH as loading control (bottom panel). The bar graphs represent the means of three independent experiments and show gephyrin expression after normalization to the corresponding expression of GAPDH; ratio measurement in wild type (WT) was set at 100% compared to ratio in R6/1 mice. In 6-month-old R6/1 mice, experiments show significant decrease in the thalamus, the hippocampus, and the cortex while no significant change in the striatum of 2- and 6-month-old R6/1 mice was detected (n=4). B, labeling with an antibody recognizing neuroligin 2 showed a significant decrease in the cortex of 6-month-old R6/1 mice while no significant change in the striatum, thalamus, and hippocampus was measured (n=3). ***p<0.001.
Figure 3-6. Fluorescent multiple-labeling in sagittal section through the CPu of WT mice. Fluorescent labeling for GABA,R a1 subunit (A-F) and parvalbumin (PV, A), or calretinin and somatostatin (Calr and Som, B), or choline acetyltransferase (ChAT, C), or parvalbumin and NeuN (D), or DARPP-32 (E). A, PV interneuron cell membrane (arrow) and proximal dendrites (arrowhead) are decorated by a1 subunit labeling. B, C; calretinin (B, arrow) and somatostatin (B, arrowhead) or cholinergic neurons (C) do not express a1 on their cell membrane. D, Neurons labeled by NeuN antibodies (arrows) revealed putative MSNs (star) that do not express a1, parvalbumin positive neurons (empty arrowhead), and unidentified neurons (white arrowhead): Note the moderate a1 expression level in the parvalbumin neuron compare to the high level in the unidentified neuron. E, DARPP-32 labeling revealing MSNs (star) does not label neurons expressing high level of a1 (asterisk) displaying dendrites with varicosities (arrow). F, A stack of confocal z-section images of the neuron (asterisk) shown in (E) were projected to create one single image showing dendrites with varicosities (arrows). All the panels (A-F) have the same enlargement: scale bar, 10 µm.
Figure 3-7. GABA₄R α1 subunit labeling in sagittal section through the CPu of WT and R6/1 mice. Fluorescent labeling for GABA₄R α1 subunit revealed PV positive neurons in 2- and 6-month old WT mice (A, B). Asterisks indicate DARP-32 positive MSN neurons (staining not shown for clarity). In the CPu of R6/1 mice, these neurons were still labeled at 2 months while few neurons were labeled at 6 months after disease onset (C, D). E, Quantitative analyses of experiments shown in A-D showing the means of the ratio between the number of neurons labeled by both α1- and PV-antibodies versus number of neurons labeled by PV antibodies is dramatically decreased in 6 month-old R6/1 mice (n=138, from 3 animals each genotype). F, G, epifluorescence images of non-PV neurons expressing high level of α1 subunit in the striatum, showing large dendritic tree (arrows). In 6-month-old R6/1 mice, mainly sparse dendritic labeling are detected (G) due to the very low number of such neurons as quantified in H (n=3 animals). **p<0.01; ***p<0.001. Scale bar, A-D: 10 µm; F,G: 50 µm.
Figure 3-8. Western blotting analysis of Globus Pallidus (GP) extracts from 2 month- or 6 month-old WT and R6/1 mice with antibodies revealing GABA synapse markers. Membrane preparations extracted from GP were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted. A, Representative Western blot out of four shows α1 subunit immunoreactivity (top panel). The blot was also probed for GAPDH as loading control (bottom panel). The bar graphs represent the means of three independent experiments and show α1 subunit expression after normalization to the corresponding expression of GAPDH; ratio measurement in wild type (WT) was set at 100% compared to ratio in R6/1 mice. Experiments show a significant decrease in expression level in the GP of 2-month- and 6-month-old R6/1 mice (n=4).

B, A significant decrease in expression level of β2 subunit in the GP of 2-month- and 6-month-old R6/1 mice (n=4) was detected. C, γ2 subunit expression did not change in the GP at 6 months (n=3).

D, Labeling with an antibody recognizing both GABA synthesizing enzymes GAD 65 and 67 showed a significant decrease in the GP of 6-month-old R6/1 mice (n=3).

E, In the GP of 2- and 6-month-old R6/1 mice (n=4), a significant decrease of GAD 67 expression was measured. F, The expression of GAD 65 did not change significantly in the GP of 2- month-old R6/1 mice (n=4) while a significant decrease was measured at 6 month.

G, Vesicular transporter Vgat immunoreactivity experiments showed a significant decrease in 2-month-old R6/1 mice (n=4) and a trending increase at 6 months (n=4).

H, Labeling with an antibody recognizing neuroligin 2 showed a significant decrease in GP of both 2- and 6-month-old R6/1 mice.

