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Hao Xu

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Dopaminergic modulation of bidirectional endocannabinoid plasticity at corticostriatal synapses

Par Hao XU
Thèse de doctorat de neuroscience

Dirigée par Dr. Laurent VENANCE

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Hao XU
RESUME

The basal ganglia are involved in the adaptive control of behavior and procedural learning. The striatum, the primary input nucleus of basal ganglia, receives afferents from both cortical glutamatergic and midbrain dopaminergic neurons, and constitutes a major site of synaptic plasticity. It is well demonstrated that synaptic plasticity at corticostratial synapses is a key substrate for procedural learning and is affected in Parkinson’s disease (PD). The corticostratial transmission and plasticity are modulated by various neurotransmitter systems, such as the endocannabinoid (eCB) and dopaminergic (DA) systems. Thus it is of critical importance to characterize the involvement of eCB-DA interactions in the striatal plasticity in both physiological and pathophysiological conditions. Here, we mainly investigated the spike-timing dependent plasticity (STDP), a synaptic hebbian learning rule in which the precise timing between pre- and postsynaptic activity affects the sign and magnitude of synaptic strength. The major aim of this project was to identify and characterize the synaptic plasticity which could be the substrate for rapid learning that involve only few spikes. Then we investigated the DA dependency of this plasticity and whether it is altered in PD.

Using electrophysiological recordings in rat/mouse brain slices, we unraveled a homosynaptic spike-timing-dependent potentiation (tLTP) mediated by eCBs and DA. We show that at the single-cell level:

1. A very low number of spikes (~5-10) is sufficient to induce eCB dependent tLTP.
2. This eCB-tLTP depends on the activation of type-1 cannabinoid receptor (CB1R).
3. eCB-tLTP occurs in both Dopamine type1 receptor (D1R)- and Dopamine type2 receptor (D2R)-expressing MSNs.
4. Dopamine, through presynaptic D2R located in cortical striatum afferents, is required for eCB-tLTP induction.
5. eCB-tLTP is impaired in a rat model of PD (6-OHDA lesion model) and is restored by chronic L-DOPA treatment.

Thus, while usually considered as only depressing synaptic function, eCBs constitute a versatile system underlying bidirectional plasticity. Moreover, eCB-plasticities are deeply affected in the pathophysiology of the basal ganglia.

In addition, we investigated another form of corticostriatal plasticity: the eCB-dependent long-term depression (LTD) induced by low frequency stimulation (LFS). We report that inhibition of the contractile non-muscle actomyosin II, fully inhibits this form of plasticity. This finding suggests a new mechanism whereby eCB-induced presynaptic actomyosin contraction would induce a retraction of vesicles from the presynaptic sites, thus leading to LTD.

In summary, the results presented in this thesis confirm and further extend a new form of interplay between eCB and DAergic systems involved in synaptic plasticity. It considerably extends their spectrum of action in physiological and pathophysiological plasticity processes.
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ABBREVIATIONS

Δt - Temporal timing interval
2-AG - 2-arachidonoylglycerol
6-OHDA - 6-hydroxydopamine
ACSF - Artificial cerebrospinal fluid
AC - Adenyl cyclases
AEA - Anandamide
bAP - backpropagating action potential
BDNF - Brain-derived neurotrophic factor
cAMP - cyclic adenosine monophosphate
CaN - Calcium-sensitive phosphatase calcineurin
CB1R and CB2R - Type-1 and type-2 cannabinoid receptors
CNS - Central nervous system
DAT - Dopamine transporter
DSI - Depolarization-induced suppression of inhibition
DSE - Depolarization-induced suppression of excitation
eCBs - Endocannabinoids
FSI - Fast-spiking interneuron
EPSC - Excitatory postsynaptic current
GPCRs - G protein-coupled receptors
GPI and GPe - Internal and external segments of globus pallidus
HFS - High frequency stimulation
LFS - Low-frequency stimulation
LTP - Long-term potentiation
LTD - Long-term depression
MFB - Medial forebrain bundle
mGluR-I - Group I metabotropic glutamate receptors
MSN - Medium-sized spiny neurons
NAc - Nucleus accumbens
PKA - Protein kinase A
PLC - Phospholipase C
PLTS - Persistent low-threshold spiking
SNC - substantia nigra pars compacta
SNR - Substantia nigra pars reticulata
STN - Subthalamic nucleus
TH - Tyrosine hydroxylase
TRPV1 - Transient receptor potential vanilloid-type-1
TBS - Theta-burst stimulation
VSCC - Voltage-sensitive calcium channels
VTA - Ventral tegmental area
CHAPTER 1. The basal ganglia

The basal ganglia (BG) are an ensemble of subcortical nuclei, which are tightly interconnected with all functional areas of the cerebral cortex, some nuclei of the thalamus and the brainstem. Historically, the basal ganglia have been initially proposed to be associated with motor control and habit formation. However, more recently, numerous evidences from rodents, primates and humans show that the basal ganglia play a crucial role in the various cognitive functions, such as working memory, decision making and even indicated in declarative memory retrieval (Packard and Knowlton, 2002; Haber, 2003; Graybiel, 2005; Yin and Knowlton, 2006; Scimeca and Badre, 2012; Grillner and Robertson, 2015). The dysfunction of neural circuits within basal ganglia lead to prominent neurological disorders including Parkinson’s disease (DeLong and Wichmann, 2007; Obeso et al., 2008), Huntington’s disease (Eidelberg and Surmeier, 2011), addiction (Berke and Hyman, 2000) and psychiatric disorders such as obsessive-compulsive disorder (Graybiel and Rauch, 2000). Thus, the studies within the framework of circuits internal to the basal ganglia attract increasing attention. These circuits are tightly modulated by monoaminergic inputs from the midbrain and by cortico- and thalamo-basal ganglia pathways, and lead into pathways toward the brainstem or recurrently toward the thalamus and back to the cerebral cortex (Gerfen, 1992a; Graybiel, 2005; Gerfen and Bolam, 2010).

1.1 The different nuclei constituting the basal ganglia

The basal ganglia constitute evolutionarily conserved structures with similar organization throughout vertebrates. The main subnuclei composing basal ganglia are highly organized in anatomo-functional territories: the dorsal and ventral striata, the subthalamic nucleus (STN), the globus pallidus (the internal segment (GPi) and the external segment (GPe)), the substantia nigra pars compacta (SNC) and the substantia nigra pars reticulata (SNr) (Figure 1,2). Briefly, these six nuclei are classified as follow:
- Two input nuclei (striatum and STN), which receive mainly excitatory afferences from cerebral cortex and thalamus;
- Two output nuclei, GPi and SNr, which project to the thalamus and brain stem;
- Two relay nuclei, GPe and STN; note that STN has a double function as an input nucleus as well as a relay nucleus.
- One neuromodulatory nucleus, SNC.

![Basal Ganglia Nuclei Circuit](image)

**Figure 1. The basal ganglia nuclei circuit.** Abbreviations: Ctx, cortex; Str, striatum; GP, globus pallidus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

The striatum

The striatum, the largest nucleus among the basal ganglia nuclei, can be divided into two main substructures which are the dorsal and the ventral striatum. In this thesis manuscript, we are interested in the dorsal portion of the striatum which integrates cortical and thalamic excitatory afferents and serves as the major input of basal ganglia (Graybiel et al., 1994; Wall et al., 2013). One of the main features of the striatum is that it is composed of GABAergic output neurons, which represent more than 90% of the striatal neurons in rodents, the so-called
medium-sized spiny neurons (MSNs). The striatum is also composed of a small proportion of GABAergic and cholinergic interneurons, which will be detailed in next chapter. It has been proposed that MSNs would act as coincidence detectors of cortical and thalamic activities. The intrinsic membrane properties (e.g. the very hyperpolarized membrane potential, around -90 mV \textit{in vivo}, the strong inward rectification of the I/V curve, the delay to first spike (Charpier and Deniau, 1997)) of MSNs make them to act as efficient detectors and integrators of different patterns of cortical and thalamic activities, which allows the extraction of behaviorally relevant information from the background noise. Upon sustained and synchronized cortical and thalamic activity, MSNs shift from a silent state to a bursting activity and relay the information toward the output nuclei of basal ganglia, i.e. the SNr and GPe in the rodents (Wickens and Wilson, 1998; Deniau et al., 2007). In addition to glutamatergic inputs, the dorsal striatum also receives a dense dopaminergic innervation from the SNc. The dopamine (DA) affects striatal neuron activity through the dopamine receptors expressed in MSNs, as well as different groups of striatal interneurons and corticostriatal afferents (Bamford et al., 2004; Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012).

In the standard model of the basal ganglia, the MSNs in striatum separately belong to the so-called direct and indirect trans-striatal pathways according to the projection sites. The direct-pathway MSNs express dopamine receptor type 1 family (D1R, D5R), and project monosynaptically to the basal ganglia output nuclei, the GPi and SNr. The indirect-pathway MSNs express the dopamine receptor type 2 family (D2R, D3R, D4R) and send their primary projections through two intermediate relay nuclei, the GPe and STN, before reaching the output structures, the GPi and SNr (Gerfen, 1992a; Surmeier et al., 1996; Valjent et al., 2009). (see below in the section 1.2 for the organization and function of these direct and indirect pathways)
Figure 2. Schematic organization of the basal ganglia and associated structures. In blue are represented the glutamatergic (Glu) structures, in red the GABAergic (GABA) nuclei and in yellow the dopaminergic (DA) nucleus. GPe: external part of the globus pallidus; GPI: internal part of the globus pallidus; SNr: substantia nigra pars reticulata; SNC: substantia nigra pars compacta; dp: direct pathway; ip: indirect pathway; hp: hyperdirect pathway. Modified from (Fino and Venance, 2010)

The subthalamic nucleus

The STN, a lens-shaped structure, exhibits an unique feature within basal ganglia: it is the only glutamatergic nucleus while all the other basal ganglia nuclei are GABAergic. In current conceptual models of the basal ganglia circuits (Alexander and Crutcher, 1990), the STN occupies a dual functional position. First, the STN acts as a relay nucleus in the so-called indirect trans-striatal pathway that connects the striatum to the basal ganglia output nuclei (Joel and Weiner, 1997; Nambu et al., 2000). Second, it receives inputs directly from the cortex,
bypassing the striatum, then conveys excitatory signal to SNr/GPi, thus constituting the fast conducting hyperdirect pathway (Maurice et al., 1999; Nambu et al., 2002). Most of the cortical afferents to the STN arise from the prefrontal cortex in rodents and primary motor cortex in primates (Nambu et al., 2000). Contrarily to MSNs, the STN neurons are tonically active and their firing pattern can be easily modulated by cortical activity. A recent study showed that STN innervates the cerebral cortex, indicating the existence of a functional cortico-STN loop circuit (Degos et al., 2008). The STN also receives glutamatergic projection from thalamus (Hamani et al., 2004), which confirms again that the STN is another input of basal ganglia besides striatum. There are also evidences demonstrated that the convergence of GABAergic pallidal and glutamatergic cortical/thalamo afferents in the STN could be modulated by DA projected from the SNc (Brown et al., 1979; François et al., 2000).

The external segment of the globus pallidus

The external segment of the globus pallidus is classically considered as a component of the indirect pathway that relays striatum inputs to the SNr/GPi (Smith et al., 1998; Obeso et al., 2008). The retrograde tracing studies revealed that approximately 30% of GPe neurons project to the striatum (Kita et al., 1999). In addition, the GPe is the major source of GABAergic inhibition in the STN mediated by strong multiple synaptic contacts (Smith et al., 1998; Baufreton et al., 2009). Meanwhile, it receives glutamatergic afferent fibers from the STN. Together, such topographic arrangements of GPe-striatum and GPe-STN loops suggest the existence of feedback and feedforward control on signaling between these nuclei (Kita, 2007; Gittis et al., 2014).

The substantia nigra pars reticulata and internal segment of the globus pallidus

The SNr and GPi (or entopeduncular nucleus in rodents) are the output structures in basal ganglia network (Deniau et al., 2007). Neurons in these nuclei are all GABAergic and tonically active. The most salient neurophysiological feature of SNr and GPi projection neurons is their depolarized membrane potential and spontaneous high frequency tonic activity under resting...
condition. Generally, SNr neurons fire at 25–65 Hz while GPi neurons display spontaneous activity at 10–110 Hz in awake rodents or primates (Nambu, 2007; Zhou and Lee, 2011). The major affereces to SNr and GPi projection neurons come from the striatum, GPe and STN (Smith et al., 1990; Smith and Bolam, 1991), which then control their tonic firing rate. For instance, the GABA synaptic inputs from direct pathway MSNs in the striatum and GABA neurons in the GPe inhibit and even silence SNr/GPi neurons. On the contrary, glutamate inputs from the STN can enhance burst firing of SNr/GPi neurons (Gerfen and Surmeier, 2011; Zhou and Lee, 2011). Finally, the SNr and GPi convey integrated information back to the motor and prefrontal cortices through the thalamus or to the various brainstem structures, such as the pedunculopontine nucleus and the superior colliculus.

The substantia nigra pars compacta

The SNC, composed of DA neurons, is one of the major sources of DA in the brain (~50% in rodents and >70% in primates). This dopaminergic system increases in size and complexity along evolution. In mammals, one can found that in the rats that the number of DA cells in the SNC is ~ 11,000-12,000 (per hemisphere), in monkeys is 60,000-100,000 per side, and in young humans is over 200,000 per side (Björklund and Dunnett, 2007a). Compared to adjacent SNr neurons, the SNC DA neurons fire at a much lower frequency (1-4 Hz) and display long duration spikes (~2.5 ms) (Guzman et al., 2009). These slow-spiking DA neurons keep a sustained tonic tone of DA release which sets a background level of DA receptors stimulation (Grace, 1991). Along the nigrostriatal pathway, SNC DA neurons project predominantly to the sensorimotor striatum (Bolam et al., 2000) and potently modulates the neuronal excitability (Nicola et al., 2000; Gerfen and Surmeier, 2011) as well as the processing of corticostriatal information (Reynolds and Wickens, 2000a; Wickens, 2009). Furthermore, not only the striatum but also several other basal ganglia nuclei receive DA innervation. Some studies reported collateral branches of DA axons projecting to the GPe and STN on their way to the striatum (Hassani et al., 1997; Gauthier et al., 1999; Prens and Parent, 2001). The neuromodulation of the basal ganglia by DA has been proved critical to a broad of
physiological functions including movement control, psychomotor and cognition (Schultz, 2007a; Wise, 2009; Cools, 2011).

1.2 The cortico-basal ganglia information processing

The basal ganglia are mainly involved in motor control, goal directed behavior and procedural learning and memory (Haber, 2003; Graybiel, 2005; Yin and Knowlton, 2006). In the current models of the basal ganglia circuit, the striatum and the STN are the two input nuclei through which cortical signals are transmitted toward the output nuclei of the basal ganglia, the GPi and SNr (Alexander and Crutcher, 1990). It is classically admitted that there are three main information processing pathways in the cortico-basal ganglia circuits: the hyperdirect pathway via the STN, and the direct and indirect pathways via the striatum. In vivo electrophysiological studies in rats and non-human primates demonstrated that a cortical activation induces three phases of responses in SNr/GPi neurons, as a result of the sequential involvement of the three aforementioned pathways: a early excitation, due to the hyperdirect pathway activation, followed by an inhibition and a late excitation, respectively due to the activation of the direct and the indirect pathways (Maurice et al., 1998, 1999; Nambu et al., 2000; Kolomiets et al., 2003) (Figure 3). Thus, the signals (of course looping several times) through this sequential information processing, control the initiation, execution and termination of the selected motor program according to the context (Nambu et al., 2002).

The cortico-subthalamo-nigral hyperdirect pathway

The cortico-STN and STN-SNr/GPi glutamatergic projections consist in the cortico-STN-SNr/GPi pathway which conveys excitatory afferences from the motor and prefrontal cortex to the SNr/GPi, bypassing the striatum (Smith et al., 1998). It refers to the 'hyperdirect pathway' (Nambu et al., 2002), which contributes to the first excitation phase of the response to cortical activations observed in SNr neurons. Some pioneering studies characterized the features of this relative new pathway (Maurice et al., 1998, 1999; Smith et al., 1998; Nambu et al., 2000; Kolomiets et al., 2003). Because each nucleus in the basal ganglia
presents exclusively glutamatergic or GABAergic output neurons (the STN being the sole glutamatergic nucleus while the other nuclei are GABAergic), pharmacological blockade method was used to determine the respective contribution of the trans-striatal (direct and indirect pathways) and trans-subthalamic (hyperdirect pathway) circuits in the SNr responses evoked by cortical activation (Maurice et al., 1999). For instance, pharmacological blockade of the glutamatergic transmission within the STN markedly decreased the early and late excitatory responses, while it increased the duration of the inhibition. Thus, the STN, the structure is critically involved in both hyperdirect and indirect pathway and thus contributes to the two peaks of excitation recorded in the SNr neurons. However, blockade of the cortico-striatal glutamatergic transmission and the GABA transmission in the GPe did not affect the early excitatory response while it impaired both the inhibition and the late excitation, demonstrating that it is the glutamatergic cortico-STN-SNr pathway forming the first excitation (Maurice et al., 1999). In addition, the excitatory response induced by the trans-subthalamic pathway appears ~10 ms earlier than inhibition and ~30 ms earlier than second excitation induced by direct and indirect pathway, respectively (Maurice et al., 1999; Kolomiets et al., 2003). These observations demonstrated that the trans-subthalamic pathway has much shorter conduction time (due to less synaptic relay) compared to the two trans-striatal pathways. In accordance with that, simultaneous recordings of the neuronal evoked responses in the GPI and STN have also shown that the cortical stimulation induced an early, short-latency excitation in STN neurons preceding that in GPI neurons (Nambu et al., 2000).

Output neurons belonging to the SNr and the GPI are GABAergic. Thus, the activation of the hyperdirect pathway drives an increase of the inhibition on their targets (thalamus and brain stem premotor areas) and promotes a pause of the ongoing motor programs in process.
Figure 3. The three cortico-basal ganglia pathways and a triphasic synaptic response in SNr neurons. (Right panel) Schematic view of the cortico-basal ganglia information processing through the hyperdirect (1), direct (2), and indirect (3) pathways. (Left panel) The major pattern of the responses observed in SNr neurons after stimulation of prefrontal cortex consisted of two excitatory peaks often separated by a brief inhibitory period. The numbers indicate the hyperdirect (1), direct (2) and indirect (3) pathways activation. From (Maurice et al., 1999).

*The striatonigral direct pathway and the striatopallidal indirect pathway*

The striatum receives excitatory cortical and thalamic inputs, then projects to the basal ganglia output nuclei SNr/GPi through the so-called direct and indirect pathways (Albin et al., 1989; Alexander and Crutcher, 1990; Gerfen and Surmeier, 2011). The direct pathway arises from GABAergic striatal MSNs co-expressing the D1-family dopamine receptors, the substance P, the dynorphin and the muscarinic M4 acetylcholine receptors, and projecting monosynaptically to the SNr/GPi (Valjent et al., 2009). The indirect pathway arises from
MSNs expressing the D2-family dopamine receptors, the A2a adenosine receptors and the enkephalin. The latter MSNs project polysynaptically to the SNr/GPi through synaptic relays in the GPi and STN (Valjent et al., 2009). The segregation of information processing into the direct and indirect pathways is the basis of the standard model of the basal ganglia (Alexander and Crutcher, 1990). It has been confirmed by using BAC transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of D1R or D2R promoters (Gong et al., 2003), showing approximately 50-50% D1R- and D2R-MSNs with only a small proportion of D1R and D2R co-expressing MSNs in the dorsal striatum of mice (–5–6%) (Bertran-Gonzalez et al., 2010) (Valjent et al., 2009; Gangarossa et al., 2013). However, anatomical studies in rats and primates report a much higher overlap (~30–80%) of MSNs in both direct and indirect pathways than in mice in which most of the studies are performed (Kawaguchi et al., 1990; Surmeier et al., 1996; Aizman et al., 2000; Wu et al., 2000).

The cortical stimulation evokes an inhibitory effect onto the activity of the GABAergic output neurons of the SNr/GPi mediated by the direct pathway, which in turn disinhibits the thalamus (the mediodorsal and ventromedial nuclei) and the brain stem (pedunculopontine nucleus and the superior colliculus). Shortly after, an excitatory transmission through the indirect pathway activates the SNr/GPi output neurons, which result in an increased inhibition onto the thalamus and the brain stem (Maurice et al., 1999). Thus the striatonigral and striatopallidal pathways exert an opposing neuronal effect in sequence, which is consistent with their bidirectional influences on the selection of actions (Albin et al., 1989; DeLong, 1990). To test the specific function of these circuits in behaving animals, *in vivo* studies used optogenetics to specifically drive the activity of the direct- and the indirect-pathway belonging MSNs, combined with single-unit recordings in the SNr. The harvested results indicated that the activation of the direct pathway facilitates actions correlated with the inhibition of cellular responses in SNr neurons, while the optogenetic activation of the indirect pathway inhibits behaviors by excited SNr output neurons (Kravitz, 2010; Freeze et al., 2013).
1.3 Role of the basal ganglia in the procedural learning and memory

A growing body of evidences strongly indicate that the basal ganglia carry out an important role not only in motor control, but also in learning and memory, especially in habit formation (Packard and Knowlton, 2002; Yin and Knowlton, 2006; Kreitzer and Malenka, 2008; Helie et al., 2013). To establish habits, it is required an extensive repetition (generally mimicked by a heavy training in behavioural task) of the behavioural procedure, leading to gradual, incremental learning of certain associations of actions sequences, often in the absence of conscience awareness.

Packard and McGaugh used the place / response learning task, in which clearly identified hippocampus dependent place learning (the declarative memory) and basal ganglia (specially, dorsal striatum) dependent response learning (aka the non-declarative or the procedural memory, also called: habit memory), respectively (Packard and McGaugh, 1996; Packard, 1999). In this task, rats were trained to obtain a food pellet in goal box of a T-maze surrounded by various environmental cues (Figure 4). Following a light or an intensive training, a probe test was given in which rats were placed in the start box opposite the one used during training. They observed that the rats which receive a light training made a different turning response and entered the baited maze arm (i.e., approached the place where food was located during training) while the rats with an intensive training entered the unbaited maze arm (i.e., made the same turning response as during training, i.e. make a egocentric response). Thus, these findings indicated a shift from place learning to response learning with an extended training. Moreover, upon pharmacological inactivation of the dorsal striatum by lidocaine injection, the rats tend to show the place learning despite the intensive training, whereas, rats upon pharmacological inactivation of their hippocampi showed impaired the place learning and an accelerated shift to a response learning (Packard and McGaugh, 1996). Thereby, the dorsal striatum and the hippocampus mediate distinct memory processes in a competing manner.
Figure 4. T-maze tasks for estimating procedural and spatial memory.

In the place/response task, rats are trained to retrieve food from one arm of a T-maze or cross maze. The content of learning can be assessed by moving the starting arm to the other side of the maze on a probe test. The animal may enter the arm corresponding to the location of the reward during training (place strategy) or the arm corresponding to the turning response that was reinforced during training (response strategy). Modified from (Yin and Knowlton, 2006).

Given the large size of the dorsal striatum, later studies further investigated the heterogeneous function of different subregions of the dorsal striatum, such as dorsomedial striatum and dorsolateral striatum receiving inputs from association and sensorimotor cortex, respectively. Lever press task and T-maze were tested on rats with neurotoxic lesions of the dorsomedial or dorsolateral striatum (Yin and Knowlton, 2004; Yin et al., 2004, 2005). In conclusion, these results showed that the dorsomedial striatum is necessary for flexible goal-directed behavior in which performed actions are driven by reward, whereas the dorsolateral striatum regulates rigid habitual control which no longer depends on reward values (Yin and Knowlton, 2006; Redgrave et al., 2010).