I, Anchoring protein gephrin immunoreactivity showed a significant decrease in 2- (n=4) and 6-month-old R6/1 mice (n=3). *p< 0.05; **p<0.01; ***p<0.001.
Figure 3-9. Spontaneous GABAergic transmission in GP neurons is altered in R6/1 at 2 and 6 months. **A,B.** Representatives traces showing spontaneous Inhibitory post-synaptic currents (sIPSCs) recorded in GP neurons from WT (A) and R6/1 (B) mice aged of 2 months. **C,D.** Composite distributions indicating that there is a significant increase \((p < 0.05)\) in sIPSCs interevent intervals \((C)\) and a significant reduction in amplitude \((D)\) in R6/1 mice \((\text{red traces})\) compared to WT mice \((\text{black traces})\) at 2 months. **E,F.** Population data summarizing the reduction of frequency and amplitude of sIPSC in R6/1 mice aged of 2 months. **G,H.** Representatives traces showing sIPSCs recorded in GP neurons from WT \((G)\) and R6/1 \((H)\) mice aged of 6 months. **I-J.** Composite cumulative distributions indicating that there is a significant increase \((p < 0.05)\) in interevent intervals in sIPSCs from R6/1 mice \((\text{red trace})\) to WT mice \((\text{black trace})\) at 6 months. **J.** The cumulative probability of sIPSC amplitude depicting that there is a significant decrease \((p < 0.05)\) in the amplitude of sIPSCs from R6/1 mice \((\text{red trace})\) compared to WT mice \((\text{black trace})\) at 6 months. **K,L.** Population data summarizing the reduction of frequency and amplitude of sIPSC in R6/1 mice aged of 6 months. Recordings were performed with CsCl-based internal solution at a holding potential of -60 mV and in presence of, DNQX (20µm), AP-5 (50µM) and CGP55845 (1µM) to block AMPA, NMDA and GABA<sub>B</sub> receptors, respectively. Cumulative distributions were compared using Kolmogorov-Smirnov test and population data using Mann-Whitney U test. *\(p < 0.05\)
Figure 3-10. Quantal GABAergic transmission in GP neurons is reduced in R6/1 at 2 and 6 months. A,B. Representatives examples of miniature inhibitory post-synaptic currents (mIPSCs) recorded in GP neurons from WT (A) and asymptomatic R6/1 (B) mice aged of 2 months. C, D. Composite cumulative distributions indicating that there is a significant increase \((p < 0.05)\) in mIPSCs interevent intervals in R6/1 mice (red trace) compared to WT mice (black trace) at 2 months (C) but no difference in mIPSCs amplitude (D). E,F. Populations graphs showing the significant reduction in mIPSCs frequency \((p < 0.05)\) and the absence of modification of mIPSCs amplitude between R6/1 and WT mice aged of 2 months. G,H. Representatives examples of miniature inhibitory post-synaptic currents (mIPSCs) recorded in GP neurons from WT (G) and symptomatic R6/1 (H) mice aged of 6 months. I-J. Composite cumulative distributions showing that there is a significant increase \((p < 0.05)\) in mIPSCs interevent intervals in R6/1 mice (red trace) compared to WT mice (black trace) at 6 months (I) but no difference in mIPSCs amplitude (J). K,L. Populations graphs showing the significant reduction in mIPSCs frequency \((p < 0.05)\) and the absence of modification of mIPSCs amplitude between R6/1 and WT mice aged of 6 months. All recordings were performed with CsCl-based internal solution at a holding potential of -60 mV and in presence of TTX \((0.5\mu M)\), DNQX \((20\mu M)\), AP-5 \((50\mu M)\) and CGP55845 \((1\mu M)\) to block action potential-dependent synaptic transmission, AMPA, NMDA and GABA\(_B\) receptors, respectively. The numbers of recorded neurons are indicated in the column bars. Cumulative distributions were compared using Kolmogorov-Smirnov test and population data using Mann-Whitney U test. *\(p<0.05\)
Figure 3-11. High resolution imaging of the whole GP. Sagittal sections of mouse brain were immunolabeled to detect parvalbumin (PV), GABA_A R α1 subunit (α1), and vesicular transporter (VGAT). Fluorescence labeling were detected using optimized settings for high resolution confocal microscopy. A, the whole GP in a brain section imaged by creating a mosaic of high resolution images. A1-3, Zoom in images indicated by white frames in the mosaic showing PV positive perikarya (five-pointed stars) or dendrite (arrow); four-pointed star indicates an α1-positive PV-negative cell while an asterisk indicates an α1-positive PV-negative cell. B, quantification of PV positive neurons showing that there is no change in the GP of R6/1 mice compare to WT.
Figure 3-12. Quantitative analysis of the number of GABAergic synapses in the GP. Sagittal sections of mouse brain were immunolabeled to detect parvalbumin (PV), GABA_A R α1 subunit (α1), and vesicular transporter (VGAT). High resolution mosaic images were analyzed with the “Imaris” software. Synapses were identified as juxtaposed VGAT-α1 subunit labeling. Part of a mosaic is shown (A), white frame detailed in B and white square detailed in C. Asterisks point to PV cells; synapses are identified and quantified as juxtaposed spots and represented as red (VGAT) and green (α1) spheres (arrows). Some labeling contained 2 post- and 1 pre-synaptic spots (white arrowhead) and was counted as 2 α1 and 1 VGAT synapse; some contained 1 post- and 2 pre-synaptic spots (empty arrowhead) and was counted as 1 α1 and 2 VGAT synapse. D, Quantitative analyses synapse in the GP of 6-months old WT and R6/1 mice showing a decrease number of synapses in HD model. E1, E2, alternatively, such analyses of high resolution mosaic images can be performed with the “ImageJ” software.
Discussion

4-1 Phenotypic changes of R6/1 mice

Huntington’s disease (HD) is an inherited and progressive neurodegenerative disease with motor dysfunctions, cognitive disturbances and energy metabolic deficits (Martin and Gusella, 1986, Browne and Beal, 2004). It is well recognized that one of the most important hallmarks of HD is the selective loss or atrophy of GABAergic MSNs (medium spiny neurons) in the CPu (Van Raamsdonk et al., 2006, Guo et al., 2012), which may contribute to the chorea and akinesia symptoms. This disease hallmark has been observed in both human HD patients and in HD mice models, including R6/1 mice (Graveland et al., 1985, Mangiarini et al., 1996, Glass et al., 2000). Besides the CPu atrophy, a reduction of total brain mass and the loss of animal body weight have also been reported in R6/1 model, in agreement with our findings in phenotypic studies. A small reduction of GP volume is also observed, which is consistent with previous study in human HD brains (Wakai et al., 1993).

Interestingly, our AChE staining experiments suggest a decreased AChE activity in 6 months R6/1 mice. The level of acetylcholine (Ach) in the CPu is exclusively dependent on cholinergic interneurons which receive corticostriatal and thalamostriatal inputs and modulate the functions of MSNs. In previous post-mortem human HD studies, the decreased activity of ChAT and reduced protein expression of VACHT have been reported in the CPu (Suzuki et al., 2001, Massouh et al., 2008). Due to the fact that ChAT is a key enzyme in the synthesis of Ach and VACHT is the major transporter these findings indicate a down-regulated of cholinergic activity in human HD patients and altered function of cholinergic interneurons. AChE is the most important enzyme involved in Ach degradation. The decreased activity of AChE in R6/1 mice may suggest an adaption to reduced Ach level and compensation to weakened cholinergic neurotransmission in the CPu. Considering that AChE in the CPu is not only produced in cholinergic interneurons but also from striatal cholinceptive neurons and neurons outside the CPu (like dopaminergic neurons from SNC, corticostriatal neurons and thalamostriatal neurons) (Bernard et al., 1995), the decreased AChE activity may also be the result of altered nigrostrial pathway or abnormal cortico (thalamo) striatal neurotransmissions.
4-2 Decreased dopamine in R6/1 mice

Dopamine (DA) is an important neuromodulator controlling basal ganglia circuit, especially the MSNs in the CPu which receives abundant dopaminergic nigrostriatal inputs from the SNc. Striatal MSNs express either D₁ (direct pathway) or D₂ (indirect pathway) dopamine receptors. Abnormal dopaminergic tone may affect the functions of MSNs, leading to motor dysregulations such as PD (Parkinson’s disease) or HD. Biphasic DA level changes have been reported in human HD patients: increased DA level occurs in the cerebrospinal fluid of HD patients at early stage (Garrett and Soares-da-Silva, 1992); reduced DA level is observed from postmortem study of late stage patients (Kish et al., 1987). However, this biphasic change is not imitated in transgenic mice. A recent study demonstrated that the DA level in the CPu is already significantly decreased even in asymptomatic (4 weeks) R6/2 mice (Mochel et al., 2011). Our findings from HPLC revealed the similar situation in R6/1 mice. As early as presymptomatic age (2 months), the levels of DA and DOPAC (3,4-Dihydroxyphenylacetic acid, an important metabolite of DA) are largely reduced. This reduction progresses with the disease progression. The turnover of DOPAC/DA is also progressively changing and significantly increased in R6/1 at late stage (6 months), indicating a further depletion of DA in the CPu. Additionally, we measured the levels of DA and DOPAC in the SN from where the majority of striatal dopamine comes. There is also a reduction or tendency of reduction of both markers. These data indicate a gradual loss of DA modulation in the CPu which may contribute to the development of motor symptoms in R6/1 mice. This finding is in line with a PET (positron emission tomography) study in humans, which shows that the density of D₁ and D₂ receptors are reduced even in asymptomatic patients and the loss of receptors progresses by 3-5% each year (Andrews et al., 1999).