At the cellular and synaptic levels, these skill-learning processes occur accompanying with neuronal activity changes in the basal ganglia (e.g. striatum). It has been reported that the striatal forelimb neurons fired with lever press action early in training, then declined after repeated sessions, until totally ceased upon an over-training in the task (Carelli et al., 1997).
This observation indicates that the striatum plays a pivotal role in early stage of skill acquisition and its involvement decreases with intensive training. During acquisition, it was suggested that information chunking in the basal ganglia provides a mechanism for the expression of action repertoires (Graybiel, 1998). In support of this proposal, long-term recordings from neurons in the dorsal striatum was performed to monitor the neural activity signaling the initiation and termination of action sequences during learning in a T-maze (Barnes et al., 2005; Jin and Costa, 2010). Furthermore, combination of in vivo multi-channels extracellular recordings in behaving mice and ex vivo recordings in striatal brain slice directly taken from mice which received a light or heavy training, demonstrated a long-lasting synaptic plasticity in the striatum during the acquisition and consolidation of a skill (Yin et al., 2009a). In vivo results showed a region-specific changes in neural activity during the different phases of skill learning, with the dorsomedial striatum being preferentially involved in early training and the dorsolateral striatum being involved in later training (Figure 5A,B) (Yin et al., 2009a). Ex vivo recordings of MSNs showed an increase of the NMDA/AMPA ratio (admitted to be the signature of a LTP) significantly in the dorsolateral striatal neurons of extensive trained mice (Figure 5C), especially in the MSNs of the indirect pathway rather than the MSNs of the direct pathway. This revealed that the changes observed in vivo corresponded to a regional and training-specific changes in excitatory synaptic transmission in the dorsal striatum (Yin et al., 2009a). Taking together, these important findings indicate that a long-lasting potentiation of the cortical glutamatergic transmission in the striatum strongly contributes to basal ganglia-dependent skill learning.

More recently, new evidences demonstrated the involvement of the basal ganglia in decision making under dopamine signaling modulation. Indeed, optogenetic specific activation of either D1-MSNs or D2-MSNs showed opposing roles of the two trans-striatal direct and indirect pathways in the basal ganglia. The direct pathway activation induced an associative reward response and the indirect pathway activation acted as transient punishment (Hikida et al., 2010; Kravitz et al., 2012; Tai et al., 2012). Although many behavioural assays and neurobiological analysis have been performed to demonstrate the crucial role of the basal ganglia in procedural learning and memory, the detailed molecular and cellular mechanisms...
underlying basal ganglia related neural circuit function and behavior remain to be further elucidated.

Figure 5. In vivo (A,B) and ex vivo (C,D) recordings of neuronal activity in the DLS during the different phases of skill learning. (A) Example of a single-unit waveform, interspike interval and cluster separation from noise. (B) Example of a putative MSN that increased firing rate (impulses per second) when the animal was running on the rotarod versus baseline (intertrial period). (C) NMDA/AMPA ratio in DLS during the different phases of skill learning. Representative traces are shown on the left. The number of neurons and number of animals (between brackets) are shown for each condition trials. Note: Yoked animals were handled and placed in the rotarod in the same manner as the trained animals, but without the rotation of the rod. From (Yin et al., 2009a)
CHAPTER 2. The striatum

The striatum is a major forebrain nucleus that receives glutamatergic innervation from almost all the cerebral cortices and from several thalamic nuclei. In turn, the MSNs send their axons toward the basal ganglia output structures (McGeorge and Faull, 1989; Smith et al., 2004). This integration of information signaling is modulated by the dopamine afferences from midbrain nuclei (Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012). Considering the convergence of such various sets of inputs, it is not surprising that the striatum plays a central role of diverse physiological functions, such as motor performance, action selection and reward-related behaviors (Liljeholm and O’Doherty, 2012). The crucial role underlie by striatum is dramatically illustrated by pathologies. Indeed, many of the dysfunctions affecting the striatum (among other structures) are generally related to severe movement disorders, including Parkinson’s disease and Huntington’s disease with pathological hallmark like loss of striatal dopamine and loss of striatal MSNs, respectively (Gittis and Kreitzer, 2012).

2.1 The functional anatomy of the striatum

The striatum is composed by the ventral and the dorsal substructures (Figure 6A) (Voorn et al., 2004). The ventral striatum (also named nucleus accumbens), which can be further divided into the shell and the core (Figure 6B), mainly plays a crucial role during reward. The dorsal striatum likewise can be divided into dorsomedial striatum (DMS) and dorsolateral striatum (DLS) (Figure 6B) participating to different functions. The dorsomedial striatum is critical for the goal-directed instrumental learning, while the dorsolateral striatum is related to the movement-related activity like habitual learning (Packard and Knowlton, 2002; Yin et al., 2008). The distinction of such subregions of the dorsal striatum is mainly based on their specific physiological role and afferent/efferent circuitry; however, it should be noted that it does not exist clear anatomical boundary between these substratal regions. Because this PhD focused on the synaptic plasticity of neuronal circuits between the somatosensory cortex and the dorsolateral striatum, the following introduction will be concentrated on the dorsal region of the striatum.
Figure 6. Anatomy of the striatum.

(A) The schematic sagittal view of a rat brain with the striatum. (B) The major functional domains of the striatum. An illustration of the striatum from a coronal brain hemisphere section. Note that these four functional subdivisions are anatomically continuous, including nucleus accumbens shell and core (limbic striatum), dorsomedial (DMS, association) striatum, and dorsolateral (DLS, sensorimotor) striatum. cc: corpus callosum.

Note: The ventral striatal regions (e.g. areas posterior to the nucleus accumbens) are not included here. Modified from (Lerchner et al., 2007) and (Yin et al., 2008)

Although the striatal regions have very similar basic structures, the behavioral, neuroanatomical and neurophysiological findings indicate several ways of subdividing the striatum, (see review: Hilário and Costa, 2008) (Figure 7). The DMS has been shown to receive most of its inputs from the associative cortex, while the DLS receives inputs from the sensorimotor cortex, which may explain their functional difference (Voorn et al., 2004) (Figure 7A). The projection of the dopaminergic neurons into the striatum also follows a anatomical gradient, showing the dopaminergic innervation from the SNc (A9) targeting more the DLS, and dopaminergic innervation from the ventral tegmental area (VTA) (A10) targeting more the ventromedial striatum (Moore et al., 2001) (Figure 7B). Interestingly, sensitization with amphetamine, by acting on the dopamine transporter, can increase dendritic spine density in the MSNs belonging to the DLS (Jedynak et al., 2007) and simultaneously decreases spine density in the DMS (Figure 7C). Furthermore, the expression of cannabinoid receptor type 1 (CB1R) across the striatum displays a medial-lateral gradient of increased expression, with the highest expression in the dorsolateral striatum (Gerdeman et al., 2003; Herkenham et al., 1991) (Figure...
7D), which suggest a potential role of endocannabinoid pathways in habit learning (Hilário and Costa, 2008).

**Figure 7. Gradients of function across the striatum.** (A) Scheme depicting the striatal regions shown to be involved in the goal-directed actions (A-O) and habit formation (S-R). DMS-dorsomedial striatum; DLS-dorsolateral striatum. (B) Representation of the striatal areas innervated by dopaminergic neurons from the VTA and the SNc in the rat. (C) The number of dendritic spines in MSNs increases in DLS and decreases in DMS after chronic exposure to methamphetamine. (D) Gradient of expression of CB1 receptors in the striatum. From (Hilário and Costa, 2008).

### 2.2 The striosome and matrix compartments of the dorsal striatum

In addition to the subdivision into the ventral/dorsal substructures and the direct/indirect pathways (see previous chapter), the dorsal striatum exhibits another layer of heterogeneity with two anatomo-functional compartments: the striosome (also termed as patch) and the matrix compartments, based on divergent expression of neurochemical markers and input/output connections (Pert et al., 1976; Graybiel and Ragsdale, 1978; Herkenham and Pert, 1981; Gerfen, 1984, 1992a). It has been reported that a large number of proteins (>60) are segregated in the striosomes or the matrix (Graybiel, 1990). For instance, striosome compartment is enriched in the mu-opioid receptor, dopamine receptor type 1 and cholinergic receptors, while matrix compartment is enriched in acetylcholinesterase, somatostatin, calcium-binding protein, cannabinoid receptor type 1 and tyrosine hydroxylase (for review, see (Crittenden and Graybiel, 2011; Fujiyama et al., 2015)).
The afferents and efferents of striosome and matrix compartments

A broad view is that the vast majority of the pyramidal cells from the neocortex project to both striosomes and matrix, but the strength of the projections may be different depending on the two compartments. The striosomes receive predominant afferents from more evolutionarily conserved regions of the cerebral cortex, especially the limbic cortex. Whereas the matrix are preferentially innervated by associative and sensorimotor cortices (Figure 8) (Gerfen, 1989; Ragsdale and Graybiel, 1990; Crittenden and Graybiel, 2011). Correspondingly, the midline paraventricular nuclei of the thalamus, which are highly interconnected with the nucleus accumbens and the amygdala, have been shown to terminate mainly onto the striosomal MSNs. By contrast, the intralaminar nuclei of the thalamus, which have strong interconnections with sensorimotor cortices, projected primarily to the matrix (Ragsdale and Graybiel, 1991; Crittenden and Graybiel, 2011).

The matrix contains approximately 85% of the MSNs of direct and indirect pathways considering that the matrix is much larger (~90% in rodent) than the total striosomes area (~10%) (Johnston et al., 1990). Interestingly, most of striosomal MSNs belong to the direct pathway rather than the indirect pathway (Figure 8) (Lévesque and Parent, 2005). In addition, a striosome-specific pathway has been proved by a recent study using single-neuron labeling, showing that the striosomal MSNs target directly the dopaminergic neurons in SNC (Figure 8) (Fujiyama et al., 2011). This definitive evidence about the connections between the striosomes and the SNC is in concert with earlier studies using classical anterograde and retrograde tracing methods (Gerfen, 1985; Jimenez-Castellanos and Graybiel, 1989) and electrophysiological recordings in rats (Lee and Tepper, 2009). Therefore, striosomal MSNs may exert a powerful influence on the balance of the signaling between the direct and indirect pathways through their projection to the dopaminergic neurons in the SNC.

In summary, despite extensive morphological studies showing the striatal mosaic organization of the striosomes and matrix, it remains unconfirmed how these striatal compartments contributes to the specific behavior, largely due to technical reasons (e.g., a lack
of specific striosome-targeting or matrix-targeting manipulation). Based on the anatomical connections, the hypothesis was proposed that striosome–matrix domains participate in modular reinforcement learning and decision making. Namely, the matrix would perform the action selection through the basal ganglia output nuclei whereas the striosomes represent a dopaminergic and limbic system-related circuit responsible for the reward prediction errors (Houk et al., 1995; Amemori et al., 2011). Recently, this hypothesis has been partially proved in dorsomedial associative striatum. The optogenetic manipulation showed that striosome-targeting corticostriatal circuits controls neural processing of decision making under cost-benefit conflict (Friedman et al., 2015).

In pathological condition, imbalances between striosome and matrix functions have been reported in most basal ganglia-associated disorders, including Parkinson's disease, L-DOPA-induced dyskinesias (a severe side effect of dopamine replacement therapy for Parkinson's disease), Huntington's disease, dystonia and drug addiction (Crittenden and Graybiel, 2011).
Figure 8. A simplified diagram of striosome and matrix compartmental organization of corticostriatal, striatonigral and striatopallido pathways. Model of the direct, indirect, and striosome-specific striatal projection pathways from the dorsal striatum. Striosomes are shown in blue, and the extra-striosomal matrix in orange. Shading of the striatum from medial (right) to lateral (left) schematically indicates limbic, associative, and sensorimotor striatal domains. Arrows flowing into the striatum are colored to represent the relative abundance of inputs from limbic cortical regions to striosomes and from sensorimotor and associative regions to the matrix. Arrows exiting the striatum represent GABAergic efferent connections from the medium-sized spiny projection neurons (MSNs) in the striosome and matrix compartments to their respective downstream target nuclei. The nucleus accumbens is shown in gray. GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus (entopeduncular nucleus, in rodents); SNr, substantia nigra pars reticulata; SNC, dopamine-containing substantia nigra, pars compacta; AC, anterior commissure. From (Crittenden and Graybiel, 2011)
2.3 Neuronal subtypes in the striatum

From anatomical, histochemical and electrophysiological studies, the striatal projection neurons, the so-called medium-sized spiny neurons (MSNs) are recognized to make up the vast majority of all the neurons in the rodent striatum, while the remaining neurons comprise diverse populations of aspiny interneurons, which are made up of the GABAergic and the cholinergic interneurons (Kawaguchi et al., 1995; Kreitzer, 2009; Tepper et al., 2010). At present, the use of transgenic mice which selectively express fluorescent proteins under the control of specific promoters has accelerated the progresses in our understanding of more refined characterization of the role of the various subtypes of striatal neurons.

The striatal output neurons (the medium-sized spiny neurons, MSNs)

The principal neurons of the striatum are the GABAergic MSNs, which represent around 75-80% of the striatal neurons in primates and 90-95% in rodents (Graveland and DiFiglia, 1985; Rymar et al., 2004). These output neurons are characterized by a medium-sized cell body (~10-15 μm) and a heavy investment of dendritic spines (Wilson and Groves, 1980). The MSNs dendritic trees spread out spherically ~300-400 micrometers around the cell bodies. The axons of the MSNs arising from the soma or from a large dendritic trunk near the soma mainly project downstream toward the basal ganglia output structures (Smith et al., 1998). It also exists some electrical and chemical (GABAergic) transmission between MSNs within the striatum through the distal dendrites and the axon collateral plexus, respectively (Venance et al., 2004). Interestingly, electrical and chemical synapses are mutually exclusive. In addition, MSNs display several specific electrophysiological properties, such as a very hyperpolarized resting membrane potential (~90mV in vivo and ~80mV ex vivo, a low input resistance, a marked inward rectification of the I/V curve, and a long delay to initial spike (Kita et al., 1984; Nisenbaum and Wilson, 1995; Charpier and Deniau, 1997). Such intrinsic membrane features are mainly shaped by inwardly rectifying potassium channels (Kir3) expressed on the MSNs membrane (Uchimura et al., 1989; Nisenbaum et al., 1996; Mermelstein et al., 1998; Mahon et al., 2000).
If MSNs exhibit a relatively similar morphological appearance at a first glance, they are commonly divided into two main sub-populations based on the segregated expression of dopamine receptors and neuropeptides associated to their distinct axonal projection targets. The D1-striatonigral (dopaminergic D1 receptor-positive) neurons are enriched in the neuropeptides substance P and dynorphin, and in M4 muscarinic acetylcholine receptor whereas the D2-striatopallidal (dopaminergic D2 receptor-positive) neurons specifically express the neuropeptide enkephalin and the adenosine A2A receptor (A2AR) (Gerfen, 1992a; Surmeier et al., 1996; Valjent et al., 2009). Early brain slice electrophysiological studies were mainly performed in rats and mixed different types of MSNs. The development of bacterial artificial chromosome (BAC) transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of promoters for the D1 and D2 receptors led to a big leap forward in identification of two groups of MSNs (Gong et al., 2003; Valjent et al., 2009). Recent works characterized the intrinsic properties of transgenic mice electrophysiological and anatomically (Figure 9) (Gertler et al., 2008; Planert et al., 2013). Although most of the electrical properties (passive and active) were found to be similar, D2-MSNs are more excitable than D1-MSNs. Namely, the rheobase (current necessary to inject to evoke the first spike) is lower in D2-MSNs than in D1-MSNs. In accordance, D2-MSNs evoked more spikes than D1-MSNs upon suprathreshold conditions (Figure 9A,B) (Gertler et al., 2008). Similar dichotomy in excitability has also been found in rats (Planert et al., 2013). Moreover, D1- and D2-MSNs differed in their somatodendritic morphology. A three-dimensional Sholl analysis was used on the reconstructed MSNs filled with biocytin, showing that the total length of the dendrites of the D1-MSNs was significantly greater than that of the D2-MSNs. Such difference is due to a significantly more primary dendrites, branch points and tips (terminal endings) in D1-MSNs, but the mean dendritic length in two types of MSNs is similar (Figure 9C,D) (Gertler et al., 2008).
Figure 9. Electrophysiological and morphological characterizations of D1- and D2-MSNs. (A) Sample responses to intrasomatic current injection revealed that rheobase is significantly higher in D1-MSNs. (B) Up: membrane responses to intrasomatic current injection revealed a significant subthreshold divergence. Down: firing rate of D1- and D2-MSNs to intrasomatic current steps demonstrated increased excitability in the D2-MSNs. (C) Fan-in diagrams displayed no apparent preferred orientation in either the D1- or D2-MSNs. (D) Dendrograms displaying in two dimensions the length, number, and connectivity of dendritic segments in sample neurons. (from Gertler et al., 2008)

Although MSNs are hyperpolarized and silent under resting conditions, early in vivo recordings have demonstrated the existence of subthreshold transitions between hyperpolarized potentials (-90 to -70 mV) to more depolarized potentials (-60 to -40 mV) (Wilson and Groves, 1981), which were subsequently investigated and termed "Down" and "Up" states (Wilson and Kawaguchi, 1996; Stern et al., 1997, 1998; Plenz and Kitai, 1998; Wickens and Wilson, 1998; Mahon et al., 2001). A spiking activity was usually triggered by noisy fluctuations in the Up state (Figure 10 A,B) (Stern et al., 1997; Wickens and Wilson, 1998). The Down state of MSNs is attributable to the high expression of inwardly rectifying K⁺ channels which allow keeping MSNs quiescent near the K⁺ equilibrium potential and limit the membrane depolarization in response to excitatory synaptic inputs for cerebral cortex or thalamus (Nisenbaum and Wilson, 1995). The Up state near the spike threshold depends on a temporally convergent excitatory synaptic inputs from cortex and thalamus, interacting with voltage-gated intrinsic membrane
conductances (Figure 10 C) (Wilson and Kawaguchi, 1996; Blackwell et al., 2003). Recent work showed that these transitions from Down to Up state are mainly due to the activation of NMDA receptors and voltage-dependent Ca^{2+} channels (Plotkin et al., 2011). Moreover, up and down states were bidirectionally regulated by dopaminergic receptor signaling. The somatic Up state was increased by the activation of D1 receptors in MSNs belonging to the direct-pathway whereas it was shortened by activation of D2 receptors in MSNs forming the indirect-pathway (Plotkin et al., 2011). Importantly, Up and Down states are found to be much less prominent in awake animals than under different anesthesia and during slow-wave sleep (Mahon et al., 2001, 2006). During the awaking state, MSNs displayed continuous and irregular membrane potential fluctuations together with random action potential discharges (Mahon et al., 2006). Contrasting with the conventional bistable activities in the anesthetic conditions, the finding of novel style of spontaneous synaptic activities in the awake head-restrained animal indicates that the membrane potential fluctuations and firing patterns of the MSNs are much more versatile than expected, and strongly depend on the state of vigilance. Although the neural function of this complex cellular behavior remains unclear and the neural activities could differ from that occurring during natural behaviors (particularly in sensorimotor specific tasks), these findings in the awake animal provide the natural intracellular activities of MSNs during wakefulness and suggest multiple capabilities of information processing in the basal ganglia.
Figure 10. Up and Down states in MSNs. A: Intracellular recordings from a silent MSN displaying up and down subthreshold membrane potential fluctuations. B: Intracellular recordings from a spontaneously firing MSN. Both neurons (A and B) displayed subthreshold membrane potential fluctuations between a depolarized Up state and a hyperpolarized Down states, but only one fired action potentials while being in the Up state. C: The membrane potential values of MSNs oscillate between Up state and Down state, depending on the degree of cortical activity. D: MSNs intracellular recordings
(bottom trace) together with the corresponding electromyographic (EMG) and electroencephalographic (EEG) activity during wakefulness. Note that Up and Down states were absent. Modified from (Wickens and Wilson, 1998; Mahon et al., 2006; Calabresi et al., 2007).

**The striatal interneurons**

The striatal interneurons can be classified into medium-sized GABAergic interneurons and large cholinergic interneurons (Kawaguchi et al., 1995). Despite representing only ~5% of all striatal neurons in rodents, these different groups of aspiny interneurons form the local feedforward microcircuits and critically regulate the integration of cortical and thalamic inputs and the striatal output (Tepper et al., 2004, 2010; Witten et al., 2010).

**The GABAergic interneurons**

The GABAergic interneurons can be further categorized neurochemically (based on calcium-binding protein expression) at least into three main subtypes: the parvalbumin-positive, the somatostatin-positive and the calretinin-positive interneurons (Kawaguchi, 1993; Tepper and Bolam, 2004; Kreitzer, 2009).

**The parvalbumin-positive interneurons** (also named fast-spiking interneurons) represent about 1-2% of the striatal neurons in rodents (Kawaguchi, 1993; Rymar et al., 2004). They are characterized by an irregular round shaped cell body with a diameter around 16 µm. They display a highly ramified dendritic tree and a dense arborization of local axonal collaterals, which favors numerous local contacts (Figure 11B left) (Kawaguchi et al., 1995). Physiologically, these interneurons exhibit a hyperpolarized resting membrane potential (~ -70 to -75 mV) and a low input resistance similar to that of MSNs (50-150 MΩ). The most distinctive electrophysiological profile is that the action potentials evoked by a depolarizing injection current are of short duration (<0.5 ms), with a pronounced fAHP associated with a reduced sAHP and display high-frequency firing rates (Figure 11B right) (Kawaguchi, 1993; Fino and Venance, 2011). The fast-spiking interneurons receive monosynaptic glutamatergic
innervations from cortex and target MSNs preferentially on the soma (Kita et al., 1990; Bennett, 1994; Mallet et al., 2005; Fino et al., 2008). They can delay or even prevent the discharge in MSNs (Plenz and Kitai, 1998; Koós and Tepper, 1999; Planert et al., 2010). This strong inhibitory effect is mediated by GABAa receptors expressed on MSNs (Koós and Tepper, 1999; Koos et al., 2004).

The somatostatin-positive (or nNOS) interneurons also make up 1% of the striatal neurons population (Rymar et al., 2004). They express various neuropeptides and enzymes, including neuropeptide Y (NPY), somatostatin (SOM) and nitric oxide synthase (NOS). Generally, nNOS interneurons display various somatic morphologies such as round, polygonal, or fusiform shapes with diameters varying from ~9 to 25 µm. The dendritic tree exhibits only 3-4 main dendrites and a sparse axonal arborization (Figure 11C left) (Kawaguchi, 1993; Tepper et al., 2010). Physiologically, they have a relatively depolarized resting membrane potential (~60-to -55 mV), a very high input resistance (>500 MΩ) and a low-threshold action potentials with a long duration (1 ms at half amplitude) (Tepper et al., 2010). Typically, they exhibit a doublet of action potential at rheobase followed by a persistent discharge (Figure 11C right); Accordingly, they are also named "persistent and low-threshold spike (PLTS)" interneurons (Kawaguchi, 1993; Fino and Venance, 2011). Compared to fast-spiking interneurons, nNOS interneurons contact MSN dendrites mainly on the neck of the spines, form weaker inhibitory synapses (Kubota and Kawaguchi, 2000; Gittis et al., 2010). It has been showed that a burst of spikes in a nNOS interneuron induced large inhibitory postsynaptic currents (IPSCs) which delayed the depolarization-induced firing at postsynaptic MSN (Tepper and Bolam, 2004). However, the main function of PLTS interneurons appears to modulate the activity of MSNs more efficiently via the release of neuromodulators. Indeed, nitric oxide release exerts an inhibitory influence on MSN excitability (West and Grace, 2004).

The calretinin-positive interneurons have been barely studied although they represent around 0.8% of striatal neurons based on stereological cell counts (Rymar et al., 2004). Early studies in rats described calretinin-positive interneurons as medium-sized cells, with aspiny
and infrequently branching dendrites, and relatively sparsely present in the caudal region of the striatum (Bennett and Bolam, 1993; Tepper and Bolam, 2004). Subsequent studies in mice and primates describe at least three or four morphologically distinct types of these neurons, ranging from small to large in somatic sizes (Wu and Parent, 2000; Tepper et al., 2010). Physiologically, it is considered that the calretinin-positive interneurons may also exhibit some features of low-threshold spike interneurons (Tepper and Bolam, 2004), but detailed electrophysiological characteristics remain to be determined.

Figure 11. Anatomical and electrophysiological characteristics of the different striatal interneurons compared to MSNs. Biocytin injections and current-clamp recordings in rat brain slices: (A) the medium-sized spiny neurons (MSNs), (B) the fast-spiking GABAergic interneurons (FS), (C) the neuronal nitric oxide synthase interneurons (nNOS) and (D) the cholinergic interneurons (Chol). From (Fino and Venance, 2011)

The cholinergic interneurons

The cholinergic interneurons are the only non-GABAergic cells within the striatum and constitute 0.3–2% of the striatal neurons in rodents (Rymar et al., 2004; Kreitzer, 2009). They are also known as giant aspiny neurons because of their large cell bodies (~50 µm) and their widespread axonal fields (up to 1 mm) (Figure 11D left). The cholinergic interneurons are electrophysiologically characterized by their depolarized resting potential (~-60mV in vitro), prominent afterhyperpolarization and high input resistance (~ 300MΩ) (Kawaguchi 1993) (Figure 11D right). Driven by the combined action of the persistent Na+ currents and
hyperpolarization-activated cation currents (Ih), cholinergic interneurons spontaneously fire at 2-10 Hz in vivo (Bennett et al., 2000). Because of this functional characteristic (which is quite unusual in the silent striatum), they are also named "tonically active neurons" (TANs) (Bennett et al., 2000). Another strong feature of cholinergic interneurons in vivo is their pause in their tonic firing in response to salient sensory cues predictive of reward, which is driven by dopaminergic inputs (Aosaki et al., 1994; Morris et al., 2004; Apicella, 2007). Indeed, D2 receptors are highly expressed by cholinergic cells, whose activation slows down the autonomous pacemaking and reduces neurotransmitter acetylcholine (ACh) release (Yan et al., 1997a; Morris et al., 2004). In turn, cholinergic interneurons modulate MSNs activity through muscarinic receptors positioned at corticostriatal synapses (Calabresi et al., 2000b; Eskow Jamaraj et al., 2015). Two families of muscarinic receptors (M1 and M4) are broadly distributed on both classes of MSNs. Striatonigral MSNs express both M1 (excitatory) and M4 (inhibitory) receptors while striatopallidal MSNs only express M1 receptors (Akins et al., 1990; Galarraga et al., 1999). However, muscarinic agonists (acetylcholine or muscarine) have mainly an excitatory effect on MSNs by increasing their evoked discharge (Perez-rosello et al., 2005) or EPSC amplitude (Lin et al., 2004) due to postsynaptic M1 receptor activation. Cholinergic interneurons also modulate GABAergic interneurons since acetylcholine potently depolarizes and excites fast-spiking interneurons via the activation of ionotropic nicotinic ACh receptor (Koós and Tepper, 2002). They are thought to modulate nNOS cells since their expression of M1 and M2 receptors (Bernard et al., 1998). For a long period, cholinergic interneurons were considered to be the only source of acetylcholine within the striatum. However, a recent study described that the pedunculopontine nucleus (PPN) and the laterodorsal tegmentum (LDT) nuclei in the brain stem send prominent cholinergic afferents to dorsolateral and medial striatum, respectively (Dautan et al., 2014). These cholinergic terminals target both MSNs and interneurons.

Thus, a significant heterogeneity exists among the striatal interneurons in the striatum (Kawaguchi et al., 1995; Kreitzer, 2009; Tepper et al., 2010). Based on their chemical and physiological phenotypes, each of these interneurons differentially influences the striatal microcircuits and regulates excitability of MSNs. All the GABAergic interneurons produce
strong feed-forward inhibitory effect on MSNs, and control the precise timing and the pattern of firing of MSNs (Wilson, 2007). On the other hand, the cholinergic interneurons modulate the sub- and supra-threshold responses of MSNs to cortical and/or thalamic afferents, particularly in reward-related behaviors (Calabresi et al., 2000b; Ding et al., 2010). Together with axon collaterals between MSNs (Venancio et al., 2004), the GABAergic and cholinergic feed-forward action on MSNs exert a critical role in cortico-basal ganglia information processing (Tepper and Bolam, 2004; Tepper et al., 2004; Kreitzer, 2009).
CHAPTER 3. Synaptic plasticity as the main biological substrate for learning and memory

Synaptic plasticity is the ability to modify the strength of connections between neurons transiently (short-term plasticity) or persistently (long-term plasticity). Evidences supporting a strong relationship between synaptic plasticity and learning and memory is growing continuously (Martin et al., 2000; Neves et al., 2008; Johansen et al., 2011; Takeuchi et al., 2014), though there also exists several alternative mechanisms for learning processes like changes in intrinsic excitability (Kim and Linden, 2007), neurogenesis (Leuner et al., 2006; Deng et al., 2010) and structural plasticity (Yuste and Bonhoeffer, 2001; Caroni et al., 2012). Here, in the present manuscript, we mainly focused on a form of synaptic plasticity: the spike-timing dependent plasticity (STDP) which is a synaptic Hebbian learning rule (Caporale and Dan, 2008; Sjöström et al., 2008; Feldman, 2012)

3.1 A brief history of synaptic plasticity

Around 1890, Santiago Ramón y Cajal proposed that the dendritic spines might serve as contacting sites between neurons. This proposal opened the door for suggesting a mechanism of learning that did not require the formation of new neurons but the effectiveness change of their communication. Over half a century later, this view was further challenged about how the cells may change the connection by Donald Hebb in his book The Organization of Behavior (see review for history of Hebb postulate, Sejnowski, 1999).

"When an axon of cell A is near enough to excite B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased." (Hebb, 1949); this postulate was later on summarized by Carla Schatz in the famous “fire together, wire together”.

Since then this postulate (the so-called Hebb Postulate) was demonstrated in numerous in vitro and in vivo experimental studies. The first electrophysiological evidence for an Hebbian
plasticity was found in the dentate gyrus of the rabbit hippocampus, in which the excitatory postsynaptic potential (EPSP) of granule cells evoked by electrical stimulation increased in amplitude following repeated tetanic stimulation at the perforant path synapses (Bliss and Lomo, 1973). This constitutes the first report for the existence of long-term potentiation (LTP). Because hippocampus was known to be critical for long-term memory formation (particularly the declarative memory), early studies addressing LTP focused on the hippocampus, then expanded later on to other brain regions such as the cerebral cortex, cerebellum, striatum and the extended amygdala (Weinberger et al., 1993; Morris and Frey, 1997; Martin et al., 2000; Maren, 2005; Kretz and Malenka, 2008; Yin et al., 2009b).

However, because the weakening of the neuronal connection is not included in Hebb's postulate. Gunther Stent suggested a supplementary hypothesis to the Hebbian postulate.

“*When the presynaptic axon of cell A repeatedly and persistently fails to excite the postsynaptic cell B while cell B is firing under the influence of other presynaptic axons, metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is decreased.*” (Stent, 1973)

A decade later, the inverse form of LTP was discovered in Purkinje cells of the cerebellar cortex: a persistent decrease of synaptic efficacy, the so called long-term depression (LTD) following the conjunctive stimulation of the climbing fibers and the parallel fibers (Ito et al., 1982). Thus the fact that the synaptic efficacy can be significantly modified in a bidirectional manner, paved the way for the idea that synaptic plasticity may be deeply involved in learning and memory processes, and namely be the main cellular substrate for learning and memory.

### 3.2 The short-term synaptic plasticity

Functionally, not all the synaptic weight in the brain require long-lasting changes. So the short-term synaptic plasticity could well explain the fast response of neural circuit to sudden context changes and allows a full recovery afterward without any recall (contrarily to working
memory); then short-term memory should not be confused with the working memory. For example, during sensory adaption, repeated whisker deflections temporarily decrease the thalamocortical synaptic transmission, and this depression recovered simultaneously with the recovery of sensory responsiveness (Chung et al., 2002). This result strongly suggests that short-term synaptic depression has a crucial role on the dynamic regulation of neuronal sensitivity during rapid changes in sensory inputs.

Paired pulse depression (PPD) and paired pulse facilitation (PPF) are the two ways and forms of short-term synaptic plasticity which are synapse-activity dependent. Two postsynaptic potentials or currents (PSPs or PSCs) are evoked by presynaptic stimulation in rapid succession, with a time interval of $\Delta t$. If the ratio of the amplitude of the second and the first responses is $>1$, it indicates a facilitation (PPF) while a ratio $<1$ indicates a depression (Figure 12). In addition, paired-pulse ratio (PPR) is an important and convenient way to monitor the probability of transmitter release at the synapse (Zucker and Regehr, 2002; Stevens, 2003; Regehr, 2012). PPR revealed different properties depending on different types of synapses, i.e., PPD is prominent at synapses between cerebellar climbing fibers and Purkinje cells, whereas PPF is observed at synapses between cerebellar parallel fibers and Purkinje cells, whereas it exists a biphasic short-term plasticity at corticostriatal synapse dependent on the intervals between two stimuli (Blitz et al., 2004; Xu-Friedman and Regehr, 2004; Goubard et al., 2011; Blackman et al., 2013).

![Figure 12](https://via.placeholder.com/150)

**Figure 12.** Simulated experiments show the properties of PPD and PPF. Modified from (Regehr, 2012) ($\Delta t$: presynaptic axon is stimulated twice with a short time difference, usually lasts for hundreds of milliseconds.)
Sustained presynaptic high-frequency stimulation (HFS) can induce more prominent depression at some synapses that recovers more slowly (Figure 13A). Meanwhile, tetanic activation may also profoundly enhance synaptic strength for tens of seconds up to minutes known as augmentation and post-tetanic potentiation (PTP) (Figure 13B).

![Diagram](image)

Figure 13. Simulated experiments show the properties of long-lived depression and PTP. (A) At some synapses low-frequency stimulation results in a stable synaptic response, while sustained high-frequency stimulation results in a depression that persists for tens of seconds. (B) Augmentation or post-tetanic potentiation of synaptic responses lasting tens of seconds or minutes after tetanic stimulation is observed at other synapses. Modified from (Regelhr, 2012)

Different forms of short-term plasticity exist depending on a combination of mechanisms, including presynaptic vesicle depletion, residual calcium accumulation as well as postsynaptic desensitization and saturation (Zucker and Regelhr, 2002; Regelhr, 2012). According to the simplified depletion model of the short-term depression, the preceding stimulus is associated with a high initial probability of release of the neurotransmitter which causes a lack of release-ready vesicles within presynaptic cells when the second stimulation is delivered, subsequently leading to short-term depression. If the presynaptic cell has a low initial release probability, then the first activation will cause a small postsynaptic response, but the residual calcium in the presynaptic terminal from the initial stimulation will lead to an increased release probability upon the following stimulation and as a result, a greater transmitter release accompany with facilitation of postsynaptic response (Zucker and Regelhr, 2002; Regelhr, 2012).
After a stronger stimulation, this short-term facilitation disappears with intraterminal calcium concentration returning to its resting levels.

Postsynaptic mechanisms can also mediate short-term plasticity. Under sustained exposure to the neurotransmitter, the postsynaptic AMPA and NMDA receptors desensitize and become unable to respond to subsequent stimulation, leading to short-term decreases in synaptic responses (Regehr, 2012); note that AMPA receptors desensitize after 1 ms of glutamate exposure, whereas NMDA receptors desensitize on the time scale of hundreds of milliseconds. For the receptors with high affinity for the released transmitter, a portion of receptors remains bound with the neurotransmitter and therefore fewer receptors are available to the new bulk of transmitter released in response to the subsequent stimulus (Blitz et al., 2004). In several brain regions (e.g., in visual and somatosensory cortex), short-term plasticity switches from depression to facilitation over development due to the both neuronal maturation and experience activity (Blackman et al., 2013).

### 3.3 The long-term synaptic plasticity

Long-term synaptic plasticity, i.e. the persistent strengthening or the weakening of a synaptic connection on a long-time scale, has been found in many areas of the CNS with multiple mechanisms depending on induction protocols (Kauer and Malenka, 2007; Shouval et al., 2010). Typically, HFS (tetanic stimulations at 100Hz repeated 1 to 4 times) mainly yields to LTP, whereas low-frequency stimulation (LFS, 1Hz for 5 to 15 minutes) produces LTD (Figure 14A). The early phase of LTP which lasts for 1–3 hours does not require protein synthesis, whereas the late phase which lasts for at least one day requires both translation and transcription (Kandel, 2001).

The best understood form of LTP depends on postsynaptic N-methyl-D-aspartate receptor (NMDAR)-mediated Ca\(^{2+}\) influx (Figure 15a). These ionotropic glutamate receptors are normally blocked by Mg\(^{2+}\). When the postsynaptic neuron is depolarized by a strong
presynaptic stimulation (Figure 14A) or purposely clamped (Figure 14B), the Mg$^{2+}$ is forced out of the channel and Ca$^{2+}$ can enter the postsynaptic cell. The elevation of postsynaptic Ca$^{2+}$ level initiates the calcium/calmodulin-dependent protein-kinase II (CaMKII) pathway that causes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) trafficking to the postsynaptic membrane. Increased number of AMPARs leads to an additional binding of more glutamate released from the presynaptic side, and consequently an increased in the PSP/PSC (namely the LTP).

LTP does not always require NMDARs activation. Depending on brain area or neuronal subpopulations, HFS inducing rise of presynaptic Ca$^{2+}$ can also activate adenylyl cyclases (AC) pathway to produce cyclic AMP, with subsequent activation of protein kinase A (PKA). This in turn leads to a persistent increase in the amount of glutamate released which induces presynaptic LTP (Figure 15b) (Nicoll and Schmitz, 2005). Since theta frequency was found in vivo in the hippocampus during active behavior (Levy and Steward, 1983). To mimic more naturalistic conditions, theta-burst stimulation (TBS) composed by short high-frequency bursts is also classically delivered to promote LTP (Figure 14C) (Larson and Lynch, 1988).

Synaptic efficacy can be modified in a bidirectional manner, which obviously would avoid synaptic saturation to occur. LTD, the “reverse” of LTP, is classically induced by prolonged LFS (Figure 14A). A moderate depolarization of the postsynaptic cell paired with LFS (in a Hebbian paradigm) can also result in LTD with a shorter stimulation duration (Figure 14B). One mechanism known to cause LTD involves AMPARs. Due to a low stimulation rate of the presynaptic neurons, low Ca$^{2+}$ influx through NMDARs initiates calcineurin and protein phosphatase 1 (PP1)-dependent cascade, which results in the partial removal of AMPARs from the postsynaptic membrane. With the decrease in AMPARs, the postsynaptic neuron becomes less responsive to the glutamate released from the presynaptic neuron and a decrease of the synaptic weight is then observed (Figure 15c). Beside the NMDAR-dependent LTD, it has been described that the activation of the metabotropic glutamate receptors (mGluRs) can also lead to a postsynaptic LTD in the cerebellum, hippocampus and neocortex (Figure 15d) (Malenka and Bear, 2004). In addition, at many CNS excitatory and inhibitory synapses, strong postsynaptic
Ca\textsuperscript{2+} influx triggers the synthesis of endocannabinoids (eCBs), which travel retrogradely across the synapse to bind to presynaptic cannabinoid type-1 receptors (CB1Rs) and cause LTD (eCB-LTD) (Figure 15e) (Chevaleyre et al., 2006); its mechanism is detailed in chapter 4.

Different with the classical frequency-dependent inducing long-term plasticity, a timing-dependent plasticity has been found in more recent years (Bell et al., 1997; Magee and Johnston, 1997; Markram et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Zhang et al., 1998a; Feldman, 2000). Because the order and precise temporal interval between presynaptic and postsynaptic spikes determine the sign and magnitude of plasticity, this novel plasticity was termed spike-timing-dependent plasticity (STDP) (Song et al., 2000). Low-frequency stimulation can be used to induce both LTP and LTD within a narrow temporal window (<40ms) (Figure 14D). In the canonical form of STDP, LTP is induced when presynaptic stimulation precedes postsynaptic spiking (\(\Delta t>0\), pre-post pairings, typically 60–100 pairings) within a narrow window of several tens of milliseconds, whereas spiking of the opposite order (\(\Delta t<0\), post-pre pairings, typically 60–100 pairings) leads to LTD (Markram et al., 1997; Bi and Poo, 1998; Feldman, 2000; Fino et al., 2008). The STDP appears to exist \textit{in vitro} as well as \textit{in vivo}, from invertebrates to mammals, making it considered as a genuine Hebbian synaptic learning rule that may underlie neural circuit remodeling and behavioral activities (Dan and Poo, 2006; Caporale and Dan, 2008; Shouval et al., 2010; Feldman, 2012).
Figure 14. Classical induction protocols for long-term synaptic plasticity. (A) Changing the frequency of extracellular stimulation affects the direction and strength of synaptic plasticity. Left: HFS results in LTP whereas LFS produces LTD. Right: frequency vs. plasticity curve. (B) LFS paired with voltage clamping of the postsynaptic cell can also result in LTP or LTD depending on the postsynaptic voltage. Left: moderate depolarization produces LTD whereas large depolarization produces LTP. Right: depolarization vs. plasticity curve. (C) In the hippocampus of awake behaving animals a theta-frequency oscillation can be observed and was subsequently used to promote plasticity.
(right). Left: LTP induced by TBS protocol (D) STDP protocols are induced by precisely stimulating the presynaptic afferents at a specific time ($\Delta t$) before or after a postsynaptic spike. Right: The precise $\Delta t$ determines the direction and strength of synaptic plasticity. Each circle represents the plasticity amplitude recorded from one cell. From (Shouval et al., 2010)

Figure 15. Well-described mechanisms of LTP and LTD. Highly simplified diagrams of the induction and expression of long-term synaptic plasticity in the rodent brain. From (Kauer and Malenka, 2007)
3.4 Multiple forms of spike-timing dependent plasticity

As a universal “first rule” for associative plasticity, STDP has been widely studied throughout the CNS at the single-cell level, showing different properties depending on the brain region, cell type, developmental state and experimental conditions (Dan and Poo, 2006; Caporale and Dan, 2008; Sjöström et al., 2008; Feldman, 2012).

Canonical STDP is bidirectional and temporally order-dependent, which is in concert with an Hebbian rule. Early studies showed that a spike-timing dependent LTD (t-LTD) is obtained when postsynaptic action potential precedes the presynaptic stimulation, while a spike-timing dependent LTP (t-LTP) was induced when postsynaptic action potentials followed the presynaptic activation (Markram et al., 1997; Bi and Poo, 1998). This phenomenon was then prevalently found at excitatory synapses in various brain regions, including neocortex (Feldman, 2000; Sjöström et al., 2001; Froemke et al., 2005; Nevian and Sakmann, 2006) (Figure 16A), hippocampus (Nishiyama et al., 2000; Wittenberg and Wang, 2006), and striatum (Fino et al., 2005, 2008, 2009) (Figure 16A). In most of the cases, the post-pre t-LTD window (~0 to 20–100 ms) is longer than that for t-LTP induction (~0 to 25 ms) leading to the term of asymmetric Hebbian STDP (Debanne et al., 1998; Feldman, 2000; Sjöström et al., 2001; Froemke et al., 2005) (Figure 16B). The reason could be that a lower level of Ca\textsuperscript{2+} influx through NMDARs is required for t-LTD induction compared to t-LTP. As the amount of Ca\textsuperscript{2+} influx decreases with the pre-post interval duration, it is expected that it passes through the range appropriate for t-LTD induction before reaching the baseline (Dan and Poo, 2006).

However, beyond the initial characterization of the canonical form of STDP (i.e., bidirectional asymmetric Hebbian STDP), various other new forms of STDP have emerged, displaying an anti-Hebbian polarity at some synapses (Figure 16C and D) (Han et al., 2000; Fino et al., 2005, 2008; Letzkus et al., 2006). In anti-Hebbian bidirectional STDP, pre-post pairings induce t-LTD and post-pre pairings induce t-LTP. In fact such STDP constituted the first report for a canonical STDP (Bell et al., 1997) and was observed in the electric fish but has been largely ignored for more than a decade since being anti-Hebbian. Afterwards, the
anti-Hebbian STDP have been observed in different area of mammalian brain, such as GABAergic cartwheel neurons in the dorsal cochlear nucleus (Tzounopoulos et al., 2004), at excitatory synapses onto striatal medium-sized spiny neurons (Fino et al., 2005, 2010) (Figure 16C) and cholinergic interneurons (Fino et al., 2008). Similar temporal order of STDP was also found at synaptic inputs from L2/3 synapses onto L5 pyramids in somatosensory cortex. The polarity of this STDP is dependent on the electrotonic distance of the synapse from the soma, namely distal inputs induced anti-Hebbian plasticity while proximal inputs lead to Hebbian plasticity (Letzkus et al., 2006) (Figure 16C). In most of the cases, the so-called anti-Hebbian STDP displays only LTD, which is often temporally asymmetric (i.e., pre-post pairings preferentially induce stronger t-LTD) (Han et al., 2000; Lu et al., 2007; Safo and Regehr, 2008) (Figure 16D). It occurs at excitatory synapses onto fast-spiking GABAergic interneurons in neocortex (Lu et al., 2007) as well as at parallel fiber synapses onto Purkinje-like neurons in the electrosensory lobe of the electric fish, where it co-occurs with timing-independent LTP (Bell et al., 1997; Han et al., 2000). LTD dominance is most prominent in cerebellar Purkinje cell, with maximal t-LTD when parallel fiber activation occurs before postsynaptic spiking by 80–150 ms (Safo and Regehr, 2008).
Figure 16. Schematic representation of various STDP phenotypes at different synapses. (A) Hebbian STDP is equally balanced between LTP and LTD. 1: at proximal dendrite of cortical L2/3 pyramidal neurons (Froemke et al., 2005). 2: Striatal fast-spiking GABAergic interneurons (Fino et al., 2008). (B) Hebbian STDP (or asymmetric Hebbian STDP) is biased toward LTD. 3: In vivo recording cortical L2/3 pyramidal neurons by whisker deprivation (Celikel et al., 2004). 4: At distal dendrite of cortical L2/3 pyramidal neurons (Froemke et al., 2005). (C) Anti-Hebbian STDP that expresses both LTP and LTD. 5: MSN in striatum (Fino et al., 2005). 6: At distal cortical L2/3 synapse onto L5 pyramidal neurons (Letzkus et al., 2006). (D) Anti-Hebbian STDP that contains only LTD (anti-Hebbian LTD). 7: Parallel fiber synapse onto Purkinje-like cells in electric fish (Han et al., 2000). 8: Fast-spiking interneuron in somatosensory cortex (Lu et al., 2007). 9: Cerebellar Purkinje cell (Safot and Regehr, 2008). From (Feldman, 2012)
3.5 The principle of spike-timing dependent plasticity

STDP is the synaptic efficacy change induced upon repeated nearly coincident pre- and postsynaptic APs (Feldman, 2012). This plasticity is dependent on the increased dendritic Ca\(^{2+}\) influx, which suggests that the back-propagating APs (bAPs) from postsynaptic neuron (induced by the somatic current injection) are critically involved in STDP (Sjöström and Nelson, 2002; Sjöström et al., 2008) (Figure 17A). Together with dendritic Ca\(^{2+}\) spikes, the AMPAR-mediated EPSPs, and NMDAR-mediated EPSPs, bAPs play a major role of the dendritic depolarizing that is necessary for plasticity expression (Kampa et al., 2007).