Since GABAergic neurons are dominant in the GP and CPu, study in alterations of GABA modulation is of interest to understand the changes in BG (basal ganglia) circuits in HD. As demonstrated before, HD is a progressive neurodegenerative disorder; it could be beneficial to compare presymptomatic animals with late stage symptomatic animals. Due to technical limitations, we cannot measure GABA content by HPLC directly. However, we combined several approaches to assess the GABAergic neurotransmission during the disease progression.
4-3 Alterations of GABAergic neurotransmission in the CPu of R6/1 mice

As discussed above, one of the major hallmarks of HD is the atrophy of CPu which is mainly composed of GABAergic neurons. Therefore, study in alterations of GABAergic neurotransmission in HD may improve our understanding of the disease’s origination and progression.

Our results of Western blot show increased β2 subunit expression and a tendency of increased α1 subunit in the CPu of 2 months R6/1 mice, while the GABAergic scaffold protein gephyrin and anchor protein NL2 are not changed. This result implies that the composition of GABA\(_A\)R may start changing before motor symptoms occur. At 6 months when full HD symptoms are developed, the alteration of GABA\(_A\)R composition becomes more obvious by the evidence of significantly raised α1, γ2 and a trend of increased β subunits while gephyrin and NL2 are not affected. According to a recent study of GABA\(_A\)R subunits distribution in mouse brain, the most concentrated subunits in the CPu are α2, α4, β3 and δ, instead of the most widely distributed α1, β2 and γ2 (Hortnagl et al., 2013). In order to reveal the details of the alteration of GABA\(_A\)R composition, studies of these striatal abundant subunits are reequired, although we have already found the expression of β3 subunit is not affected no matter the age of R6/1 mice. Interestingly, a small decrease of δ subunit is found in the CPu of presymaptomatic R6/1 and this decrease becomes significant at symptomatic age. Because δ subunit is exclusively localized in extrasynaptic membranes and control neuronal activity in response to low concentration of ambient GABA in extrasynaptic space, it is considered to control tonic GABA current in the CPu (Bright et al., 2011, Brickley and Mody, 2012, Egawa and Fukuda, 2013a). The decreased δ subunit indicates reduced tonic currents in HD, which is in line with a recent finding that the tonic GABA activity is decreased in indirect striatal MSNs of R6/2 HD model (Cepeda et al., 2013). It is recognized that tonic GABA current is neuroprotective, therefore, the reduced tonic GABA activity in the MSNs of indirect pathway may result in their higher vulnerability in HD (Cepeda et al., 2008, Brickley and Mody, 2012, Cepeda et al., 2013, Wojtowicz et al., 2013). Another GABA\(_A\)R subunit α5 is also reported to regulate tonic inhibition, although it is not expressed abundantly in the CPu (Prenosil et al., 2006, Hortnagl et al., 2013). With Western blot, we observed a dramatic increase of α5 in the
CPu of 6 months R6/1 mice, as well as a tendency of increased α5 in presymptomatic mice. It has been shown that α5 subunit is involved in cognition and memory: increased α5 activity leads to impaired memory and reduced cognitive performance, while α5 inverse agonists could restore memory deficits and enhance cognitive behavior (Rudolph and Mohler, 2013). However, these memory and cognitive effects are mainly depending on the functions of α5 in the hippocampus and cerebral cortex. The influence of increased α5 in the CPu has not been studied yet.

Altered composition of GABA₆R subunits may lead to abnormal GABAergic synaptic currents in the CPu. For example, both frequency and amplitude of sIPSCs of MSNs in the CPu are increased in R6/2 mice, as well as the kinetic and amplitude of intrastriatatal stimulation evoked IPSCs (Cepeda et al., 2013). These electrophysiological data also indicate an increased GABA release probability. Because the GABAergic neurotransmission does not only depend on postsynaptic GABA₆Rs but also on the synthesis and release of GABA from presynaptic compartment, we tested the expression of the key synthetase of GABA (GAD65 and GAD67) and the major GABA transporter (VGAT). We did not detect any change of these markers in the CPu of both presymptomatic and symptomatic mice, besides a small reduction of GAD67 at 2 months. According to a recent paper, GABA in the CPu could be released in a VGAT independent but VMAT2 dependent way (Tritsch et al., 2012). Nevertheless, we did not find any difference of VMAT2 expression in the CPu. The reason for this could be the changes of the GABAergic markers are too weak and cannot be detected by Western blot. Additionally, the decisive GABAergic regulation within CPu is determined mainly by GABAergic interneurons (Koos and Tepper, 1999). However, the number of those interneurons is small, so that their alteration of GABA synthesis/release is “shadowed” by the abundant MSNs and the GABAergic projections from the GP (Mallet et al., 2008).