At present, combined soma/dendritic recordings and calcium imaging techniques have provided quantitative measurements for bAPs in a variety of mammalian neuronal types, such as hippocampal CA1 pyramidal cell, cortical pyramidal neuron, striatal MSN, thalamocortical neuron, motoneuron and granule cells in the olfactory bulb (Häsässer et al., 2000; Waters et al., 2005; Sjöström et al., 2008). The bAPs in the dendrites were observed both in vitro and in vivo traveling on a long distance range, with decreased amplitudes and increased halfwidth duration upon their back-propagation along the dendritic tree (Sjöström et al., 2008; Clopath and Gerstner, 2010) (Figure 17A and C). However, the AP back-propagation efficacy spans a wide range of dynamic characteristics depending on the different dendritic structures and ionic channel expression (Johnston and Narayanan, 2008) (Figure 17B).

The attenuation of the bAPs (speed, amplitude and duration) is controlled by dendritic morphology (like dendritic length and diameter, nodes, spines) and distribution of dendritic ion channels (Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\)) (Waters et al., 2005; Johnston and Narayanan, 2008). For instance, in the highly branched dendritic tree of Purkinje cells, the bAP rapidly diminishes to 0 in less than 200 \(\mu\)m. Meanwhile, single-channel recordings reveal that Na\(^{+}\) channels are absent on dendrites of Purkinje cells, highly expressed in the apical dendrites of mitral cells, and at intermediate densities in pyramidal neurons, which directly impact on the efficacy of AP back-propagation (Figure 17B).
Figure 17. Summary of back-propagation features in different cell types. (A) Induction of STDP by pairing presynaptic spikes and associated EPSPs with postsynaptic spikes. bAP, back-propagating spike. (B) Dendritic AP amplitude normalized to the somatic AP amplitude and plotted as a function of the distance from the soma. Based on recordings from apical dendrites of mitral cells, CA1 pyramidal cells, L5 pyramidal cells, L2/3 pyramidal cells, spinal cord motoneurons, cerebellar Purkinje cells and dopaminergic substantia nigra neurons. (C) Simulated back-propagation in a reconstructed L5 pyramidal neuron. Pseudo-color representation of peak dendritic AP amplitude. Modified from (Waters et al., 2005; Feldman, 2012)

AP back-propagation can also be modulated by synaptic inputs and by neuromodulators (Waters et al., 2005). Indeed, pairing of excitatory inputs in distal dendritic regions with bAPs amplified both dendritic APs and the evoked Ca$^{2+}$ influx near the site of the synaptic inputs. Such pairing of EPSPs with bAPs also induced a robust LTP (Magee and Johnston, 1997). On the other hand, inhibitory inputs in mitral cell dendrites blocked bAPs (Lowe, 2002; Xiong and Chen, 2002). Neuromodulators such as dopamine and acetylcholine can activate distinct second messengers (e.g. cyclic AMP, diacylglycerol and Ca$^{2+}$), thus resulting in a change in the phosphorylation state of ion channels and subsequently affecting the AP back-propagation.
features (Sjöström et al., 2008). Such impact of the neuromodulators on bAP and dendritic excitability could further explain the discovery that STDP depends critically on neuromodulators (Seol et al., 2007; Pawlak et al., 2010). For examples, activation of muscarinic receptors coupled to adenylyl cyclase and phospholipase C are necessary for cortical STDP induction (Seol et al., 2007) and activation of dopamine receptors also strongly regulates corticostriatal STDP (Pawlak and Kerr, 2008; Shen et al., 2008; Cui et al. 2015).

A growing body of information suggests that increasing or decreasing excitability in dendritic branches through propagation of bAPs modulation regulates the temporal window of STDP expression, and its polarity and magnitude. In L5 pyramidal cells in neocortex, high-frequency EPSP-AP pairings evoke LTP at proximal inputs (<100 mm from soma), but the very same protocol results in LTD at most distal inputs (>500 mm) due to decremental bAP. However, if the distal dendrite is depolarized sufficiently, accompanying with boosted bAP propagation, the plasticity of distal inputs is converted from LTD to LTP (Letzkus et al., 2006; Sjöström and Häusser, 2006). Similar results were found in CA1 pyramidal cells, bAP enhancement also promotes Hebbian-LTP (Watanabe et al., 2002). Taking together, firing rate and depolarization regime, together with the relative timing of EPSPs and bAP feature (due to electrotoneic properties of dendrites) control the polarity and magnitude of STDP.

3.6 Cellular signaling for spike-timing dependent plasticity

STDP at excitatory synapses is mediated roughly by similar signaling pathways, which mediate classical HFS-induced LTP and LFS-induced LTD. As illustrated in Figure 18, it exists three major cellular mechanisms for STDP: the NMDAR-dependent t-LTP, the NMDAR-dependent t-LTD, as well as mGluR-dependent and/or CB1R-dependent t-LTD (Feldman, 2012). During all these processes, the elevation of postsynaptic Ca²⁺ level is the key parameter (Schiegg et al., 1995; Graupner and Brunel, 2010, 2012). Compared with presynaptic stimulation or steady-state postsynaptic depolarization (in conventional protocols), postsynaptic spiking (in STDP protocol) induces Ca²⁺ influx with different effectiveness and
magnitudes (Dan and Poo, 2006). Fluorescence Ca\textsuperscript{2+} imaging experiments showed that the Ca\textsuperscript{2+} transient through NMDARs and voltage-sensitive calcium channels (VSCC) in an active spine evoked by pairing EPSPs and bAPs, with a $\Delta t < \pm 50$ ms, was larger in pre-post order than in post-pre order (Koester and Sakmann, 1998; Nevian and Sakmann, 2004). These observations are consistent with the Hebbian STDP and the general idea that high and brief Ca\textsuperscript{2+} influx generates LTP whereas prolonged and modest Ca\textsuperscript{2+} variations induces LTD (Yang et al., 1999; Graupner and Brunel, 2010, 2012). This has been further demonstrated by the evidence that pre-post pairings under partial pharmacological blockade of NMDARs in CA1 hippocampus reduced t-LTP or even converted t-LTP to t-LTD (Nishiyama et al., 2000). There are two main reasons, which could explain why post-pre pairings generates weaker Ca\textsuperscript{2+} influxes. Firstly, the postsynaptic AP is followed by an after-depolarization component that lasts tens of milliseconds (note that this duration varies greatly among neuronal subtypes), thus the EPSP is rather paired with the after-depolarization instead of the bAP itself (the peak component), generating modest NMDAR-mediated Ca\textsuperscript{2+} increase (Karmarkar and Buonomano, 2002; Shouval et al., 2002). Secondly, at some synapses, Ca\textsuperscript{2+} influx through VSCC causes Ca\textsuperscript{2+}-dependent inactivation of postsynaptic NMDARs, leading to less NMDAR current evoked by presynaptic release (Rosenmund et al., 1995; Tong et al., 1995) (Figure 18A). NMDAR-dependent t-LTP and t-LTD, the major forms of STDP, have been reported at CA3-CA1 hippocampal synapses (Nishiyama et al., 2000) and synapses on neocortical L2/3 pyramidal cells (Froemke et al., 2005).

Although NMDAR acts as a molecular coincidence detector in STDP, accounting for both t-LTP and t-LTD at many synapses, such single coincidence detector model (see for example Shouval et al., 2002) cannot explain all the order-specific STDP observed so far. The existence of multiple coincidence detectors was predicted by biophysical models (Karmarkar and Buonomano, 2002) and then proved by experimental findings of CB1R-dependent t-LTD in L2/3 and L5 of somatosensory cortex, and at corticostriatal synapse (Sjöström et al., 2003; Bender et al., 2006; Nevian and Sakmann, 2006; Fino and Venance, 2010; Fino et al., 2010). These studies have revealed a cellular signaling underlying CB1R-dependent t-LTD: activation of postsynaptic mGluRs (mGluR5) activates phospholipase C (PLC), which triggers Ca\textsuperscript{2+}
release from VSCCs and IP3 receptor-gated postsynaptic internal Ca\(^{2+}\) stores, then induces on demand eCBs synthesis and release. eCBs travel retrogradely to activate presynaptic CB1Rs and drive a sustained decrease in transmitter release probability at the presynaptic terminal (Chevaleyre et al., 2006) (Figure 18B). This CB1R-dependent t-LTD sometimes also requires activation of presynaptic NMDARs, but not postsynaptic NMDARs (Sjöström et al., 2003; Bender et al., 2006; Rodriguez-Moreno and Paulsen, 2008), possibly through glutamate released by astrocytes (Min and Nevian, 2012).

Altogether, bidirectional STDP induction is governed by both postsynaptic and presynaptic molecular coincidence detectors, including NMDARs, PLC, and IP3Rs which are engaged in distinct pathways. Some key elements of the cascade signaling such as VSCCs, mGluRs and CB1Rs play crucial roles in these processes.

**Figure 18.** Cellular mechanisms for STDP (A) Biochemical signaling pathways for major forms of STDP. N and A, NMDA and AMPA receptors. Red, depolarization. For mGluR-CB1-LTD, the proposed presynaptic coincidence detector is in green, and the postsynaptic coincidence detector is in blue. A, astrocyte. Signals conveying pre- and postsynaptic spike timing in each model are labeled. From (Feldman, 2012)
3.7 The corticostriatal spike-timing dependent plasticity

The striatum, the primary input within the basal ganglia, receives a large convergence of glutamatergic afferents from different regions of the cortex and from some thalamic nuclei. Excitatory corticostriatal synapses onto striatal MSNs and interneurons present both LTP and LTD (Mahon et al., 2004a; Fino and Venance, 2011). These changes in neuronal activity are thought to be the key substrates for various aspects of neural functions including behavioral control and procedural learning and memory. The first evidence of bidirectional corticostriatal long-term synaptic plasticity in MSNs were reported in vitro on brain slices (Calabresi et al., 1992b, 1992c). Then similar results have been repeated afterwards (Lovinger et al., 1993; Walsh, 1993; Calabresi et al., 1996; Centonze et al., 2001), which demonstrated that the HFS in association with postsynaptic neuronal spikes induced LTD in the presence of a physiological Mg\(^{2+}\) concentration while same protocol induced LTP in Mg\(^{2+}\)-free medium. The fact that NMDAR antagonists did not alter corticostriatal LTD but fully blocked LTP, indicated that NMDAR activation was critical for LTP whereas another mechanism governs corticostriatal LTD. Because CB1Rs are highly expressed in the striatum (Herkenham et al., 1991) and its activation inhibits glutamate release (Huang et al., 2001), CB1R was a good candidate for corticostriatal LTD. Conclusive evidence for eCB-LTD was then reported by showing that LTD induction was blocked by the CB1R antagonist and by CB1R gene deletion (Gerdeman et al., 2002).

*In vivo* forms of corticostriatal plasticity were observed in anesthetized rat (Charpier and Deniau, 1997; Charpier et al., 1999; Reynolds and Wickens, 2000a). LTP was induced by ipsilateral cortical HFS (100Hz) or LFS (5Hz) coupled with postsynaptic depolarization (Charpier and Deniau, 1997; Charpier et al., 1999). However, LTD occurred when HFS protocol of the contralateral cortex was paired with a “just”-suprathreshold depolarizing current pulse (Reynolds and Wickens, 2000a). The cellular mechanisms of the *in vivo* corticostriatal plasticity remain to be elucidated, but it is expected that it should be quite similar to those observed *in vitro* in brain slices.
The early studies at corticostriatal synapses suggest that long-term plasticity induction needs coincidental activities in both pre- and postsynaptic components (reviewed in: Kreitzer and Malenka, 2008; Di Filippo et al., 2009), as the Hebb’s postulate. The critical role of the precise timing of spiking activity was investigated by few studies in dorsal striatum using STDP protocols (Fino et al., 2005, 2009b, 2010; Pawlak and Kerr, 2008; Shen et al., 2008). Due to different experimental conditions such as stimulation protocols, presence or absence of GABAa receptor antagonist in the ACSF, there have been some controversial conclusions concerning the anti-Hebbian versus Hebbian polarity of the corticostriatal STDP. Corticostriatal STDP was first explored by using a horizontal brain slice preserving the corticostriatal connections at the level of the somatosensory area (Fino et al., 2005). Both t-LTP and t-LTD occurred at the same synapses, in which the polarity depended exclusively on the order of pre- and postsynaptic stimulation. The t-LTP was obtained when APs were evoked in MSN before the cortical stimulations (100 post-pre pairings at 1 Hz), while the t-LTD was induced when postsynaptic APs followed the cortical stimulations (100 pre-post pairings at 1 Hz) (Fino et al., 2005; Fino and Venance, 2010) (Figure 19A). Conversely, paired stimulations with the opposite temporal order was found to evoke t-LTP (60 pre-post pairings at 0.1 Hz) and t-LTD (60 post-pre pairings at 0.1 Hz) with blockade of GABAa receptors (Pawlak and Kerr, 2008) (Figure 19B).
In addition, Pawlak and Kerr (2008) showed that dopamine influences STDP. Both LTD and LTP were prevented when blocking dopamine D1/D5 receptors, whereas blocking dopamine D2 receptors changed the temporal structure of STDP. The requirement of dopamine receptor activation in STDP raises the question of the STDP in different dopamine receptor-expressing MSN. Another study using modified STDP protocol (theta-burst frequency, 5 Hz) reported a Hebbian STDP in D1 or D2 receptor co-expressed green fluorescent protein (GFP) mice, and a lack of STDP at D1R-MSNs after post-pre pairings (Shen et al., 2008). The complexity of the effects of the dopamine in STDP will be detailed in chapter 5.

The in vivo experiments showed that the existence of a bidirectional corticostriatal STDP displaying an anti-Hebbian polarity, with physiological GABAergic transmission (Schulz et al., 2010). Repetitive electrical stimulation in contralateral motor cortex (with 60 pairings, at
0.2 Hz) evoked PSPs, which were paired with postsynaptic spike discharge induced by an intracellular current pulse by a time interval of 5 to 10 ms.

Although few studies investigated the presence of STDP at corticostriatal synapses, each of them differed in the experimental conditions, including the frequency of the pairing (0.1 Hz, 0.2 Hz, 1 Hz and 5 Hz), the species used (rats versus mice of different genetic backgrounds), the presynaptic stimulation locus (in the somatosensory cortex, in the corpus callosum, within the striatum or in the contralateral motor cortex), the number of evoked action potential (single or burst), the pharmacological conditions (with or without GABAaR blockade) and the recording method (in vitro versus in vivo) (Fino et al., 2005; Pawlak and Kerr, 2008; Shen et al., 2008; Schulz et al., 2010). These differences possibly account for distinct dendritic activities causing the shift in timing requirements for STDP. Recently, a study reconciled the apparent discrepancy between the different studies reporting different polarity of the corticostriatal STDP as depending on the blockade (Hebbian STDP; Pawlak and Kerr, 2008; Shen et al., 2008) or not (anti-Hebbian STDP; Fino et al., 2005; Schulz et al., 2010) of GABAergic transmission (Paille et al., 2013). It demonstrated that GABAaR blockade was able to completely reverse the temporal order of corticostriatal STDP in rats and mice. Thus, GABAergic signaling acts as a Hebbian/anti-Hebbian switch at corticostriatal synapses.

The corticostriatal STDP comprises diverse signaling pathways which are similar to those requires for HFS- or LFS-induced corticostriatal plasticity. Depending on the experimental conditions, different receptors and intracellular pathways appear to underlie the MSN STDP (Fino and Venance, 2010). Pawlak and Kerr (2008) showed both t-LTP and t-LTD relied on a single coincidence detector, the NMDAR, associated with D1 receptor activation. On the other hand, the t-LTP and t-LTD induced by 1 Hz pairings were mediated by distinct coincidence detectors through independent signaling pathways. Namely, t-LTP relies on the NMDAR, while t-LTD is mGluR-CB1R-dependent and is mediated by retrograde endocannabinoid signaling, which requires multiple molecular coincident detectors: the PLCβ, the IP3R-gated Ca\(^{2+}\) stores and the diacylglycerol lipase α (DGLo) (Fino et al., 2010).
Endocannabinoids (eCBs), a prominent neurotransmitter system, play key roles in normal brain function and are responsible for the psychotropic and behavioral effects of marijuana (Lichtman et al., 2002; Hashimotodani et al., 2007; Moreira and Lutz, 2008). eCBs have emerged as a major actor in learning and memory because of their powerful influence on synaptic plasticity (Freund et al., 2003; Chevaleyre et al., 2006; Heifets and Castillo, 2009; Kano et al., 2009; Katona and Freund, 2012). The eCB system is mainly composed of active bio-lipids synthesized and released on-demand, which act as retrograde neurotransmitters on presynaptic cannabinoid receptor type-1 (CB1R) and postsynaptic transient receptor potential vanilloid type-1 (TRPV1) (Piomelli, 2003; Piomelli et al., 2007; Di Marzo, 2008; Alger and Kim, 2011). It has been characterized five different eCBs and the two best characterized eCBs, i.e. anandamide (AEA) and 2-arachidonoylglycerol (2-AG), mediate short- and long-term synaptic plasticity (mainly depression) at both excitatory and inhibitory synapses in different brain regions. eCBs constitute a retrograde signaling system since they are synthetized and released from the postsynaptic neuronal element and act presynaptically. However, it exists noticeable exceptions in which eCB could also act by non-retrograde pathway or via the interaction between neurons and astrocytes (Castillo et al., 2012; Katona and Freund, 2012; Kano, 2014). These forms of eCB-mediated synaptic modulation contribute to a variety of physiological processes, including learning and memory, motor control, depression, feeding behaviors and pain, to cite a few (Kano et al., 2009; Mechoulam and Parker, 2013). Here, in the experimental study of this thesis, we focused on the role of eCBs on bidirectional synaptic plasticity and its interaction with dopamine in striatum.

4.1 Cannabinoid receptors

The cannabinoid receptors (here we consider all the receptor that bind eCBs) are classified as : (1) CB1R and CB2R, both belong to the G protein-coupled receptor (GPCR) family, (2) TRPV-1, (3) so-called “CB3R” (unknown receptors), and (4) GPR55 (Kano et al., 2009; Battista et al., 2012). The CB3R and GPR55 are proposed as novel cannabinoid receptors, of
which functional roles in the brain are not clear. CB2Rs are expressed mainly in the immune system. Thus, we will detailed the two main receptors in the central nervous system (CNS) which bind eCBs: CB1R and TRPV1.

The cannabinoid type-1 receptor (CB1R)

CB1R was identified as a 473-amino acid cannabinoid receptor in 1990 (Matsuda et al., 1990), which is one of the most abundant G protein-coupled receptors in the brain. Later on, a human homolog of 472 amino acids and a mouse homolog of 473 amino acids have been reported. These CB1Rs share 97–99% amino acid sequence identity (Kano et al., 2009). CB1Rs are preferentially expressed at presynaptic axon terminals in most of brain structures, especially in the basal ganglia, neocortex, hippocampus, cerebellum and amygdala, but are absent in the brainstem (Herkenham et al., 1990; Katona et al., 1999; Mátys et al., 2006). Although CB1Rs are express at both excitatory and inhibitory synapses, inhibitory synapses generally display higher levels of CB1R than excitatory synapses within the same regions. For example, the density of CB1R immuno-labeling on inhibitory synaptic elements is 30 times higher than excitatory synapses for hippocampal CA1 pyramidal cells, 6 times for cerebellar Purkinje cells, and 3-4 times for striatal medium spiny neurons (Kano et al., 2009). More specifically, in the striatum, CB1Rs are localized in three distinct neuronal elements: the glutamatergic terminals of cortical afferents, the GABAergic fast-spiking interneurons and the axon terminals of MSN (Piomelli, 2003). This strongly suggests a potential role for eCBs in the modulation of basal ganglia-dependent motor functions.

The CB1Rs are coupled to the Gi/o subtypes of G-proteins which activation leads to inhibition of adenylyl cyclase (AC), thus decreases cyclic adenosine monophosphate (cAMP) accumulation and reduces protein kinase (PKA) activity (Piomelli, 2003; Heifets and Castillo, 2009). The CB1Rs also act on voltage-gated calcium channels (VGCCs), K⁺ channels, including voltage-dependent Kᵥm and Kₐ channels and voltage-independent G protein-coupled inwardly rectifying K⁺ (GIRK) channels (Schlicker and Kathmann, 2001). The activation of CB1Rs inhibit the release of neurotransmitter, such as GABA, glutamate, acetylcholine and
dopamine (Cadogan et al., 1997; Szabo et al., 1998; Gerdeman and Lovinger, 2001). Previous studies have revealed that the inhibitory effect on transmitter release involves sites downstream of the voltage-dependent Ca\textsuperscript{2+} channels, i.e., the CB1R-mediated suppression of GABA release is via N-, but not L-, P- or Q-, type Ca\textsuperscript{2+} channels in rat striatal or hippocampal slices (Schlicker and Kathmann, 2001). A series of studies using the blocker of potassium channel (BaCl\textsubscript{2} or 4-aminopyrididine) and activator of adenylyl cyclase (forskolin) abolished this inhibitory effect of CB1R agonists (WIN55,212-2) on the EPSCs or IPSCs, which indicates the possibility of the other CB1R-mediated signal pathways in regulating transmitter release (Schlicker and Kathmann, 2001). Several strand of CB1R knockout mice are now available and are extensively used to help understanding eCB system in brain physiology and pathology (Zimmer et al., 1999).

Interestingly, the CB1Rs levels in rats are globally increased during the transition from adolescence (postnatal day 35-37) to adulthood (postnatal day 70-72), which is opposite to most other neuropeptide systems undergoing pruning during this period (Verdurand et al., 2011). This suggests that distinct time periods during adolescence may have different sensitivity to cannabinoid relevant to specific function, which needs further investigation.

**Transient receptor potential vanilloid-type-1 (TRPV1)**

TRPV1 is a nonselective cation channel that is activated by external transient stimuli, such as temperature, mechanical or osmotic stimuli, light, PH change and electrical charge (Starowicz et al., 2007). Besides the classical cannabinoid receptors, there is growing evidence that TRPV1 channels also participate in eCB signaling (Ross, 2003) since AEA binds both CB1R and TRPV1. TRPV1 expression was observed in the cortex, hippocampus, cerebellum, amygdala, striatum, thalamus, SNC and many other brain regions (Cristino et al., 2006). Compared to the presynaptic CB1R, TRPV1 controls synaptic signaling at the postsynaptic side. Thus TRPV1 signaling is defined as non-retrograde pathway (Castillo et al., 2012). Both endogenous ligands AEA and 2-AG interact with CB1R, whereas only AEA activates TRPV1 (Starowicz et al., 2007; Puente et al., 2011a).
4.2 Endocannabinoids signaling

The major eCBs were identified in the 90s, first the anandamide (Devane et al., 1992) and then the 2-AG (Mechoulam et al., 1995; Sugiuira et al., 1995). The details about these discoveries can be found in this review concerning the historical steps of the cannabinoids and endocannabinoids research (Di Marzo, 2006). eCBs comprise also other bio-active lipid molecules such as the 2-arachidonylglycerol ether (noladin), N-arachidonoyl-dopamine and O-arachidonoyl-ethanolamine (virodhamine). However, their physiological role remains pretty much unclear. Different to the other classic neurotransmitters and neuropeptides, eCBs cannot be stored in intracellular vesicles due to their lipidic nature and yet they are produced upon neuronal stimulation and released from neurons immediately after synthesis. Therefore, eCBs are synthesized and released in a “on-demand” manner (Alger and Kim, 2011). That implies that eCBs can be delivered quite quickly upon sustained neuronal activity.