Besides the CPu, we also tested the markers of GABAergic neurotransmission in several other brain regions which may be involved in HD, including the thalamus, hippocampus and motor cortex, at symptomatic age. The significant decreases of α1 subunit, VGAT, gephyrin and NL2 have been observed in the motor cortex, which is indicating a weakened GABAergic inhibition. The decreased inhibition in motor cortex may lead to hyperactivity of cortico-striatal glutamate release (Lievens et al., 2001, Nicniocaill et al., 2001), therefore, contribute to the classic excitotoxicity hypothesis of HD. In addition to the motor cortex, we observed decreased expression of α1 subunit, gephyrin and GAD67/65 in
the thalamus, which is suggesting an abnormal thalamic GABAergic activity that may influence thalamostriatal glutamatergic projections.

As mentioned above, in respect that Western blot has technical limits to investigate the properties of low-density-GABAergic interneurons in R6/1 mice we applied fluorescent immunohistochemistry with multiple fluorophores. The interneurons in the CPu are not many in number and can be biochemically divided into four subgroups: three GABAergic, one cholinergic. The three GABAergic interneuron subtypes can be distinguished neurochemically: one expresses PV (parvalbumin); one expresses CR (calretinin); the last one coexpresses NPY (neuropeptide Y), SOM (somatostatin) and nNOS (neuronal nitric oxide synthase) (Rymar et al., 2004). The PV interneurons are also classified as FS (fast-spiking) interneurons while the SOM/NPY/nNOS interneurons are PLTS (persistent and low-threshold spike) interneurons (Kawaguchi, 1993, Ibanez-Sandoval et al., 2011). The cholinergic interneurons are a group of large aspiny neurons expressing specific enzyme ChAT (choline acetyltransferase). Our fluorescent immunohistochemistry experiments have identified these subtypes of interneurons by antibodies recognizing PV, CR, SOM and ChAT. We studied these interneurons with GABA_A R α1 subunit and NeuN which is a biomarker for most neurons. Our result indicates that in wild-type mice and presymptomatic R6/1 mice most striatal PV interneurons have evident α1 expression on cell membrane and the portion of α1-expressing striatal PV neurons is dramatically decreased in symptomatic R6/1 mice. The decreased α1 expression on PV interneurons corresponds with the study of optogenetic paired recording on FS (PV)-MSN pairs (Cepeda et al., 2013). This study shows that in the paired MSNs, the amplitude and kinetics of GABA currents induced by light stimulation of corresponding PV interneurons are both significantly increased in R6/2 mice. Considering the number of connected pairs is only slightly increased and the success rates are not significantly changed, the increased firing kinetics of paired MSNs could be the result of increased GABAergic activity of PV interneurons. In our R6/1 model, the reduced proportion of α1 express on striatal PV interneurons may lead to decreased inhibition of these neurons, therefore, probably contribute to the increased stimulated activity. The increased α1 expression in the CPu, mostly on MSNs, may partially induce the elevated amplitude of GABAergic currents in paired MSNs responding to PV interneurons. Additionally, PV interneurons receive glutamatergic inputs from the cerebral cortex and, GABAergic inputs from local MSNs as well as PV positive neurons from the GP, therefore their increased activity may be a
systemic compensation to cortico-striatal excitotoxicity and be neuroprotective. It is reported that in post-mortem human HD brains, the number of PV interneurons in the CPu is significantly lowered and the degree of PV neuron loss coincidences with the appearance/severity of dystonia (Reiner et al., 2013). A recent study shows that selective inhibition of striatal FS interneurons by blocking their glutamatergic inputs will lead to reduced FS firing and result in robust dystonia-like symptoms (Gittis et al., 2011). It is tempting to speculate that the increased FS firing in HD model could be compensatory to HD dystonia behaviors and be beneficial to reduce the disease motor symptoms.