2-arachidonoylglycerol (2-AG)

2-AG acts as a full agonist for CB1R and CB2R. In brain, 2-AG content is approximately 200-fold higher than that of AEA (Piomelli, 2003). 2-AG is a retrograde messenger, since it travels backward across the synaptic cleft from postsynaptic side to presynaptic terminals where it binds CB1Rs (Figure 20). The activation of CB1R is capable of inducing reduction or even suppression of neurotransmitter release either on a short-term or long-term time-scale and further modulating synaptic strength (Kano et al., 2009; Castillo et al., 2012). Blocking the 2-AG-mediated synaptic modulation significantly suppresses the extinction of aversive memory and spatial memory, cerebellum-dependent eye blink conditioning as well as feeding behavior, which indicate the involvement of this eCB signaling in a variety of physiological processes throughout of the brain (Kano et al., 2009; Cachope, 2012; Mechoulam and Parker, 2013). Moreover, many pharmacological evidences have revealed the importance of the eCB system under pathological conditions, such as the neuroprotective effect of 2-AG in brain ischemia, proinflammatory stimuli and traumatic brain injury (Zhang and Chen, 2008; Xu and Chen, 2014).
The 2-AG synthesis from postsynaptic sites is triggered by neuronal activation (Figure 20), which evokes either a marked increase in intracellular Ca$^{2+}$ or robust activation of $G_{q11}$-GPCRs (e.g. mGluR5). Combined activation of phospholipase C (PLC) by $G_{q11}$-GPCRs and of the synthetic enzyme diacylglycerol lipase (DGL), phosphatidylinositol bisphosphate (PIP2) is finally converted into 2-AG (Piomelli et al., 2007; Alger and Kim, 2011). After release into the extracellular space, 2-AG binds to presynaptic CB1R and ~85% 2-AG is rapidly hydrolyzed into arachidonic acid and glycerol primarily by the monoacylglycerol lipase (MGL) expressed presynaptically in axon terminals and the remaining 15% is mostly catalyzed by other degradative enzymes such as ABHD6 (Kano et al., 2009).

Although 2-AG appears to be the principal eCB required for Ca$^{2+}$-driven retrograde signaling, the relative (or distinct) contribution of 2-AG and AEA to synaptic transmission is still not clear yet. Moreover, a CB1R-autocrine signaling has been reported in cortical GABAergic low-threshold spiking (LTS) interneurons by repetitive stimulation (Bacci et al., 2004; Marinelli et al., 2008, 2009). This slow self-inhibition is due to the rises of intracellular Ca$^{2+}$ through VSCCs, which activates DGLα thus facilitates 2-AG production. 2-AG activates postsynaptic CB1Rs (Bacci et al., 2004), which targets the downstream G protein-coupled inward K$^+$ channel leading to a hyperpolarization of the membrane potential and the inhibition of neuronal excitability.

![Figure 20. 2-AG synthesis and retrograde signaling](image)

In a conventional on-demand synthesis model, 2-AG synthesis is tightly linked to demand, which is a sustained neuronal activation. Stimulation causes intracellular Ca$^{2+}$ elevation and/or G-protein ($G_{q11}$)
activation, leading to activation of the synthetic enzyme for 2-AG, the DGL. Once released into the synaptic cleft, 2-AG binds to presynaptic CB1Rs and suppresses synaptic transmission. Presynaptic MGL is a major degradative enzyme for 2-AG, while another degradative enzyme, ABHD6 is located postsynaptically. From (Alger and Kim, 2011)

Anandamide (AEA)

AEA, the other major eCB besides 2-AG, is a partial agonist of CB1R but a full agonist of TRPV1 (Ross, 2003; Starowicz et al., 2007). The biochemical pathways for AEA synthesis seem to be more complex compared to that of the 2-AG synthesis. Postsynaptic depolarization and intracellular Ca\(^{2+}\) influx contribute producing AEA (similarly to 2-AG production), but the downward mechanisms remains unclear and quite debated. Early studies suggested that N-acyltransferase and N-arachidonoyl phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) play a critical role for AEA biosynthesis. However, later studies showed that AEA formation persisted in NAPE-PLD-knockout mice. It implied that alternative biosynthetic pathways should exist in a NAPE-PLD independent manner (Okamoto et al., 2007). Subsequent studies revealed that the pathway combined \(\alpha/\beta\)-hydrolase 4 (Abh4) and phosphodiesterase GDE1 might participate in the formation of anandamide, or the pathway which comprises a PLC-like enzyme with lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages also generate anandamide (Wang and Ueda, 2009). The major enzyme catalyzing anandamide degradation is the fatty acid amide hydrolase (FAAH). FAAH is localized in the soma and dendrites of neurons, and exhibits an anatomical segregated pattern with the CB1R expression; i.e., CB1R-immunoreactive axon terminals are presynaptic to the FAAH-immunoreactive principal cells (Cravatt et al., 1996; Egertová et al., 2003). The analysis of FAAH-knockout mice showed a severe impairment in degradation of AEA (Cravatt et al., 2001).

Apart from participating of the classic eCB retrograde signaling, AEA also signals in a non-retrograde manner by activating postsynaptic TRPV1 (Castillo et al., 2012). The non-retrograde synaptic signaling originates from postsynaptic mGluR activation, via PLC and
Ca\textsuperscript{2+} efflux from the intracellular stores, leading to AEA production that activates postsynaptic TRPV1 channels. Such TRPV1 activation causes AMPARs endocytosis and a decrease of the synaptic efficacy. Furthermore, co-expression of CB1Rs and TRPV1 receptors in the striatum and hippocampus can potentially result in the enhancement of the effects induced by their agonists (Cristino et al., 2006). Although AEA and 2-AG are differentially involved in different brain regions, both of them mediate similar synaptic modulation via CB1R, indicating that the two transmitters may work in a cooperative way. Nevertheless, it has been reported (in cellular extracts) that elevation of AEA concentration inhibits 2-AG production in striatum (Maccarrone et al., 2008). The functional interactions between AEA and 2-AG in different brain structures remain largely to be explored.

4.3 Endocannabinoid-mediated short-term plasticity

Depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE)

The retrograde eCB signaling was first evidenced by sustained depolarization of the postsynaptic neuron which caused eCB-mediated transient (typically <1 min) inhibition of transmitter release from presynaptic terminals (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Alger, 2002). This short-term plasticity depending on the inhibitory or excitatory nature of the synapses was classified into two groups: the depolarization-induced suppression of inhibition (DSI) and the depolarization-induced suppression of excitation (DSE). In the hippocampus, depolarization of CA1 pyramidal cells triggers eCB-mediated DSI through transient depression of GABA release from axon terminals of GABA interneurons (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Meanwhile, eCB-mediated DSE was found at glutamatergic synapses in cerebellum (Kreitzer and Regehr, 2001). Similar to DSI, DSE is induced by a sustained neuronal depolarization (around 10 seconds) which involves a retrograde eCB signaling (Figure 21). This inhibition of neurotransmission could be mimicked by CB1R agonists and blocked by CB1R antagonist. Other studies have documented DSI and DSE in various brain structures besides the
hippocampus and cerebellum. These include the cerebral cortex, amygdala, hypothalamus, striatum and substantia nigra pars reticulata (Kano, 2014).

**Figure 21. Models for the mechanisms of DSE and DSI.** Postsynaptic depolarization induces $\text{Ca}^{2+}$ influx into the soma and dendrites of the neurons through voltage-gated $\text{Ca}^{2+}$ channels. Elevation of intracellular $\text{Ca}^{2+}$ concentration triggers biosynthesis of eCBs (possibly 2-AG). eCBs are then released from postsynaptic neurons, activate presynaptic CB1Rs, which is coupled to Gi/o proteins, and suppress glutamate (DSE, left) or GABA (DSI, right) release. Modified from (Hashimotodani et al., 2007)

**Other eCB-mediated short-term plasticity**

DSI and DSE are dependent on postsynaptic $\text{Ca}^{2+}$ influx. Because voltage-gated $\text{Ca}^{2+}$ channel is not the only route for $\text{Ca}^{2+}$ influx in postsynaptic neuron, NMDAR is considered as another important candidate for calcium influx promoting eCB synthesis. It has been demonstrated that a transient suppression of inhibitory transmission (i.e. DSI) was induced solely by NMDARs activation with blockade of $\text{Ca}^{2+}$ channel in cultured hippocampal neurons (Ohno-Shosaku et al., 2007). This study indicates that $\text{Ca}^{2+}$ influx through NMDAR can also
contribute to sufficiently enhance eCB release in order to be fully responsible for the induction of the short-term depression of the presynaptic release.

The eCB-mediated short-term depression is not only driven by Ca^{2+} elevation but also by activation of G_{q11}-coupled receptors including group I metabotropic glutamate receptor (mGluR), muscarinic receptor (M1R/M3R) and serotonin receptor (5-HTR) (Kano, 2014). In these forms of short-term plasticity, the eCB release is induced by prolonged stimulation of these receptors without the need of postsynaptic Ca^{2+} influx (Kano et al., 2009). For instance, the application of the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) induced a short-term depression on cerebellar Purkinje cells, which was abolished by cannabinoid antagonists, but not affected by including the Ca^{2+} chelator BAPTA in the recording pipette (Maejima et al., 2001). This phenomenon was then found to occur in the hippocampus, striatum and nucleus accumbens (Kano et al., 2009).

### 4.4 Endocannabinoid-mediated long-term plasticity

Involvement of eCBs in long-term plasticity was first discovered at excitatory synapse in the striatum by the evidence that the HFS induced LTD was blocked by CB1R antagonist and lost in CB1R knockout mice (Gerdeman et al., 2002). Then the eCB-LTD were widely found at both excitatory and inhibitory synapses, in the dorsal striatum, nucleus accumbens, cortex, cerebellum, extended amygdala and hippocampus, as shown in Table 1 (Heifets and Castillo, 2009; Kano et al., 2009).

The mechanism of eCB-LTD is quite similar among distinct synapses. It is the best-characterized example of presynaptic forms of long-term plasticity dependent on retrograde eCB signaling. In most cases, eCB-LTD typically originates from a pronounced and long-lasting presynaptic activity, triggering release of eCBs from the target neuron via PLCβ and MAGL activation. Then eCBs travel retrogradely across the synapse and activate presynaptic CB1Rs. eCB-LTD is classically induced by tetanic or theta burst stimulation combined (or not) with postsynaptic depolarization (so in a hebbian or non-hebbian mode), and
also by STDP protocol (Kano et al., 2009; Castillo et al., 2012). This associative pattern of both pre- and postsynaptic activities ensures the induction of eCB-LTD only at the active synapses. For example, repetitive stimulations of synaptic inputs activate $G_{q11}$-coupled receptors coinciding with postsynaptic $Ca^{2+}$ elevation. This $Ca^{2+}$ flux (influx and eflux) originates from L-type voltage-sensitive calcium channels (VSCCs) activated by bAP (e.g., during STDP protocols), NMDARs, or released from the endoplasmic reticulum (IP3R activation via PLCβ activation and its resulting IP3 production). Subsequently, 2-AG is produced at postsynaptic side and travels backward to presynaptic side. It should be noted that in some synapses (e.g., hippocampal GABAergic synapses), induction of eCB-LTD by afferent-only stimulation protocols can occur independently of postsynaptic $Ca^{2+}$ (Chevaleyre and Castillo, 2003). At the presynaptic terminal, the CB1R inhibits adenylyl cyclase via $G_{s0}$, reducing protein kinase A (PKA) activity. Induction of eCB-LTD may also require a presynaptic $Ca^{2+}$ rise through presynaptic VSCCs or NMDARs or release from $Ca^{2+}$ internal stores. Activation of the $Ca^{2+}$-sensitive phosphatase calcineurin (CaN), in conjunction with the reduction of PKA activity, shifts the phosphorylation and dephosphorylation balance towards dephosphorylation direction. Consequently, a decrease in phosphorylation of the target protein (which remains to be identified) induces a decrease of transmitter release from presynaptic terminals (Figure 22) (Heifets and Castillo, 2009).

2-AG also acts in some alternative signaling pathways to efficiently modulate the synaptic plasticity. For instance, postsynaptic activity-dependent elevation of intracellular $Ca^{2+}$ facilitates 2-AG production (as previously described); then, 2-AG activates postsynaptic CB1Rs which are coupled to a G protein-coupled inwardly rectifying $K^{+}$ (GIRK) channel, constituting a self-inhibition effect on the synaptic transmission (Bacci et al., 2004; Marinelli et al., 2008). In another study, Yasuda et al. (2008) suggested the involvement of presynaptic $K^{+}$ channels in a hetero-synaptic LTD in the developing hippocampus.

Although most early evidences suggest that eCB-LTD is largely mediated by 2-AG (Heifets and Castillo, 2009; Kano et al., 2009), accumulated recent results reveal that AEA may also play a non-negligible role. Since the first finding that the postsynaptic TRPV1 activation
can trigger LTD in the nucleus accumbens (Grueter et al., 2010). AEA became another candidate capable of driving LTD as well as 2-AG. In addition, a CB1R-independent and TRPV1-dependent LTD exclusively mediated by AEA was found in dentate gyrus (Chávez et al., 2011). Study in the extended amygdala showed AEA mediates TRPV1-dependent LTD whereas 2-AG mediates the short term depression (Puente et al., 2011a). Moreover, in D2R-MSNs of the dorsal striatum, 2-AG mediates the LTD induced by LFS, in contrast, HFS induces CB1R-dependent LTD that is mediated by AEA (Lerner and Kreitzer, 2012). Taking together, the diversity of eCB-LTDs at play in the brain suggests that eCB system plays specific roles in different behaviorally function.

It is well known that eCBs modulate LTD in both GABAergic and glutamatergic synapses (Chevaleyre et al., 2006; Heifets and Castillo, 2009; Kano et al., 2009). However, some noticeable evidences suggest that eCBs may also facilitate the induction of STP or LTP (Carlson et al., 2002; Zhu and Lovinger, 2007; Lin et al., 2011; Xu et al., 2012). At hippocampal schaffer collateral-CA1 synapses, LTP was lost in pharmacological and genetic inhibition of CB1R. Furthermore, an increase of 2-AG level facilitates LTP while a decrease of 2-AG level prevented this potentiation (Xu et al., 2012). Lastly, we found that a eCB-LTP could be induced with a soft cell conditioning protocol (very few STDP pairings) at corticostrialal synapse (Cui et al., 2015; see Article 2).
Figure 22. Summary of the classical eCB-LTD induction mechanism. One of the most common initial steps of induction is the activation of postsynaptic mGluR-I, following repetitive activation of excitatory inputs. These receptors couple to PLC via Goαq/11 subunits and promote DAG formation, which is converted into the 2-AG by DGL. 2-AG is then released from the postsynaptic neuron by a mechanism that presumably requires an EMT and binds presynaptic CB1Rs that mediates a long-lasting reduction of transmitter release. For clarity, eCB-LTD mediated by AEA, and the contribution of other GPCRs in mobilizing eCBs, is not shown. ER: endoplasmic reticulum, DAG: diacylglycerol, DGL: diacylglycerol lipase, PI: phosphatidylinositol, IP3: inositol 1,4,5-trisphosphate, EMT: eCB membrane transporter, AC: adenyl cyclase, PKA: protein kinase A, CaN: Ca2+-sensitive phosphatase calcineurin, T: target (unknown protein at this day). From (Hefts and Castillo, 2009)
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<th>Induction</th>
<th>Dependence</th>
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<td>CB₁, I-mGluR, PLC, DGL</td>
<td>NMDAR, Ca²⁺</td>
<td>(Chevaleyre and Castillo, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MFS, TBS</td>
<td>CB₁</td>
<td></td>
<td>(Chevaleyre and Castillo, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TBS</td>
<td>cAMP/PKA (pre), RIM1a</td>
<td>PKA (post)</td>
<td>(Chevaleyre et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E Hetero-HFS</td>
<td>CB₁, mGluR1/5, K⁺ channel (pre)</td>
<td>NMDAR, Ca²⁺</td>
<td>(Yasuda et al., 2008)</td>
</tr>
</tbody>
</table>

Ac, nucleus accumbens; PFC, prefrontal cortex; BLA, basolateral amygdala; MSN, medium spiny neuron; PyC, pyramidal cell; V, visual cortex; S, somatosensory cortex; E, excitatory; PF, parallel fiber; I, inhibitory; HFS, high-frequency stimulation; Depol, postsynaptic depolarization; MFS, medium-frequency stimulation; PS, pairing stimulation; LFS, low-frequency stimulation; TBS, theta-burst stimulation; Hetero, heterosynaptic; I-mGluR, group I metabotropic glutamate receptor; VSCC, voltage-sensitive Ca²⁺ channel; L, L-type; P/Q, P/Q-type; N, N-type; pre, presynaptic; post, postsynaptic; NMDAR, NMDA receptor.
4.5 Cognitive role of the endocannabinoid system

Reflecting the prevalence of eCB-mediated synaptic modulation in vivo and in vitro, it strongly indicates the involvement of eCB system in neural functions, in different brain regions, such as cerebral cortex, hippocampus, amygdala and striatum. Behavioral studies combined with pharmacological or genetic manipulation (mainly CB1R, MAGL and FAAH) of retrograde eCB signaling have demonstrated that eCB system is involved in various aspects of neural functions, including learning and memory, mood and anxiety, drug addiction and feeding behavior (Kano et al., 2009; Mechoulam and Parker, 2013). Here, we will focus on the cognitive role of eCB system.

Marijuana smoking is considered to impair human learning and memory, but the mechanisms that underlie this process remain unclear. In concert with the human observations, most studies using rodents demonstrated that cannabinoid treatment disrupted the performance in alternation T-maze task, two-component instrumental discrimination task, delayed-match (or non-match) instrumental task and water maze, consistent with the selective impaired short-term memory or working memory (Lichtman et al., 2002; Mechoulam and Parker, 2013). However, the retrieval of previous memory (long-term or reference memory) remained intact. The functional role of eCB system are closely related to other neurochemical systems because the ability of cannabinoid agonists to inhibit glutamatergic, GABAergic and cholinergic transmission, each of which has been strongly implicated in learning and memory (Lichtman et al., 2002).

The findings that CB1R agonists damage the working memory suggest that CB1R inhibition may enhance memory. A few experiments supported this view by the evidence that CB1R-knockout mice are able to retain memory much longer than the normal mice in an object recognition test (Reibaud et al., 1999). The effects of CB1R blockade on hippocampus-dependent spatial memory have been examined by using the Morris water maze. Both the CB1R-knockout mice and the CB1R antagonist-treated mice exhibited similar rate of acquisition with the control mice in the standard fixed hidden platform task; in contrast, they
were unable to learn new rules and showed deficits in the normal extinction processes (Varvel and Lichtman, 2002; Varvel et al., 2005). These data indicate that the eCB signaling is involved in behavioral flexibility and oblivion. Contextual and auditory fear conditioning was used to decipher the distinct aspects of aversive memory. In auditory fear conditioning, mice were trained to associate a tone with a foot-shock. After training, animals froze when re-exposed to the tone, and this behavior is highly dependent on the amygdala activity. While in contextual fear conditioning, mice learned the association between the conditioning chamber and the paired shock, which involves hippocampus. A cannabinoid agonist impaired the acquisition of contextual fear conditioning, but not auditory fear conditioning in rats (Pamplona and Takahashi, 2006). On the other hand, CB1R-knockout mice showed normal acquisition but intensively impaired extinction of the auditory fear memory (Marsicano et al., 2002). Meanwhile, LTD was also disrupted and LTP was enhanced at inhibitory synapses in amygdala of the CB1R deficit mice (Marsicano et al., 2002). Together, this study indicated that eCB system has a significant contribution in the extinction of aversive memories and a negligible function in aversive memory acquisition. In basal ganglia, in which one of the highest densities of CB1R among brain structures was observed (Piomelli, 2003; Mártyás et al., 2006), a strong impairment of complex motor behaviors were observed after acute administration CB1R agonists (Rodriguez de Fonseca et al., 1998). eCBs release in the striatum is necessary for the induction of long-term synaptic plasticity in dorsolateral striatum which is the critical brain region involved in habit formation. Indeed, CB1R-knockout mice and CB1R antagonist treated mice showed impaired habit formation of instrumental lever pressing (Hilário et al., 2007). In another study, Rueda-Orozco et al. (2008) trained rats in a reversal T-maze task, i.e., after the mice learned to enter the goal arm (standard training phase), the rats were trained to enter the opposite goal arm which was now reinforced (the reversal phase). The specific intra-striatal administration of a CB1R antagonist immediately after reversal training delays the extinction process and reversal learning (Rueda-Orozco et al., 2008). These results evidenced that manipulating the eCB system profoundly influences dorsal striatum-dependent learning and memory.
CHAPTER 5. The dopaminergic system

The dopaminergic circuit is a widespread system common to different species along phylogeny like primates, rodents, birds or even insects such as drosophila (Puig et al., 2014 see Figure 23). The two major sources of dopamine in the central nervous system are the substantia nigra pars compacta (SNC) and the ventral tegmental area (VTA) which are located in the midbrain. These two nuclei project mainly to the dorsal striatum and the nucleus accumbens (NAc) (are also defined as ventral striatum) making up the nigrostriatal and mesolimbic pathway, respectively (Fallon and Moore, 1978; Gerfen et al., 1987). The pioneering work of Schultz et al., (1997, 1998) proposed that the phasic dopamine signal from midbrain dopaminergic neurons encode reward processing and error prediction. However, dopamine release is more complex than the sole phasic release; it exists a tonic and background dopamine release as well (Goto et al., 2007). Dopamine also plays a critical role in motor control, habit learning and working memory due to different projection sites from midbrain (Nicoullon and Coquerel, 2003; Vijayraghavan et al., 2007; Cools, 2011; Hosp et al., 2011). In this PhD, we focused on the dorsal striatum, which receives dopaminergic afferents from SNC. In striatum, dopamine potently modulates neuronal excitability (Nicola et al., 2000) and corticostriatal information processing (Reynolds and Wickens, 2002; Wickens et al., 2007), thus contributing to the formation of sensory-motor linkages allowing selection of adapted motor behavior in response to environmental cues.

The dysfunction of the dopaminergic system has been evidenced to lead to devastated neurological and psychological disorders such as Parkinson’s disease, drug addiction, obsessive compulsive disorders or schizophrenia (DeLong and Wichmann, 2007). At a synaptic level, corticostriatal plasticity has been shown to be dramatically impaired in rodent model of Parkinson’s disease (Kreitzer and Malenka, 2008; Di Filippo et al., 2009) thus illustrating the key role of dopamine into synaptic plasticity. Understanding the physio-pathological contribution of dopamine is of fundamental value to further elucidate the mechanism of synaptic plasticity and eventually lead to novel therapeutic targets.
Figure 23. Dopaminergic projections (in red) from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) to the PFC/NCL and striatum in the brain of a primate (human), a rat, and a pigeon. Pallidal (cortical) areas across species are shaded in gray, the hatched area denotes the PFC/NCL (the nidopallium caudolaterale), striatal areas are shaded in blue. Note that, in all species, DA neurons in both dopaminergic nuclei project to several subregions of the PFC/NCL and striatum. From (Puig et al., 2014)

5.1 Dopamine receptors

Dopamine receptors belong to the family of the G-protein coupled receptors (GPCRs) and are widely expressed in the central nervous system. On the basis of the structural, biochemical, and pharmacological properties, five different subtypes of dopamine receptors have been characterized and are now classified in two major families: the D1-like and D2-like families (Table 2) (Missale et al., 1998; Neve et al., 2004; Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012).

Basic properties of dopamine receptors

The D1-like family comprises D1 and D5 receptors, which are positively coupled to adenyllyl cyclase thus enhancing cAMP levels. On the contrary, D2-like family comprises the D2, D3 and D4 receptors, which negatively coupled to adenyllyl cyclase thus leading to reduction of cAMP levels. The affinity of D2-like receptors for dopamine is 10- to 100-fold greater than that of D1-like receptors (Beaulieu and Gainetdinov, 2011), explaining why that high dopamine level activates D1 receptors whereas low dopamine level activates preferentially D2 receptors. The D1 and D5 dopamine receptors display 80% homology in their
transmembrane domains, whereas the D3 and D4 dopamine receptors are 75 and 53% homologous with the D2 receptors, respectively (Beaulieu and Gainetdinov, 2011). The D1- and D2-like dopamine receptors differ in genomic structure, primarily in the absence or presence of introns in their coding sequences. As summarized in Table 2, The D1 and D5 dopamine receptors genes do not contain intronic sequences in their coding regions, in contrast, the genes encoding the D2-like receptors have several introns (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). The six introns, found in the gene encoding the D2 receptors, provide the genetical basis for the transcription of heterogeneous population of D2 receptors, named D2S (S=short) and D2L (L=long) receptors (De Mei et al., 2009). D2S receptors have been found to be mostly expressed on the dopaminergic neurons and their dendrites, which are mainly involved in autoreceptor functions by regulating dopamine synthesis, release and uptake (Ford, 2014). Whereas, D2L receptors are predominantly expressed in postsynaptic non-dopaminergic neurons, i.e. MSNs (De Mei et al., 2009).

<table>
<thead>
<tr>
<th></th>
<th>D1-like Family</th>
<th>D2-like Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA receptor subtype</td>
<td>D1</td>
<td>D5</td>
</tr>
<tr>
<td>Gene name</td>
<td>Drd1</td>
<td>Drd5</td>
</tr>
<tr>
<td>Number of introns</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Splice variants</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Affinity for DA(μM)</td>
<td>1.0-5.0</td>
<td>0.2-2.0</td>
</tr>
<tr>
<td>G protein coupling</td>
<td>Gαs, Gαolf</td>
<td>Gαs, Gαq</td>
</tr>
<tr>
<td>Common family-specific agonist</td>
<td>SKF-38393, SKF-81297</td>
<td>(-) Quinpirole, Cabergoline</td>
</tr>
<tr>
<td>Common family-specific antagonist</td>
<td>SCH-23390, SKF-83566</td>
<td>(-) Sulpiride, Sipiperone, Nemonapride</td>
</tr>
</tbody>
</table>

From (Tritsch and Sabatini, 2012)

**Localization of dopamine receptors in striatum**

The expression of dopamine receptors are most prominently found in striatum and prefrontal cortex, as well as at lower levels in the substantia nigra, olfactory bulb, amygdala, hippocampus and cerebellum (Table 3) (Gerfen, 2000; Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012). At the corticostrial pathway, D1 receptors are localized on postsynaptic side (i.e. striatal neurons) exclusively (Hersch et al., 1995; Caillé et al., 1996) while D2 receptors are expressed in corticostrial afferents as well as postsynaptic sides (Sesack et al., 1994; Hersch et al., 1995; Wang and Pickel, 2002). Compared to D1 and D2
receptors, the expression of D3, D4, and D5 receptors are much less abundant in striatum and are mainly expressed in the striatal interneurons (Bertran-Gonzalez et al., 2010).

The medium-sized spiny neurons (MSNs), which represent around 90-95% striatal neurons express D1 and D2 receptors in a quite segregated manner at least in rodents. The D1R-MSNs constitute the so-called direct pathway whereas the D2R-MSNs compose the indirect pathway (Gerfen, 1992b; Surmeier et al., 1996). However, it should be mentioned that a small population of MSNs express both D1 and D2 receptors, ranging from 5 to 15% in the dorsal striatum of rodents (Valjent et al., 2009; Gangarossa et al., 2013).

The remaining 5-10% of striatal neurons is constituted by interneurons, cholinergic and GABAergic cells. Among these striatal interneuron, the giant cholinergic interneurons highly express D2 and D5 receptors, whose activation modulates acetylcholine (ACh) release (Yan et al., 1997b; Maurice et al., 2004; Wang et al., 2006). Furthermore, a recent paper reports that optogenetic activation of cholinergic interneurons could trigger dopamine release via activation of nicotinic receptors located on dopamine axons (Threlfell et al., 2012). Dopamine receptors are expressed in the nitric oxide synthase (NOS) interneurons as well. Indeed, NOS interneurons are activated in vivo by burst firing of nigrostriatal dopamine cells through a D1 and D5 receptor-dependent mechanism, and stimulation of D1 and D5 receptors on striatal NOS interneurons leads to the release of nitric oxide (Tepper et al., 2010). Similarly, fast-spiking interneurons express D1 and D5 receptors since they can be depolarized by bath application of dopamine or selective D1/D5 dopamine agonists, but not D2 agonist (Centonze et al., 2003).
5.2 The dopaminergic signaling

*Dopaminergic neurons' activity and dopamine release*

The dopaminergic signals, which regulate neural functions in the basal ganglia and cerebral cortex, are originated from midbrain dopaminergic neurons mainly distributed in SNc and VTA, respectively. In most cases, dopaminergic neurons exhibit spontaneous rhythmic firing activities with broad action potentials, which are profoundly inhibited by the dopamine agonist apomorphine. Hyland et al. (2002) demonstrated two discharge patterns of dopaminergic neurons in free moving rats without specific stimulation: a low-rate bursting mode (short bursts of 3-4 spikes with mean spike intervals of 50-70 ms) and an extremely regular (clock-like) firings. It is considered that during this background tonic mode, dopaminergic neurons maintain a steady-state level of dopamine (10-20 nM) in target areas that is vital for enabling the normal cognitive and motor functions by activating high-affinity postsynaptic D2 receptors (Grace et al., 2007; Schultz, 2007a). However, in response to various sets of input (e.g., unexpected reward or cues predicting reward), dopaminergic neurons can switch to generate transient synchronous bursts (Grace, 1991; Tobler et al., 2005; Schultz, 2007b; Bromberg-Martin and Hikosaka, 2009), which elicit phasic dopamine release from axon terminals and somato-dendritic compartments (Adell and Artigas, 2004). Such dopamine

Table 3. Cellular distribution of dopamine receptors in the cortex and striatum of rodents

<table>
<thead>
<tr>
<th>Striatum</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>dSPNs</td>
<td>+++(&gt;90%)</td>
<td>-</td>
<td>+(50%)</td>
<td>+(&lt;10%)</td>
<td>-</td>
</tr>
<tr>
<td>tSPNs</td>
<td>+(&lt;10%)</td>
<td>+++(&gt;90%)</td>
<td>+(&lt;10%)</td>
<td>+(&lt;10%)</td>
<td>+(&lt;10%)</td>
</tr>
<tr>
<td>Cholinergic interneurons</td>
<td>+(&lt;20%)</td>
<td>+(&gt;80%)</td>
<td>-</td>
<td>-</td>
<td>+(&gt;80%)</td>
</tr>
<tr>
<td>PV+ interneurons</td>
<td>+(&gt;70%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPY/SOM/NO* interneurons</td>
<td>+(&lt;10%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR+ interneurons</td>
<td>+(50%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2/L3 pyramidal neurons</td>
<td>+(&lt;20%)</td>
<td>+(&lt;10%)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>L5/L6 pyramidal neurons</td>
<td>+(20%-40%)</td>
<td>+(25%)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>L2-L6 interneurons</td>
<td>+(30%-60%)*</td>
<td>+(20%)*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

This table reports semiquantitative expression levels of various dopamine receptor subtypes (+++ highest expression; +, low expression; -, mRNA not detected) and their relative cellular distribution (in parentheses) within defined cortical and striatal neuronal populations.

*For the most part, PV+ interneurons.

Note: SPNs stand for MSNs (SPNs: striatal projection neurons)

From (Tritsch and Sabatini, 2012)
transients (>100 nM) are primarily thought to activate postsynaptic low-affinity D1 receptors (Grace et al., 2007; Surmeier et al., 2011), and high-affinity D2 receptor as well by showing evoked inhibitory postsynaptic currents (Marcott et al., 2014). Meanwhile, the dopamine autoreceptors (D2S) dominant expressed in SNC and VTA dopaminergic neurons exert a strong feedback regulation of the firing activity (Ford, 2014). Indeed, D2-like receptor agonist, quinpirole suppresses the firing rate of dopaminergic neurons by activating G-protein-gated inwardly rectifying potassium (GIRK) channels while a specific D1-like receptor antagonist or agonist did not affect the firing activity (Centenze et al., 2002; Jang et al., 2011).

The change of extracellular dopamine concentration with various time scales have been measured in brain slices or behaving animals by using fast-scan cyclic voltammetry (FSCV) or microdialysis techniques (Grace, 1991; Robinson et al., 2003; Schultz, 2007b). FSCV offers real-time measurements of rapid changes in dopamine level with time courses of a few milliseconds (Venton et al., 2003; Schluter et al., 2014). This electrochemical method uses microelectrode measuring the currents related to oxidation and reduction of dopamine. The changes occur depending on reward-related stimuli (e.g. conditioned visual and olfactory stimuli predicting food or drug, unpredicted rewards) or aversive stimuli (e.g. electrical shock, air puffs and painful pinches) (Figure 24a–c) (Schultz, 1998, 2007b). Microdialysis measurements are applied to detect slow changes (10-60 min) in dopamine concentration related to moods (e.g. stress, anxiety, hunger ...), which cover a much longer period than sensory or motivational events (Figure 24d) (Schultz, 1998, 2007b; Young et al., 2005).
Figure 24. Dopamine-mediated responses, measured by different methods, with different time courses and related to different kinds of information. (a) Reward-prediction-error response. (b) Uncertainty-related response. Uncertainty is measured by statistical variance. (c) Slower depression following aversive stimuli, such as pain pinch under anesthesia. (d) Slow dopamine concentration change following a variety of behavior-related events, as measured by microdialysis in vivo. (e) Tonic dopamine concentration influence, enabling a large variety of behavioral functions of postsynaptic neurons in the striatum and cortex. These functions are deficient after dopamine-depleting lesions in patients with Parkinson’s disease and experimental animals. Abbreviation: CS, conditioned stimulus. From (Schultz, 2007b)

Dopaminergic projections and functional roles

The dopaminergic inputs profoundly modulate a number of physiological roles mainly through the nigrostriatal and mesolimbic dopaminergic systems (Nieoullon and Coquerel, 2003; Björklund and Dunnett, 2007b; Schultz, 2007b; Tritsch and Sabatini, 2012). The nigrostriatal system originates from dopaminergic neurons in SNC and projects to the dorsal striatum, preferentially participating in motor control, habit formation and goal-directed behavior (Groenewegen, 2003; Haber, 2003; DeLong and Wichmann, 2007). The mesolimbic system
originates from the VTA and projects to the nucleus accumbens, cortex, hippocampus and amygdala, mainly controls the reward-seeking behaviors (Stuber et al., 2012; Nieh et al., 2013). Here, we will focus on the nigrostriatal dopaminergic system, which is known to degenerate in Parkinson’s disease. In addition, the dysfunction of nigrostriatal dopamine system and basal ganglia lead to depression, apathy and anhedonia (Cummings, 1993; Sheline, 2003).

Classically, the instrumental performance is divided into two associative periods. During training, performance is goal-directed and essentially controlled by an action-outcome association. This behavior (e.g., lever press or chain pull) is flexible and sensitive to outcome value (e.g., pellets or sucrose) and contingency. After extended training, the performance progressively shifts from a goal-directed action to an habit-driven action by a stimulus-response association, and the behavior becomes inflexible and directly based on experience (Dickinson, 1985; Dickinson and Balleine, 1995). The conventional idea is that the dorsomedial striatum and its dopaminergic afferents are implicated specifically in the goal-directed instrumental learning, while the dorsolateral striatum and its dopaminergic inputs are involved in the habitual or reflexive control (Packard and Knowlton, 2002; Yin et al., 2008). Such model was confirmed by the study of Faure et al., (2005), in which the habit formation in instrumental learning during extended training was disrupted after local dopamine depletion in the lateral striatum. Moreover, the responses of dopaminergic neuron appear to develop and vary during different stages of learning. Precisely, during initial learning, dopaminergic neurons show increased responses to primary reward, which are progressively transferred to the reward predicting stimulus as learning increases (Ljungberg et al., 1992). During a transient learning period, both reward and stimuli are able to activate dopaminergic neurons. However, when learning is complete, neurons become activated only by reward-predicting stimuli but not rewards themselves (Mirenowicz and Schultz, 1994).

In last several years, increasing evidences proved the critical role of nigrostriatal dopamine transmission in reward and aversion, which was originally considered to restrict to mesolimbic system (Schultz, 2007b; Wise, 2009; Ilango et al., 2014). Since there is no clear anatomical boundary between the SNC and VTA dopamine systems, the anterograde and retrograde tracing
studies showed that the dopaminergic cells have overlapping of projection fields from two of these regions. Thus it is no longer possible to think of the simple dichotomy between a nigrostriatal motor system and a mesolimbic reward and motivational system (Wise, 2009).

Compared to the phasic bursts of dopaminergic neuron due to prediction and reward, less is known about the importance of phasic pauses in spiking activity in response to negative reward prediction errors. It is considered that bursts and pauses cause different patterns of dopamine release and influence downstream structures through distinct mechanisms. In dorsal striatum, the D1-MSNss belonging to the direct pathway, which are activated by high level of dopamine released by dopaminergic neuron bursts, facilitate the selection of high-value movements (Figure 25A). Whereas, the D2R-MSNss belonging to the indirect pathway, which are activated by low concentration of dopamine resulted from dopaminergic neuron pauses, suppress low-value movements (Figure 25B) (Frank, 2005; Bromberg-Martin, 2010; Hikida et al., 2010; Kravitz et al., 2012).

**Figure 25. Dopamine control of positive and negative motivation in the dorsal striatum**

(A) If an action is followed by a new situation, which displays a better value than predicted, dopaminergic neurons fire a burst of spikes. This is thought to promote D1 receptor activation on direct pathway neurons, favoring immediate action as well as reinforcing corticostratal synapses to promote selection of that action. (B) If an action is followed by a new situation that is worse than predicted, dopaminergic neurons pause their spiking activity. This is thought to activate D2 receptors on indirect
pathway neurons, promoting suppression of immediate action as well as reinforcing corticostriatal synapses to promote suppression of that action. From (Bromberg-Martin, 2010)

In addition, recent empirical evidences from human or non-human primates demonstrated that dopamine controls the striatum for high-level cognition processes, such as working memory, complex rule learning and attention switching (Clatworthy et al., 2009; Coolls, 2011). Indeed, high-level cognitive deficits occur in late stage of Parkinson’s disease and known as parkinsonian dementia (Pagonabarraga and Kulisevsky, 2012; Gratwicke et al., 2015). The mechanism by which dopamine affects cognition is not quite clear yet but a strong hypothesis would be that it possibly involved the modulation of the fronto-striatal circuitry.

5.3 Dopamine modulates corticostriatal synaptic plasticity

Corticostriatal synaptic plasticity is essential in the regulation of learning and memory for sensorimotor and cognitive associations (Graybiel et al., 1994; Wickens et al., 2003; Costa et al., 2004; Yin et al., 2009b; Koralek et al., 2012). The activation of dopamine receptors plays a central role in corticostriatal plasticity, involving both structural plasticity (Day et al., 2006; Gerfen, 2006; Yagishita et al., 2014) and synaptic plasticity (LTP and LTD induced by HFS or STDP protocol) (Calabresi et al., 2007; Cerovic et al., 2013). Furthermore, the dopamine-mediated neurological disorders such as Parkinson disease impair severely corticostriatal plasticity (Calabresi et al., 1996; Kreitzer and Malenka, 2008; Shepherd, 2013).

To investigate the role of dopamine in corticostriatal plasticity, several classical methods are mainly used, such as application of dopamine receptor agonists or exogenous dopamine to mimic enhancement of dopamine release, blockade of the dopamine effect with antagonists or receptor knock-out to test the involvement of endogenous dopamine, and depletion dopamine in some animal models for various diseases. The effects of dopamine on presynaptic and postsynaptic activity have been extensively studied by different groups using in vivo or in vitro electrophysiological approaches (Kreitzer and Malenka, 2008; Di Filippo et al., 2009; Gerfen and Surmeier, 2011).
LTD is described to be the major form of plasticity expressed at cortico striatal synapses (Calabresi et al., 1996; Di Filippo et al., 2009). Indeed, pioneering studies in vitro first described the expression of LTD (Calabresi et al., 1992b), then subsequent studies have reported that LTD is experimentally "easier" to induce with either high-frequency or low frequency stimulation (Loving er et al., 1993; Ronesi and Loving er, 2005). Whereas, cortico striatal LTP was observed in Mg^{2+}-free medium, which indicated the involvement NMDA receptors activation (Calabresi et al., 1992c; Kerr and Wickens, 2001) and has been proven afterwards (Pawlak and Kerr, 2008; Shen et al., 2008; Fino et al., 2010). However, it was demonstrated the occurrence of both LTP and LTD at the same synapses and in the same experimental conditions by HFS vs LFS as well as with STDP paradigm (Fino et al., 2005). This finding reconciles the cortico striatal plasticity with the general features of plasticity observed in the central nervous system. Moreover, the bidirectional plasticity underlies the existence of efficient homeostatic cortico striatal plasticity.

In agreement with the early studies in vitro, the in vivo findings also showed that LTD occurs in the cortico striatal pathway by stimulation of the contralateral cerebral cortex but is reversed into LTP when receiving dopamine inputs from the ipsilateral SNC (Reynolds and Wickens, 2000b). In vivo intracellular recording studies showed that both high-frequency and low frequency stimulation in the ipsilateral cortex induced LTP in MSN (Charpier and Deniau, 1997; Charpier et al., 1999).

The role of dopamine in regulating cortico striatal synaptic plasticity has been widely studied. However, previous studies had different views about how dopamine works. Indeed, the main debate of dopamine receptor and eCB-LTD is that Kreitzer and Malenka published that eCB-LTD is restricted to D2R-MSNs (Kreitzer and Malenka, 2007a) while Calabresi, Surmeier and Loving er's labs showed LTD on both D1R-MSNs and D2R-MSNs (Calabresi et al., 1992b; Wang et al., 2006). This discrepancy may due to the different experimental protocols (i.e. cortex versus corpus callosum) that could lead to a spillover of eCBs from indirect-pathway postsynaptic neurons onto direct-pathway presynaptic terminals. Or it could be explained by the theory that eCB-LTD is mediated by D2 receptor on cholinergic interneurons (Wang et al.,
2006) (Figure 26). In addition, how D2 receptor antagonist affects the eCB-LTD also remains under debate. Early studies showed that D2 receptor was necessary for eCB-LTD expression thank to D2 receptor antagonist or D2 receptor knockout mice (Calabresi et al., 1992b, 1997). Other evidences proposed that D2 receptor modulates, but does not control plasticity by showing that D2 receptor agonist enhanced the eCB-LTD while D2 receptors could be by-passed if L-type calcium channel was activated (Kreitzer and Malenka, 2005; Adermark and Lovingier, 2007).

Using STDP, a synaptic Hebbian learning rule viewed as the "elementary" brick of plasticity (Feldman, 2012), studies demonstrates the involvement of dopamine in modulation of corticostriatal STDP (Pawlak and Kerr, 2008; Shen et al., 2008). Shen et al., (2008) observed that eCB-tLTD was induced at glutamatergic synapses onto indirect pathway D2R-MSNs, but not direct pathway D1R-MSNs. They showed that a balance between the adenosine A2A receptors and the D2 receptor governed the eCB-tLTD induction. Namely, D2 receptors blockade abolishes tLTD, but D2 receptors blockade combined with A2A activation induced tLTP. A2A receptors, which are coupled to the same second messenger cascades than D1 receptors, are robustly and selectively expressed on D2R-MSNs. Moreover, a robust NMDA dependent tLTP was observed on both D1R- and D2R-MSNs with a Hebbian polarity. The tLTP in D1-MSNs was blocked by D1 receptors antagonist, while tLTP in D2R-MSNs was disrupted by A2A receptor antagonists. Thus, D2 receptor activation promotes tLTD, while D1 and A2A receptor activation promotes tLTP. In another STDP study, Pawlak and Kerr (2008) demonstrated that D1 receptors are necessary for the induction of both tLTP and tLTD. However, D2 receptor modulates the initial phase of STDP without having an effect on the final magnitude of plasticity.

Corticostriatal synaptic plasticity are strongly implicated in dopamine related pathology (Pisani et al., 2005), such as Parkinson's disease and L-DOPA induced dyskinesia, since both LTP and LTD are impaired in dopamine depleted rodents (Calabresi et al., 1992b; Centonze et al., 1999; Kreitzer and Malenka, 2007a). Proper plasticity can be restored in non-dyskinetic parkinsonian rats after L-DOPA treatment (Picconi et al., 2003, 2008, 2011). These results
again suggest that dopaminergic signaling plays a pivotal role in the corticostriatal synaptic plasticity.

**Figure 26. Dopamine (DA) receptor interaction in the induction of corticostriatal LTD.** D1 receptors are found predominantly in the striatonigral neurons of the 'direct pathway', whereas D2 receptors are mainly expressed by the striatopallidal neurons of the 'indirect pathway'. Because the LTD induction requires the concomitant activation of D1 and D2 receptors, this form of synaptic plasticity should be inducible in neurons from only one of the two projection systems of the striatum. However, it is possible to hypothesize that in striatonigral neurons the activation of the D1 receptors modulates the cAMP–DARPP32–PP1 pathway, whereas the concomitant activation of D2 receptors on the cholinergic interneuron would be required to disinhibit Cav1.3 Ca\(^{2+}\) channels through the lowering of M1 receptor tone. By contrast, in D2R-expressing striatopallidal neurons the activation of D1 receptors would be required to enhance NO release, which, in turn, participates in the induction phase of LTD through the cGMP pathway. Abbreviations: AC, adenylyl cyclase; Ach, acetylcholine; GP, globus pallidus; PKG, protein kinase G; sGC, soluble guanylyl cyclase. From (Calabresi et al., 2007)

Characterization of the physiological and pathological action of dopamine in the modulation of long-term striatal synaptic changes is complex not only because of the
differential distribution of D1 and D2 receptors but also of the other neurotransmitters controlled by dopamine. In particular, dopamine influences the modulatory action exerted by cholinergic interneurons, NOS-positive interneurons and the eCB system (Figure 26) (Calabresi et al., 2007). The release of acetylcholine and nitric oxide controlled by dopamine receptors on striatal interneurons (see section 5.2) have been described to exert a feedforward control on the corticostratal synaptic plasticity (Calabresi et al., 1999; Wang et al., 2006). Dopamine activation has been linked to eCB release in the striatum. Indeed, in response to a local administration of the D2-like receptor agonist quinpirole, a dramatic increase (> 8 fold) of eCB (anandamide but not 2-AG) occurred, which could be prevented by D2-like receptor antagonist (Giuffrida et al., 1999). Such neurochemical result unveil an essential role of D2 receptor for eliciting eCB-dependent plasticity.