In addition to PV interneurons, we observed a type of low-density and α1 strongly expressing neurons in the CPu of WT mice. This is in line with a study of GABA_A subunits distribution in rat brain (Pirker et al., 2000). These neurons are very rare (3-6 each brain section) but they seems distributed evenly in the CPu. They usually express long and arborized dendrites with strong α1 signal. The α1 strongly expressing neurons cannot be found in the CPu of symptomatic R6/1 mice anymore, although some high α1 expressing dendrites are observed. At presymptomatic age, these neurons are not totally absent but the number seems reduced. With immunohistochemistry, we discovered that these α1 strongly expressing neurons are likely to be interneurons, because they are stained by NeuN but not DARPP-32. However, this type of neurons does not express any interneuron markers we have, including PV, CR, NPY, SOM and ChAT indicating an unidentified type of striatal interneurons. Properties of these new striatal interneurons are not clear. As the presence of the new interneurons is altered in R6/1 model, further study in these neurons may contribute to the research of HD mechanisms. We also tested the TH (tyrosine hydroxylase) expression in the CPu to identify whether these new interneurons are TH positive. However, this questions remains open as our immunolabelling experiments did not reveal TH neurons in the CPu.

4-4 Dysregulations of GABAergic neurotransmission in the GP of R6/1 mice

The GP (globus pallidus) receives massive GABAergic afferent fibres from the striatum, glutamatergic projections from the STN and some dopaminergic inputs from the SNC. It virtually projects to all the basal ganglia components including the CPu (Kita, 2007,
Jaeger and Kita, 2011). Due to the close connections between CPu and GP, the dysfunctions of striatal MSNs in HD may possibly affect the properties of GP neurons.

We evaluated the expression levels of several important proteins which are involved in GABAergic neurotransmission by Western blot. We found a dramatic decrease of α1 and β2 GABA\(_A\)R subunits in the GP of R6/1 mice at both presymptomatic and symptomatic ages. Because these two subunits are the most abundant GABA\(_A\)R subunits in the GP (Hortnagl et al., 2013), this finding indicates that the number of GABA\(_A\)Rs may possibly be reduced even at presymptomatic age when no evident alterations of protein expression in the CPu are detected. We also tested the expression of γ2 and found the expression was not changed. It is reported that γ1 subunit is also highly expressed in the GP (Hortnagl et al., 2013). This subunit can form α1β2γ1 receptor instead of α1β2γ2 complex. However, we failed to raise or find specific γ1 antibodies and could not test the expression of it. We speculate that the expression of γ1 is likely to be reduced in the GP of R6/1 mice. The altered expressions of α1 and β2 subunits are also possibly due to alternative expression of other GABA\(_A\)R subunits. The significantly reduced expression levels of NL2 and gephyrin were observed in the GP of 2 and 6 months R6/1 mice. Because gephyrin is the major scaffold protein at inhibitory synapses and NL2 is a specific inhibitory synaptic anchor protein, the decreased expression of the two markers implies the reduced GABAergic connections. However, gephyrin is not always necessary for the clustering of GABA\(_A\)Rs. It is reported that a major subset of GABA\(_A\)Rs comprising α1βγ2 receptors can accumulate and cluster at synapses independently of gephyrin (Kneussel et al., 2001, Levi et al., 2004, Luscher et al., 2011). Nevertheless, decreased expression of gephyrin together with reduced α1 subunits strongly suggest a reduction of GABA\(_A\)Rs at GABAergic synapses. Considering that GABA\(_A\)Rs need the function of the KIF5 (kinesin family motor protein 5) to traffic to synapses and the adaptor linking the GABA\(_A\)R to KIF5 has been demonstrated as HAP1 (huntingtin associated protein1), the mechanisms for decreased GABA\(_A\)Rs in HD may be due do the disruption of the GABA\(_A\)R-HAP1-KIF5 motor complex, which impairs the transport of GABA\(_A\)Rs along microtubules to synaptic compartment or the recycling of GABA\(_A\)Rs back to synapses (Twelvetrees et al., 2010, Yuen et al., 2012). It has been reported that in post-mortem human HD brain studies, the expressions of GABA\(_A\)R α1, β2, β3 and γ2 subunits in the GP are significantly increased while gephyrin is unaltered (Thompson-Vest et al., 2003, Allen et al., 2009). These studies were based only on immunohistochemistry which is not a very reliable method for quantification. In addition,
the preparation and conservation of human tissues are difficult. Moreover, the brains used in the studies in human were strongly fixed by 15% formalin and it is known that the strong fixation might alter the properties of GABA\textsubscript{A}R antigens (Schneider Gasser et al., 2006). The difference between human HD brains and R6/1 mice brains may also be due to the differences between HD in human and HD mice models.