Although a huge number of studies aimed at determining the dopaminergic control of corticostratal synaptic plasticity (mainly using HFS protocol), many controversial results exist (see Table 4) due to the different experimental conditions (ex vivo preparations, intracellular and extracellular solutions, age of the animal, brain section, rats versus mice, genetic background, cell conditioning protocols, blockade of the GABA receptor or not, ...) and complex location of dopamine receptors. It is fair to say that the dopaminergic control of plasticity is not yet fully understood. Thus, dopaminergic modulation of corticostratal information processing, including plasticity, remains to be investigated and better characterized.
<table>
<thead>
<tr>
<th>Reference</th>
<th>protocol</th>
<th>supplementary induction schematic</th>
<th>drug and plasticity</th>
<th>slice</th>
<th>brain region</th>
<th>animal</th>
<th>block GABA?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calabresi et al., 1992</td>
<td>stim in cortex or corpus callosum three trains (3 sec duration, 100 Hz frequency, at 20 sec intervals)</td>
<td>during stim, the intensity was increased to suprathreshold of postsynaptic cell</td>
<td>Control: LTD Control + APV: LTD Antagonist of glutamate metabotropic receptor: ns no postsynaptic depolarization: ns D1R or D2R antagonist: ns 6OHD A: ns 6OHD A + DA: LTD 6OHD A + D1R agonist: ns 6OHD A + D2R agonist: ns 6OHA + D1R agonist + D2R agonist LTD</td>
<td>Coronal corticostriatal slices</td>
<td>the neostriatum and the neocortex</td>
<td>Wistar rat (150–200 g)</td>
<td>most condition: no even with bicuculline: still has LTD in control</td>
</tr>
<tr>
<td>Wickens et al., 1996</td>
<td>stim: deeper layers of the cortex and adjacent white matter HFS in cortex (six trains of 20 pulses at 100 Hz) to coincide with injection of depolarizing current into MSN (220 ms pulse, adjusted to ensure action potential firing in response to cortical stim), so it 120 post at high frequency</td>
<td>(Dopamine was applied by pressure ejection to coincide with experimentally induced presynaptic (cortical) and postsynaptic (striatal) conjunction of activity.)</td>
<td>Control: LTD 5 mM DA: LTD Parahorlontal slices</td>
<td>cortex, neostriatum, and the connecting fibres</td>
<td>Male Wistar rats (150–200 g)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Calabresi et al., 1997</td>
<td>stim in cortex or corpus callosum three trains (3 sec duration, 100 Hz frequency, at 20 sec intervals)</td>
<td>during stim, the intensity was increased to suprathreshold of postsynaptic cell</td>
<td>WT: LTD D2 knock out mice: LTD D2 knock out mice + APV: ns WT (Mg-free medium): LTD D2 knock out mice (Mg-free medium): LTD</td>
<td>Coronal corticostriatal slices</td>
<td>neostriatum and neocortex</td>
<td>D2 knockout mice</td>
<td>No</td>
</tr>
<tr>
<td>Reynolds and Wickens, 2000</td>
<td>stim contralateral cerebral cortex and ipsilateral SNC. Plasticity-inducing stimulation was HFS-Cx (six trains of 20 pulses at 100 Hz, 10 s intertrain interval) paired with a suprathreshold depolarizing current pulse and LFS of the SNC (six trains of 10 pulses at 20 Hz, the HFS protocol is similar to the one used in vitro. DA release was evoked by substantia SNC</td>
<td>HFS-Cx: LTD HFS-Cx + LFS-SN: LTD HFS-Cx + LFS-SN (AMPT): LTD</td>
<td>in vivo</td>
<td>medial agranular cortical field and SNc</td>
<td>Urethane-anaesthetized Wistar rats (230 – 410 g) DA was depleted by α-methyl para-tyrosine (AMPT) 2.5 h</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Kerr and Wickens, 2001</td>
<td>stimulation of the deeper layers of the cortex and adjacent white matter HFS of the cortex (trains of 50 pulses at 100 Hz repeated 6 times at 10 s intervals) was paired with depolarization of the postsynaptic neuron using an intracellular current pulse. similar to Wickens 1996, just more pulses</td>
<td></td>
<td></td>
<td>cortex, neostriatum, and corticostriatal connecting fibers</td>
<td>Male Wistar rats (190–240 g)</td>
<td>Dopamine-depleted animals injected with alpha-methyl para-tyrosine (AMPT) 2.5 h before slice</td>
<td>No</td>
</tr>
<tr>
<td>Stimulus Parameters</td>
<td>Protocol Details</td>
<td>Cell Type</td>
<td>Temperature</td>
<td>Additional Details</td>
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<tr>
<td>100 Hz stimulation</td>
<td>100 Hz stimulation for 1 s paired with depolarization to 0 mV (repeated four times at 10 s intervals)</td>
<td>MSN</td>
<td>30–32°C</td>
<td>Sodium channel activator FPL</td>
<td>Kreitzer and Malenka, 2005</td>
<td></td>
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<tr>
<td>25 Hz stimulation</td>
<td>25 Hz stimulation in 30–32°C</td>
<td>MSN</td>
<td>30–32°C</td>
<td>Sodium channel activator FPL</td>
<td>Wang et al., 2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 Hz stimulation</td>
<td>100 Hz stimulation in 30–32°C</td>
<td>D2R-MSN</td>
<td>30–32°C</td>
<td>sodium channel activator FPL</td>
<td>Kreitzer and Malenka, 2007</td>
<td></td>
<td></td>
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<tr>
<td>Paired stimuli</td>
<td>Paired stimuli with a 50 ms interstimulus interval every 20 s in the overlying white matter</td>
<td>D1R-MSN</td>
<td>31ºC–33ºC</td>
<td>Picrotoxin (50 µM)</td>
<td>Adermark and Lovinger, 2007</td>
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</tr>
</tbody>
</table>

**Control:**
- LTD
- Control (30–32°C): LTD
- AM251: ns
- AP5: LTD
- mGluR antagonist LY341495: ns
- D2R antagonist Sulpiride: ns

**Sodium channel activator FPL**
- L-type calcium channel activator FPL (without HFS): LTD
- FPL with HFS: ns
- FPL + D2R antagonist (sulpiride): LTD
- D1 GF+FPL: LTD
- D2 GF+FPL: LTD

**Depolarization of the MSN to 0 mV**
- Depolarization of the MSN to 0 mV

**Stimulus Intensity**
- During LTD induction, the stimulus intensity was increased to mimic the large synchronous cortical input received in vivo.

**Temperature**
- All temperatures at which glutamate uptake is significantly reduced.
### Glutamatergic Afferent Fibers

Glutamatergic afferent fibers were stimulated with a small pipette close (~100 μm) to the soma of an identified MSN. Subthreshold synaptic stimulation paired with bAPs at theta frequency (5 Hz). These protocols consisted of 10–15 trains of five bursts repeated at 0.1 Hz.

### Control Slices

- **Prepo**
  - D2R-MSN (prepost)
  - Control: LTD (NMBA dependent) +D2R antagonist quinpirole: LTD
  - D2R antagonist quinpirole + A2A agonist: LTD

### Postpre

- D1R-MSN (prepost)
  - Control: LTD (NMBA dependent) +D1R antagonist SCH23390: LTD
  - D1R antagonist SCH23390 + AM251: ns

### Prepo

- D2R-MSN (prepost)
  - 6OHDA: LTD (control is LTD)
  - Reserpine + quinpirole (E2R agonist restricted LTD)

### Postpre

- D1R-MSN (prepost)
  - 6OHDA: LTD (control is LTD)
  - this LTD is eCB-dependent

### Parahorizonal Corticostriatal Slices

- Control: LTD (earlier)
  - D1R antagonist SCH23390: ns
  - D2R antagonist Sulpiride: LTD (delayed)
  - +AP5: ns

### Dorsolateral Striatum

- Control: LTD (earlier)
  - D1R antagonist SCH23390: ns
  - D2R antagonist Sulpiride: LTD (delayed)
  - +AP5: ns
5.4 Dopamine and basal ganglia pathophysiology

Dopamine plays a crucial role in the regulation of motor control, goal-directed behaviors and habit formation. Dysfunctions in dopaminergic neurotransmission lead to a wide range of pathological conditions such as Parkinson's disease, dystonia, addiction, obsessive compulsive disorders, schizophrenia and depression. Here, we are focusing on the dopamine highly related disease, the Parkinson's disease, which is linked to a severe dopaminergic cell loss in SNc (Surmeier et al., 2014). Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. It affects 1% of the population over the age of 65 years and more than 4% of the population by the age of 85 years (Bekris et al., 2010). Parkinson's disease is clinically characterized by the progressive impairment of several motor and cognitive functions (Lees et al., 2009).

**Parkinson's disease**

Many studies attempt to explain the etiopathology of Parkinson's disease. The canonical model relies on the imbalance of direct and indirect pathways with opposing influences on movement (Albin et al., 1989; DeLong, 1990). According to this model, the motor dysfunction originates from an imbalance of excitatory and inhibitory pathways in basal ganglia: the direct pathway which facilitates movements become less active with dopamine loss, while the indirect pathway which inhibits movements become more active (see Figure 27). This model is widely accepted and recent *in vivo* study using optogenetics seems to confirm it (Kravitz, 2010). In their study, bilateral striatal opto-excitation of MSNs belonging to the indirect pathway elicited a "parkinsonian state": showing increased freezing, bradykinesia, and decreased locomotor initiations. In contrast, opto-activation of MSNs belonging to the direct pathway decreased frequency of freezing and increased locomotion. In the 6-OHDA model of Parkinson's disease, direct pathway opto-activation completely rescued parkinsonian motor symptoms, such as deficits in locomotor initiation, bradykinesia, and freezing (Kravitz, 2010). Numerous electrophysiological studies targeting basal ganglia (mainly the striatum) have investigated the deficient functions in Parkinson's disease rodent models, showing significant
changes in neuronal activity compared to physiological condition. For instance, the intrinsic excitability of D2R-MSNs is down-regulated 3-4 weeks after dopamine depletion (Chan et al., 2012). mEPSC frequency recorded in MSNs was elevated in 6-OHDA-lesioned rats, pointing an impact in the striatal glutamatergic transmission (Dreyer et al., 2010). Moreover, both LTP and LTD are impaired in dopamine-depleted rodents (Calabresi et al., 1992b; Centonze et al., 1999; Kreitzer and Malenka, 2007a). Some changes could also be related to the structural adaptations triggered by dopamine depletion in Parkinson's disease model. Multiphoton imaging showed a profound loss of spines and glutamatergic synapses on D2R-MSNs but not on neighboring D1R-MSNs following by dopamine depletion (Day et al., 2006). Furthermore, the reduction in spine density was mediated by the dysregulation of Cav1.3 L-type Ca\(^{2+}\) channels expressed in the dendritic spines. This result reinforced the idea of a strong involvement of default at the level of the indirect pathway in the expression of Parkinson disease motor symptoms.

![Schematic representation of the direct/indirect pathway classical model in the physiological conditions and in Parkinson's disease](image)

Figure 27. Schematic representation of the direct/indirect pathway classical model in the physiological conditions and in Parkinson's disease. (a) In the physiological conditions, dopamine arising from the SNC is thought to activate D1R-MSNs of the direct pathway (red lines) and to inhibit D2R-MSNs of the indirect pathway (blue lines). The output nuclei GPi and SNr project to the thalamus, which in turn sends efferents that complete the cortico-basal ganglia-thalamo-cortical loop. (b) In Parkinson's disease, degeneration of nigral neurons reduces dopamine receptor stimulation in striatal
The imbalance between direct and indirect pathways results into abnormal activation of output nuclei and over-inhibition of thalamic neurons projecting to the cortex. From (Calabresi et al., 2014)

However, it is fair to note that despite the surprisingly crystal-clear aforementioned results, several studies brought different views to the direct/indirect pathway model. In contrast with the classical model suggesting opposite roles of the two pathways, it has recently been shown that optogenetic activation of the striatal direct and indirect pathway MSNs produces different cellular responses in SNr neurons, but opto-stimulation of either pathway eliciting both excitations and inhibitions (Freeze et al., 2013). Moreover, in another recent work, Cui et al. (2013) developed an innovative in vivo optogenetic imaging approach, which ensures the optical monitoring of the activity of specific cells (MSNs belonging to direct or indirect pathway). Taking advantage of this advanced technique, they presented the first evidence that MSNs of the direct and indirect pathways are concurrently activated when animals initiated actions (Cui et al., 2013). This study challenged the classic view of the basal ganglia function and provided an alternative view for understanding the origin of motor symptoms in Parkinson’s disease. It suggests that these two pathways do not always have opposing effects on movement but also act in a coordinated way during some behavior (e.g. action selection).

Animal model of Parkinson’s diseases

6-Hydroxydopamine (6-OHDA)

Parkinson’s disease is characterized by the severe loss of the dopaminergic neurons in the SNc, leading a profound loss of dopamine in the striatum (Dauer and Przedborski, 2003). One of the classical animal models for Parkinson’s disease is the neurotoxin-based models produced by 6-OHDA administration, which was introduced more than 45 years ago (Ungerstedt, 1968). Because 6-OHDA cannot cross the blood-brain barrier, dopamine depletion is achieved by local stereotaxic injection into the different sites of the nigrostriatal dopaminergic pathway: substantia nigra (SN), striatum or the medial forebrain bundle (MFB).
Compared to rats, mice have the advantage of more readily achievable genetic modifications. Recent works present the effects of unilateral 6-OHDA lesions in mice and performed simple motor behavior tasks (eg: abnormal involuntary movements, accelerating rotarod, balance beam) or complex behavior tests (eg: choice reaction time task), found that the mice with complete MFB lesions displayed a greater deficit than mice with lesions in the substantia nigra (Francardo et al., 2011; Heuer et al., 2012, 2013; Smith et al., 2012). Typically, a unilateral 6-OHDA injection is frequently used because the bilateral injection leads to severe adipsia, aphagia, and even death. One attractive feature of the unilateral 6-OHDA model is that each animal can serve as its own control as there is an unlesioned hemisphere (Blesa et al., 2012). The complete unilateral lesion gives rise to the turning behavior to amphetamine or apomorphine, which could be used to test the efficacy of new therapies for Parkinson's disease. The drug-free sensorimotor behavior also show cardinal symptoms of Parkinson's disease. Thus, this rodent model has been extensively used to investigate the behavioral, biochemical, and effects of the lack of dopamine in the Parkinson's disease. However, it is still not clear whether the mechanism by which 6-OHDA kills dopaminergic neurons shares similar molecular features with Parkinson's disease.

1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)

Like 6-OHDA, MPTP is a selective catecholaminergic neurotoxin, which induces dopamine degeneration in various animal species. As rats were found to be relatively resistant to this neurotoxin, mice became the most popular species for MPTP model besides monkey. Unlike 6-OHDA, MPTP is highly lipophilic and crosses the blood-brain barrier. The most common procedure is systemic injection (subcutaneous, intravenous) (Przedborski et al., 2001). Following the treatment, MPTP enters astrocytes in the brain and is converted into metabolite phenylpyridium ion (MPP+) by the monoamine oxidase-B (MAO-B). Once released from the astrocytes, MPP+ is transported to presynaptic dopaminergic nerve terminals via the dopamine transporter (DAT). Consequently, MPTP-induced toxicity to dopaminergic cell is dependent on the levels of DAT expressed (Bezard et al., 1999). Indeed, the number of dopaminergic neurons of the SNC was not affected by MPTP in the
homozygote DAT knockout (DAT−/−) mice, whereas a decreased loss of dopaminergic neurons was induced by MPTP in heterozygotes (DAT+/−) mice (Bezard et al., 1999). Currently, the MPTP model is mainly used in the study of dopaminergic neuronal death process in Parkinson's disease, since MPTP causes a profound loss of dopamine in SNc and striatum but not in ventral tegmental area, which is similar to that in human pathology. However, Lewy body formation, the typical neuropathologic features of Parkinson's disease, is lacking in the MPTP or 6-OHDA model.

Other models

Paraquat (N,N'-dimethyl-4-4'-bypriridinium) and rotenone are the other two neurotoxic models that produce oxidative stress and cause irreversible cell death in dopaminergic neuronal populations, but with possible ability to induce Lewy bodies (Blesa and Przedborski, 2014). Recently, several genetic mutations (α-synuclein, LRKK2, PINK1, parkin, DJ-1, ATP13A2) have been identified and used to develop genetic models of Parkinson's disease (Dawson et al., 2010; Delhay and Bezard, 2011). Importantly, only about 5% of Parkinson's disease cases are inherited, whereas the vast majority of cases have no apparent genetic linkage, and arise from unknown origins (Dauer and Przedborski, 2003). Moreover, almost all of the genetic models failed to induce significant dopaminergic neurons degeneration and the behavioral symptoms vary upon genetic background. These models are mainly used to investigate the mechanisms underlying the genetic forms of Parkinson's disease, instead of the pathological and behavioral phenotypes. Thus, the selection of a particular animal model is important for the specific goals of the different experiments.

Treatments for Parkinson’s disease

Currently the diagnosis of Parkinson's disease is based on clinical criteria of several cardinal motor signs of the disease (such as bradykinesia, rest tremor, rigidity) due to the lack of specific diagnostic biomarkers. There is still no cure for Parkinson’s disease, but the existing therapies substantially provide relief of motor symptoms mainly through medications
to increase brain's supply of dopamine or by surgical methods such as deep brain stimulation, subthalamotomy and pallidotomy (Table 5) (Lees et al., 2009; Obeso et al., 2010).

### CURRENT SYMPTOMATIC THERAPIES

<table>
<thead>
<tr>
<th>Oral medications</th>
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<tbody>
<tr>
<td>Levodopa + a dopadecarboxylase inhibitor ± a catechol-O-methyltransferase inhibitor</td>
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<tr>
<td>Dopamine agonists, including slow-release formulations such as ropinirole, pramipexole</td>
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<td>Monoamine oxidase B inhibitors: for example, selegiline, rasagiline</td>
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<td>Anticholinergics: for example, trihexyphenidyl</td>
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<td>Antiglutamatergics: for example, amantadine</td>
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<table>
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<tr>
<th>Continuous delivery therapies</th>
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<tr>
<td>Dopamine agonists: subcutaneous or intravenous, such as apomorphine and lisuride</td>
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<tr>
<td>Transdermal patch: for example, rotigotine</td>
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<tr>
<td>Intrahypodermal levodopa: for example, Duodopa</td>
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<th>Surgical therapies</th>
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<tr>
<td>Deep brain stimulation of the subthalamic nucleus, globus pallidum pars interna</td>
</tr>
<tr>
<td>Lesions: for example, subthalamotomy, pallidotomy</td>
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</table>

Table 5. Treatments for Parkinson’s disease. From (Obeso et al., 2010)

Although many progresses on the pharmacological approaches have been made the last twenty years, dopamine replacement therapy with L-DOPA is currently the most efficient therapy for Parkinson’s disease patients. This therapy relies on L-DOPA, which is a dopamine precursor, in combination with a peripheral DOPA decarboxylase inhibitor (benserazide or carbidopa). Exogenous L-DOPA could be converted to dopamine through different types of cells (mainly serotonin neurons and the surviving dopaminergic cells) and their axon terminals then resulting in the increase the dopamine level in striatum (summarized in Figure 28). However there is a severe side effect of L-DOPA treatment after the so-called « honeymoon » period (5 to 10 years of L-DOPA treatment) for which L-DOPA provides to patients deeply attenuated motor symptoms. Thanks to the availability of Parkinson’s disease models in rodents, we know that the capacity for storage and clearance of dopamine (derived from L-DOPA) is greatly impaired after a severe dopamine depletion. Consequently these results in an abnormal large increase in extracellular dopamine followed by L-DOPA intake (Abercrombie et al., 1990) and explain that the long-term administration of L-DOPA induces dyskinesia (abnormal involuntary movements) as side effects. A recent study of intrinsic and synaptic plasticity of
D1R-MSNs and D2R-MSNs in striatum of the parkinsonian and dyskinetic mice indicates that L-DOPA does not normalize the dendritic structure of striatal neurons, but produces a new aberrant striatal changes associated with dyskinesia (Fieblinger et al., 2014). Although a lot of advances have been made concerning the molecular and signaling changes induced by L-DOPA, as well as anatomo-functional organization of the basal ganglia pathways involved in dyskinesia (Cenci and Lindgren, 2007; Barroso-Chinea and Bezdard, 2010), the underlying mechanisms for L-DOPA induces dyskinesia remain elusive and need further investigation.

**Figure 28. Routes of L-DOPA uptake and conversion before (upper panel) and after (lower panel) a lesion of the nigrostriatal dopamine pathway.** Severe DA-denervating lesions greatly increase the contribution of non-nigrostriatal routes to the handling of exogenous L-DOPA. From (Cenci and Lundblad, 2006)
MATERIALS AND METHODS:

1. Experimental preparations

Animals

All experiments were performed in accordance with local animal welfare committee (Center for Interdisciplinary Research in Biology and the Université Libre de Bruxelles School of Medicine Ethical Committee) and EU guidelines (Directive 2010/63/EU). Every precaution was taken to minimize stress and the number of animals used in each series of experiments.

Sprague-Dawley rats (Charles River, L’Arbresle, France) and C57BL/6 mice (CB1R-/- and CB1R+/+, DAT-Cre, Archeorhodopsin3-GFP, Adora2a-Cre, ChAT-Cre, inducible-DTR and Drd1a-GFP) were housed in standard 12 hours light/dark cycles and food and water were available ad libitum.

Brain slice preparation

Acute rodent brain slices constituted the main experimental preparation in this PhD work. To prepare the slice, animals (rats and mice) were decapitated after anesthesia and the brains were removed rapidly from the skull, and then transferred to a vibratome (VT 1200S, Leica Microsystems, Nussloch, Germany). Brains were sliced horizontally in a ice-cold artificial cerebrospinal fluid (ACSF) (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 1 pyruvic acid bubbled with 95% CO₂/5% O₂. The slices were then transferred into the same solution at 34°C for 30min and moved to room temperature before used for electrophysiological recordings.

These horizontal slices preserve the connection between somatosensory cortex and the adjacent dorsal striatum (Fino et al., 2005). Brain slices were of thickness of 330μm for rats and
300μm for mice. Here, we used rats on postnatal days 20-25 and mice on postnatal days 17-25 except when specified (e.g., 6-OHDA rats).

2. Electrophysiological recordings

Patch-clamp whole-cell recordings were made as previously described (Fino et al., 2005, 2010; Paille et al., 2013). Borosilicate glass pipettes of 4-7MΩ resistance were filled with an intracellular solution containing in mM: 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA (adjusted to pH 7.35 with KOH). All recordings were performed at 34°C using a temperature control system (Bath-controller V, Luigs&Neumann, Ratingen, Germany) and slices were continuously superfused at 2-3 ml/min with the ACSF bubbled with 95% O₂ and 5% CO₂. Signals were amplified using EPC10-4 amplifier (HEKA Elektronik, Lambrecht, Germany). Current-clamp recordings were filtered at 2.5 kHz and sampled at 5 kHz and voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz. Acquisition of data was performed with LIH 8+8 interface (HEKA Elektronik) and Patchmaster v2x32 software (HEKA Elektronik).