With Western blot, we detected a reduced expression of GAD67 in the GP of R6/1 mice at both 2 and 6 months. This corresponds to an in situ hybridization study which shows decreased mRNA of GAD67 in the GP of R6/2 mice (Gourfinkel-An et al., 2003). Because GAD67 represents the basal GABA synthesis ability, the decreased GAD67 implies weakened GABAergic activity in the GP starting from presymptomatic age. GAD65 is another GABA synthetase which is localized mainly in nerve terminals and also involved in transport GABA into synaptic vesicles (Jin et al., 2003). We detected a significant decreased GAD65 expression in the GP of R6/1 mice at 6 months, indicating the reduced synaptic synthesis of GABA. Because the trafficking of GAD65 from the Golgi membrane to presynaptic terminals is mediated by Hip14 (huntingtin interacting protein 14), the decreased GAD65 in HD may be due to the interrupted Hip14 expression by mutant huntingtin that decreases GAD65 trafficking and increases its degradation in Golgi bodies (Huang et al., 2004, Kanaani et al., 2004, Kanaani et al., 2008). In a HD cellular model study, it is shown that subcellular localization of GAD65 is altered when mutant Htt is present, and overexpression of Hip14 can rescue the palmitoylation of GAD65 and improve GAD65 trafficking (Rush et al., 2012). Additionally, we observed a biphasic change of VGAT expression in the GP of R6/1 mice: a significant reduction of expression at 2 months followed by a trend of increase at 6 months. The mechanism of this alteration of VGAT is not clear due to the complex connections and various subtypes of neurons within the GP. The decreased VGAT at presymptomatic age may be associated with decreased GAD67; the increased VGAT at 6 months may be due to a compensatory mechanism for the reduced GAD65, because it is reported that GAD65 can form complex with VGAT controlling GABA synthesis and release (Jin et al., 2003).

Our electrophysiological analyses showed a significant decrease in the frequency and amplitude of sIPSCs (spontaneous inhibitory postsynaptic currents) in R6/1 mice compared to WT animals at 2 months and 6 months. This finding indicates that an alteration of GABAergic synaptic transmission is present in the GP of R6/1 mice before the appearance of any motor symptoms and this alteration may involve both pre and postsynaptic elements.
This is in line with our findings from Western blot. We also studied mIPSCs (miniature inhibitory postsynaptic currents) in the GP of R6/1 mice and the results showed a decrease in frequency but not amplitude at both presymptomatic and symptomatic ages. This indicates that the altered GABAergic neurotransmission in the GP of R6/1 mice may be predominantly due to impaired pre-synaptic mechanisms.

In order to distinguish whether the synaptic connections are altered in the GP, we combine fluorescent immunohistochemistry with confocal mosaic imaging and stereology to quantify the number of synapses in both WT and R6/1 mice. Previous studies show that pallidal neurons can be divided into PV positive or PPE (preproenkephalin) positive groups. PV positive neurons project mainly to basal ganglia output nuclei while most PPE positive neurons form pallidostratial projections (Kita, 1994, Hoover and Marshall, 1999, 2002, Mallet et al., 2012). Accordingly, we used antibody against PV to identify the pallidal neurons target the downstream BG nuclei while the PV negative neurons are mostly pallidostratial neurons. With antibodies against α1 subunit and VGAT, we defined the most abundant GABAergic synapses by the colocalization of juxtaposed α1/VGAT clusters. We showed that the total number of GABAergic synapses was reduced in the GP of 6 months R6/1 mice. This corresponds to our findings with Western blot and electrophysiology. However, whether the synaptic distribution and the synapses in different projection are altered have yet to be studied. With the mosaic images, we also quantified the number of PV positive neurons in the GP and did not find any alterations in 6 months R6/1 mice, indicating a relatively stable pallidal neuron number in R6/1 model.

**Perspectives**

Our study in R6/1 mice is not finished yet and some experiments are needed to supplement our results. Currently, I am working on evaluation of phenotypic alterations in 2 months R6/1 mice to support our studies at presymptomatic stage. In order to finish our experiments of evaluating the alteration of α1 expression in striatal PV interneurons, we need to increase the number of tested animals because we have tested only one group of 2 months mice (n=1) and three groups of 6 months mice (n=3). We also need to increase the number of animals in the quantification of α1 strongly expressing neurons in the CPu (currently n=1 in 2 months and n=3 in 6 months). As we have described that the expression of α5 subunit is increased in the CPu of R6/1 mice and the function of striatal α5 subunit is
not clearly defined, we are going to study the effect of an inverse agonist acting specifically on α5. In the GP, we are now working on the analysis of mosaic images for synaptic quantification, in order to increase the number of experiment (we have 3 sets of mosaic images but only finished analysis on one group). As mentioned before, we are trying to quantify the synapses on different subgroups of pallidal neurons, such as PV positive and PV negative neurons. With the collaboration of the private company “Explora Nova”, we presently have a new plug-in to their software “MorphoStrider” to solve this question. We may accomplish the analysis with the new plug-in within a short time. The quantification of synapses may also be applied on presymptomatic R6/1 mice in addition to the symptomatic mice.

The expression of other GABA\(_A\)R α subunits in the CPu, such as α2 and α4 which are highly expressed in the CPu (Hortnagl et al., 2013) could also be assessed. This may contribute to the understanding of how GABA\(_A\)Rs are regulated during HD progression and give new clues to drug selection. Considering the critical roles of interneurons within the CPu, we also suggest to measure the expressions of different GABA\(_A\)R subunits within different types of interneurons, which could possibly reveal how the changes of interneurons affect striatal MSNs in HD. Additionally, further study of the properties of the neurons expressing high level of α1 and quantification of their number (as well as the number of other interneurons) in R6/1 mice could be interesting in understanding the mechanisms of HD. In the GP, we suggest to apply our strategy of stereological quantification of synapses with other GABAergic markers, such as other GABA\(_A\)(B)R subunits, GAD65, gephyrin or NL2, to investigate alterations of GABAergic transmission. We can also use the same strategy to estimate the expression of extrasynaptic receptors, for example to use “α1/PV spots subtract α1/PV/VGAT spots” to estimate α1 expression on extrasynaptic membranes. In our laboratory, a D1-GFP/R6/1 mouse line is available. We can use this line to further study the alterations of different striatal pathways in HD progression. It could also be of interest to study the properties of selective striato-pallidal or pallido-pallidal projections in R6/1 mice by optogenetic approaches.
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Abstract

GABAergic neurotransmission is widely distributed in the CNS and is the major inhibitory neurotransmission in the brain. Two important components of the basal ganglia, Caudate Putamen (CPu) and Globus Pallidus (GP), are almost exclusively made of GABAergic neurons. Basal ganglia plays an important role in control of movement, the abnormal GABAergic neurotransmission in CPu and GP may be linked to movement deficits observed in Huntington disease (HD). This disease is caused by a mutation in the huntingtin gene leading to dysfunctions of medium spiny neurons (MSNs) in the CPu.

Using molecular methods, state-of-the-art imaging techniques combined with stereology and electrophysiology we have investigated the change of GABAergic neurotransmission during HD progression in a R6/1 HD mouse model to assess the disease progression in presymptomatic (2 months) and symptomatic (6 months) animals.

We used Western blotting to investigate the expression levels of some proteins which are involved in GABAergic neurotransmission. Those protein markers included presynaptic markers, GABA_A receptor subunits, and anchor or scaffold proteins. Our results showed alterations of those markers in the GP and/or CPu. In R6/1 mice, α1 subunit was significantly increased in the CPu of symptomatic mice and decreased in the GP at both presymptomatic and symptomatic ages; GABA_A R α5 and δ subunits were dramatically increased and decreased, respectively, in the CPu at 6 months; expression of proteins involved in the formation of GABAergic synapses were all decreased in the GP at both ages. These results indicated a developmental dysfunction of GABAergic neurotransmission in basal ganglia circuit of R6/1 mouse model. In the CPu, we used fluorescent immunohistochemistry to show that α1 subunit is expressed in PV neurons as well in a group of unidentified neurons of WT mice. In R6/1 mice, at 6 months, this specific α1 expression is dramatically decreased which might lead to enhanced inhibition of striatal MSNs. In the GP, we show a decrease of both sIPSCs (spontaneous inhibitory post-synaptic currents) and mIPSCs (miniature inhibitory post-synaptic currents) showing that GABAergic dysfunctions developed before the onset of motor symptoms. We also combined confocal microscopy, mosaic scanning and stereology to show a reduction of total number of GABAergic synapses in the GP of R6/1 model at 6 months and an alteration of GABAergic synapses on PV positive neurons. All the data demonstrate a weakened GABAergic inhibition in the GP of R6/1 mice which could result in the disinhibition of basal ganglia output nuclei and decreased motor activity.
Figure S1. 5-HT and 5-HIAA content (pg/mg) in the striatum and substantia nigra of 2-, 4-, and 6-month-old WT and R6/1. The content of Serotonin (5-HT) and metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) extracellular levels were measured by HPLC and expressed in pg/mg ± SEM, n=4, each age and each genotype. *p < 0.05
Figure S2. Western blotting analysis of thalamus and cortex extracts from WT and δ-subunit KO mice with anti-δ antibodies. Membrane preparations extracted from thalamus and cortex were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted. Western blot shows δ subunit immunoreactivity. The blot was also probed for GAPDH as loading control. The experiments show strong δ subunit expression in the thalamus and a lower expression in the cortex of wt mice. No labeling were detected in δ KO mice showing the specificity of the antibody used in this study.