Identification of the medium-sized spiny neurons in striatum

Slices were visualized on a microscope (BX51WI, Olympus, Rungis, France) using a 5x objective for the placement of the stimulating electrode and a 40x water-immersion objective for localizing cells for whole-cell recordings.

Striatal output neurons MSNs, the principal neurons in the striatum, display a hyperpolarized resting membrane potential (~81.7±0.6 mV, n=25), a low input resistance at rest (95±6MΩ, n=25), an inward rectification of I-V relationship and a long depolarizing ramp to spike threshold (Figure 29). Previous studies also noted several different electrical properties of two subtypes of MSNs (the D1R- and D2R-MSNs) (Gertler et al., 2008; Valjent et al., 2009; Planert et al., 2013). For instance, D2R-MSNs were more excitable than D1R-MSNs. In our study, the subthreshold segregation of membrane responses to injection current (I-V curve)
revealed a difference in input resistance and showed that one group of MSNs spike earlier than another. It was consistent with the earlier observation, which convinced the recording of both subtypes of MSNs (Figure 29). The GABAergic interneurons including FS, nNOS and cholinergic interneurons were characterized since they display marked different morphological and electrophysiological properties (Fino et al., 2008; Fino and Venance, 2011). The astrocytes were distinguished by a linear I-V curve and an absence of action potential (Goubard et al., 2011).

Figure 29. Characteristic membrane properties and spiking pattern of MSNs. Left panel: Canonical properties of an MSN are illustrated with a hyperpolarized membrane potential (-80mV), an inward rectification and a long depolarizing ramp to the AP threshold leading to a delayed spike discharge. Raw traces show individual voltage responses to series of 500 ms current pulses from −150 pA to +190 pA with 20 pA steps (black traces) and to +80 pA above AP threshold (gray trace). Right panel: A divergence of current-voltage response (I-V curve) reveals a difference in input resistance: namely D2R-MSNs being more excitable than D1R-MSNs. Each color represents one MSN.

**Stimulation protocols**

Electrical stimulations were performed with a bipolar electrode (Phymep, Paris, France) placed in the layer 5 of the somatosensory cortex (Fino et al., 2005; Paille et al., 2013). Electrical stimulations were monophasic at constant current (ISO-Flex stimulator, AMPI, Jerusalem, Israel). Currents were adjusted to evoke 50-200 pA EPSCs of MSNs in dorsal striatum. Repetitive control stimuli were applied at 0.1Hz, a frequency for which neither short-nor long-term synaptic plasticity was observed (Goubard et al., 2011). Series resistance was
monitored and calculated from the response to a hyperpolarizing potential (-5 mV) step during each sweep throughout the experiments and a variation above 20% led to the rejection of the experiment.

Cortico-striatal short-term plasticities were evaluated by paired-pulse ratio (PPR) and the expression of depolarization-induced suppression of excitation (DSE). Cortico-striatal long-term plasticities were investigated by applying either low frequency stimulation (LFS) or spike-timing dependent plasticity (STDP) induction protocols.

a. Paired-pulse protocol.

Repetitive paired stimuli were applied at 0.1Hz. The inter-stimulus intervals (ISIs) for paired-pulse experiments were 50, 100 and 500 ms, by which MSN displayed a bidirectional short-term plasticity (Goubard et al., 2011). 40 successive EPSCs were individually measured. Paired-pulse ratio was calculated by the mean (EPSC2 amplitude/EPSC1 amplitude). Similar to cortico-striatal PPR, the cortico-cortical PPR was estimated with the same protocol, but with stimulating in the layer II/III of the somatosensory cortex and recording the pyramidal cells in the layer V.

b. Depolarization-induced suppression of excitation (DSE) protocol

For DSE induction, MSNs were depolarized from resting membrane potential to 0 mV during 10 seconds, with bath-applied carbachol (10μM) and DHPG (50μM) (Puente et al., 2011b). The magnitudes of DSE were measured as percentage of the mean amplitudes of consecutive EPSCs after depolarization (acquired between 5 s and 25 s after the stimulation protocol) relative to five EPSCs before depolarization.

c. Low-frequency stimulation (LFS) induction protocol

Long-term depression was induced with low frequency stimulation protocol consisting in 900 cortical stimulations at 1 Hz paired with postsynaptic concomitant depolarization of the
MSN during 50 ms (Fino et al., 2005). Neurons were recorded for 10 min during baseline and for 60 min after LFS protocol; long-term synaptic efficacy changes were measured from 50 min. 30 successive EPSCs (at 0.1 Hz) were individually measured and then averaged.

d. Spike-timing dependent plasticity (STDP) induction protocols

STDP protocols consisted in pairings of pre- and postsynaptic stimulations (at 1 Hz) with the two events separated by a limited temporal interval (Δt) (Figure 30). Based on our previous work, striatal bidirectional plasticity can be reliably induced within a strict time window (-25 ms<Δt<0 ms for LTP and 0 ms<Δt<+40 ms for LTD). Presynaptic stimulations corresponded to the cortical stimulations and the postsynaptic stimulation to a single action potential evoked by a depolarizing current step (30 ms duration) in MSNs. To note, this 30 ms suprathreshold depolarization was chosen to mimic corticostriatal summation of EPSPs induced by cortical activity as observed in \textit{in vivo} studies (Charpier et al., 1999). MSNs were maintained all along the STDP experiments at a constant holding membrane potential which corresponds to their initial resting membrane potential. MSNs with RMP<-70 mV were excluded. Thus, EPSCs during baseline or after STDP protocol were measured at the same membrane potential (in voltage-clamp mode); STDP pairings (performed in current-clamp mode) were conducted also at this very same holding membrane potential. Neurons were recorded for 10 min during baseline and for 60 min after STDP protocol; long-term synaptic efficacy changes were measured from 50 min. 30 successive EPSCs (at 0.1 Hz) were individually measured and then averaged.

It should be noted that STDP protocol consisting in 5-10 post-pre pairings (with a single postsynaptic spike) were sufficient to induce potent tLTP in rat while in C57BL/6 mice 15 pairings (with 2-3 postsynaptic spikes) were necessary to trigger tLTP.
Figure 30. Experimental protocol of STDP in brain slices. Pre- and post synaptic activities are evoked in several milliseconds difference. An action potential (AP) was evoked in the MSN before (termed post–pre protocol, Δt<0) or after (termed pre–post protocol, Δt>0) a cortical stimulation.

3. Chemicals

Chemicals were bath-applied or injected only in the recorded postsynaptic neuron through the patch-clamp pipette. Y-27632 dihydrochloride (10 μM; Tocris), active (S)-(−)-blebbistatin and inactive (R)-(−)-blebbistatin enantiomers (10 μM; Santa Cruz Biotechnology), Carbamoylcholine chloride (carbachol, 10 μM; Tocris), (RS)-3,5-DHPG (50 μM; Tocris), DL-2-amino-5-phosphono-pentanoic acid (DAP5, 50 μM; Tocris), 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP hydrochloride, 10 μM; Tocris), 5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6H-pyrido[2,3-b][1,4] benzodiazepine pin-6-one dihydrochloride (pirenzepine dihydrochloride, 1 μM; Sigma), R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390, 4 μM, Sigma) and (4aR-trans)-4.4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g] quinoline hydrochloride (Quinpirole, 10 μM, Tocris) were dissolved directly in the extracellular solution and bath applied. N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidine (AM251, 3 μM; Tocris), picrotoxin (50 μM; Sigma), 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyrindinedicarboxylic acid 2-methoxyethyl 1-methylethyl ester (nimodipine, 1 μM; Tocris) and (2E)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide
(AMG9810, 1 μM; Tocris) and (S-)5-aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide (sulpiride, 10 μM; Tocris) were dissolved in ethanol and then added in the external solution at a final concentration of ethanol of 0.01–0.1%. BAPTA (1 mM; Sigma) and GDP-β-S (2 mM) were dissolved directly into the intracellular solution and applied via the patch-clamp pipette. U73122 (5 μM; Sigma) was dissolved in ethanol and then added to the intracellular solution at a final concentration of ethanol of 0.033%. Tetrahydrolipstatin (THL, 10 μM; Sigma) was dissolved in DMSO and applied internally via the patch-clamp pipette at a final DMSO concentration of 0.1%. (S)-α-methyl-4-carboxyphenylglycine (MCPG, 500 μM; Tocris) was dissolved in 1.1 equiv NaOH, and then added in the external solution. N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide (capsazepine, 10 μM; Tocris) and 2-arachidonoylglycerol (2-AG, 10 and 100 μM; Tocris) were dissolved in DMSO and then added to the external solution at final DMSO concentrations of 0.0025 and 0.1%, respectively.

Note that none of the bath-applied drugs had a significant effect on basal EPSC amplitudes. And we verified that DMSO (0.1%) or ethanol (0.033%) when applied intracellularly did not alter the LTP observed in control conditions.

4. Pharmacological experiments

Bath-applied drugs: After recording of 10 min control baseline, drugs were applied in the bath. A new baseline with drugs was recorded after a time lapse of 10 min (to allow the drug to be fully perfused) for 10 min before the STDP protocol. Drugs were present until the end of the recording (except when specified).

Drugs delivered through the patch-clamp pipette: the patch-clamp pipettes were systematically tip-filled with regular intracellular solution and back-filled with drug-containing solutions. Once the cell patched, drugs were allowed to diffuse into the cell during 15 minutes before starting the baseline recording.
Local applications of 2-AG were performed through a patch-clamp pipette placed at the vicinity (50μm) of the recorded neuron and linked to a Picospritzer II system (Parker, USA), which supplies reliable pressure pulses.

5. Statistic and analysis

Experimenters were blind to the genotype of mice during electrophysiological recordings and analysis. An off-line analysis was performed using Fitmaster (Heka Elektronik) and Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA). Statistical analysis was performed using Prism 5.0 software (San Diego, CA, USA). In all cases “n” refers to the number of repetitions of an experiment (each experiment being performed on different brain slices) from single slice. The majority of results were expressed as mean±s.e.m, and statistical significance was assessed using Mann-Whitney test or the one sample t test when appropriate at the significance level (p) indicated. With exception, sEPSCs were evaluated by amplitude or inter-event interval using the Kolmogorov–Smirnov (K–S) test on their cumulative distributions. Data were analyzed with Mini Analysis (version 6.0.3; Synaptosoft, Decatur, GA).

6. Optogenetics

We bred C57BL/6 DAT-Cre+/− with C57BL/6 Arch3-GFP+/+ mice (Jackson laboratory) leading to mice that selectively expressed Archeo-rhodopsin in DAT-expressing neurons (DAT-Cre+/− : Arch3-GFP+/+ mice); DAT-Cre−/− : Arch3-GFP+/+ mice were used as a control. 585 nm yellow-green light was delivered via field illumination using a high-power LED source (pE excitation System, CoolLED, Andover, UK).

Double immunostaining for GFP and TH. Brain slices (300μm) were fixed overnight in 2% paraformaldehyde at 4°C. Non-specific binding was blocked by incubated the slices for two hour at room temperature in 10% normal goat serum (Merk-Millipore, Molsheim, France)
in 2% BSA and 1% Triton X-100 solution. After PBS washings, brain slices were incubated with a pair of primary antibodies, chicken anti-GFP (1:2000, AB13970, Abcam, Paris, France) and mouse anti-TH (1:500, MAB318, Merk-Millipore), in 0.5% Triton X-100, 1% BSA and 2.5% normal goat serum on a shaker overnight at 4°C. After PBS washings, brain slices were incubated with the secondary antibodies, goat anti-chicken Alexa (1:1000, A11039, Life Technology-Invitrogen, Villebon-sur-Yvette, France) and goat anti-mouse IgG1 (1:200, 1070-03, Southern Biotech-Clinisciences, Montrouge, France) in 0.5% Triton X-100, 1% BSA and 2.5% normal goat serum on a shaker overnight at 4°C. Brain slices were mounted using Fluoromount-G (Southern Biotech) and stored at 4°C. Photomicrographs were acquired using a Leica TCS SP5 inverted confocal laser-scanning microscope (Leica Microsystems) with a 40x oil objective and the 488 and 561nm channels.

7. Selective ablation of D2R-MSNs and cholinergic interneurons in striatum

To assess the importance of D2 receptors (D2R) on the striatopallidal MSNs, these cells were selectively ablated by Cre-mediated expression of a diphtheria toxin receptor (DTR) and the diphtheria toxin injection. The mice were generated to express Cre recombinase under the control of the Adora2a receptor (A2AR) promoter by bacterial artificial chromosomes (BAC) transgenesis. A2AR promoter was chosen because of its specifically expression in striatum only on D2R-MSNs, as previously described (Durieux et al., 2009, 2012). C57BL/6 Adora2a-Cre+/- were bred with inducible C57BL/6 DTR+/+ (iDTR+/+) mice leading to double heterozygous: mice that selectively expressed the iDTR in D2R-MSNs (Adora2a-Cre+/- : iDTR+/-, A2A-DTR+/+ mice) and Adora2a-Cre+/-. iDTR+/+ (A2A-DTR-/- mice) used as the corresponding control. Inducible ablation allows a high spatial resolution (achieved with stereotaxic injections of the diphtheria toxin) and limits developmental adaptations. Then the Adora2a-Cre+/- : iDTR+/+ mice were bred with with Drd1a-GFP mice (Gong et al., 2003) to ensure recording of D1R-MSNs. Adora2a-Cre+/- : iDTR+/+ : Drd1a-GFP+/+ mice (and their control Adora2a-Cre+/-. iDTR+/+ : Drd1a-GFP+/+) were deeply anesthetized at the age of 4 weeks, and placed on a stereotaxic apparatus. 1μl of the diphtheria toxin (Sigma-Aldrich) (diluted in PBS 0.01M to a concentration of 100pg/μl) was
slowly stereotaxically injected at 0.25μl/min with a blunt needle in four sides of the dorsal striatum with the following coordinates (atlas of Franklin and Paxinos, with bregma as references): anterior +1.2mm, lateral ±1.5mm, ventral +3.6mm; or anterior +0.5mm, lateral ±1.8mm, ventral +3.6mm. There was a complete loss of striatal A2A receptor binding from 14 days after toxin injections (Durieux et al., 2009, 2012) indicating the total ablation of D2R-MSNs without affecting the D1R-MSNs. Electrophysiological recordings were performed from 15-20 days after injection of the diphtheria toxin.

Similar method was used to construct ChAT-ablated mice. C57BL/6 ChAT-Cre+/- were bred with inducible C57BL/6 DTR+/+ (iDTR+/+) mice leading to double heterozygous: mice that selectively expressed the iDTR in cholinergic interneurons (ChAT-Cre+/+: iDTR+/+) and ChAT-Cre-/: iDTR+/ used as the corresponding control. Then they performed stereotaxic injection of the diphtheria toxin in dorsal striatum. Electrophysiological recordings were performed from 15-20 days after injection of the diphtheria toxin.

Dr Alban de Kerchove d'Exaerde (Université Libre de Bruxelles) provided all these mice and the stereotaxic injections and immunohistochemistry were performed by Bérangère Detraux (PhD student in Alban de Kerchove d'Exaerde's lab).

8. Unilaterally substantia nigra pars compacta (SNC) or medial forebrain bundle (MFB)

6-hydroxydopamine (6-OHDA)-lesioned rats and L-DOPA treatment

6-OHDA-lesioned animals were performed as previously described (Bosch et al., 2011). Sprague-Dawley rats weighing 125-150g (Charles River Laboratories, L’Arbresle, France) were anesthetized with sodium pentobarbital (30 mg/kg ip; Ceva Santé Animale, Libourne, France) supplemented by injections of ketamine (27.5 mg/kg, im; Imalgène, Merial, Lyon, France) repeated as needed. Thirty minutes before the injection of 6-OHDA (or vehicle in sham-operated animals), all animals received a bolus of desipramine dissolved in saline (25 mg/kg, ip; Sigma-Aldrich) to prevent neurotoxin-induced damage of noradrenergic neurons. Animals were fixed in a stereotaxic head frame (Kopf Instruments, Tujunga, CA, USA). Body
temperature was maintained at 36.5°C with a homeothermic blanket (Harvard Apparatus, Kent, UK). A small craniotomy was made unilaterally (left side) over the SNc (or MFB) and the overlying dura mater was removed. A single stereotaxic injection of 6-OHDA (or of vehicle in the sham-operated animals) was delivered into the SNc on one side (stereotaxic coordinates anteriority from the interaural line (A): 3.7 mm, laterality from the midline (L): 2.1 mm, depth from the cortical surface (H): -7.55 mm. And for MFB lesion, 6-OHDA was injected into one side of medial forebrain bundle (stereotaxic coordinates anteriority from the bregma (A): 4.5 mm, laterality from the midline (L): 1.2 mm, depth from the cortical surface (H): -7.9 mm, according to the stereotaxic atlas of (Paxinos and Watson, 2007). The neurotoxin 6-OHDA (hydrochloride salt; Sigma) was dissolved immediately prior use in ice-cold 0.9% w/v NaCl solution containing 0.01% w/v ascorbic acid to a final concentration of 2.5 mg/ml. Then 4.0 μl of this 6-OHDA solution (or vehicle in sham-operated animals) was injected at a rate of 16 μl/h via a steel canula (0.25 mm outside diameter) attached to a 10μl Hamilton microsyringe (Cole-Parmer, London, UK) controlled by an electrical pump (KDS100; KD Scientific, Holliston, MA). A delay of 5 min was observed between the time the canula was inserted into the SNc (or MFB) and the onset of the 6-OHDA injection, and the canula was left in place 10 min following the end of injection before removal. After surgery, rats received an intramuscular injection of gentamicin to prevent bacterial infection (3 mg/kg, im; Gentalline, Schering-Plough, Levallois-Perret, France). Sham and 6-OHDA experiments were performed on animals of similar ages.

Chronic L-DOPA treatment.

Two weeks after the 6-OHDA stereotaxic injection in SNc, rats were split into two groups which received i.p. injection twice-daily for 10 days of either L-DOPA (10 mg/kg) and benzerazide (7.5 mg/kg) or saline. The sham-operated animals were also subjected to L-DOPA or saline injections with the same schedule (Figure 31). Animals received the last injection of L-DOPA or saline 30-60 minutes before being sacrificed for ex vivo experiments.
Figure 31. Protocols of the unilateral 6-OHDA lesions (or sham) in P_{35} rats and timescale for chronic L-DOPA treatment (or saline).

9. Tyrosine hydroxylase (TH) immunostaining.

The severity of the 6-OHDA lesions was quantified after electrophysiological whole-cell recordings by striatal TH immunostaining. Brain sections were incubated in 0.1% hydrogen peroxide and 10% methanol in PBS for 15 min at room temperature prior to incubation in primary antibody. TH immunostaining was performed by first incubation of the slices in 0.1% hydrogen peroxide and 10% methanol in PBS (15 min at room temperature) and then in a 1/500 dilution of mouse anti-TH monoclonal antibody (MAB318; Merk-Millipore, Molsheim, France) overnight at 4°C. Biotin-goat anti-mouse secondary antibody (Life Technology-Invitrogen, Villebon-sur-Yvette, France) was incubated at a dilution of 1/500 for 2.4 h at room temperature and visualized using with avidin-biotin complex (ABC Elite standard, PK-4000, Vector Laboratories, Burlingame, CA, USA) before visualization with DAB detection kit (Vector Laboratories) according to the instructions of the manufacturer.
**Figure 32.** TH staining of coronal brain slice containing SNC and VTA. In SNC, the DA neurons were depleted by 6-OHDA in left side. In VTA, the DA neurons are not impaired in either side.
General introduction of the PhD results

The basal ganglia play an important role in adaptive control of behavior and procedural learning and memory (Haber, 2003; Graybiel, 2005; Yin and Knowlton, 2006; Grillner and Robertson, 2015). The corticostriatal pathway is the main input component of the basal ganglia circuits (Gerfen, 1992a). The corticostriatal synaptic plasticity is widely considered to be the main neuronal substrate of procedural learning and memory (Costa et al., 2004; Citri and Malenka, 2008; Yin et al., 2009a; Koralek et al., 2012). The activity-dependent changes in corticostriatal synaptic efficacy may be engaged in various aspects of neural functions. Notably, neurological disorders involving basal ganglia dysfunctions such as Parkinson's disease show some impairment of the corticostriatal synaptic plasticity (Calabresi et al., 1996; Mahon et al., 2004b; Shepherd, 2013).

The corticostriatal synapse is targeted by various neuromodulatory systems, particularly the endocannabinoid and dopamine systems. Endocannabinoid-mediated LTD was first demonstrated at excitatory synapse in the striatum (Gerde et al., 2002), and is now known as one of the widespread of forms of synaptic depression in the brain (Heifets and Castillo, 2009; Kano et al., 2009; Katona and Freund, 2012). The essential role of dopamine in regulating corticostriatal long-term potentiation (LTP) and long-term depression (LTD), the two main forms of synaptic plasticity (mainly induced by high-frequency stimulation, HFS), has also been widely characterized in both physiological and pathophysiological conditions (Calabresi et al., 2007; Di Filippo et al., 2009). However several controversies still remain, probably due to the different experimental conditions (brain slices orientation, composition of the intracellular solutions, inhibition or not of the GABAergic transmission, location of the electrical stimulation: cortex vs corpus callosum vs striatum itself, ...) and the influence of interneurons on MSNs (Calabresi et al., 1992b; Wang et al., 2006; Kreitzer and Malenka, 2007a).

Compared to high- or low-frequency stimulation protocols, spike-timing dependent plasticity (STDP) appears as a quite "soft" experimental protocol and constitutes a genuine
Hebbian learning synaptic rule, which make it as an appropriate condition to test involvement of dopamine in controlling learning rules. Since our first report of the existence of a bidirectional corticostriatal STDP (assessed with the canonical form of STDP: 100 pairings at 1 Hz) (Fino et al., 2005, 2009, 2010; for review see Fino and Venance, 2010 and 2011), two studies using STDP demonstrates the prominent involvement of dopamine in modulation of such plasticity induction (Pawlak and Kerr, 2008; Shen et al., 2008).

After a systematic investigation of the capabilities of the neuronal long-term coding at corticostriatal synapse, we recently demonstrated two unidirectional forms of spike-timing dependent plasticity, i.e. LTD (at 50 pairings) and LTP (at 10 pairings) of STDP. Strikingly, the LTP induced by a low number of pairings (10 post-pre pairings) is not NMDA-dependent as classically reported for LTP, but underlain by the endocannabinoid system. Thus, we unraveled a new form of synaptic plasticity at play in the striatum, which potentially could encode for the so-called fast learning or fast mapping (Schultz et al., 2003; Pasupathy and Miller, 2005; Tse et al., 2007; Quilodran et al., 2008; Ito and Doya, 2009).

This PhD work mainly aims at characterizing the endocannabinoid-dependent LTD and LTP at corticostriatal synapses. We first determined that the classical eCB-LTD (induced with LFS cell conditioning paradigm) relies on the presynaptic acto-myosin complex. This observation brings a new vision on presynaptic mechanisms which could drive the long-term changes in the synaptic weight. We then identified a new form of plasticity, namely a eCB-tLTP induced with a very low number of pairings (5 to 10 pairings). After, the extensive characterization of eCB-mediated plasticity, we investigated the dopaminergic regulation of the eCB-tLTP in physiological and pathological conditions. To achieve this goal, we combined ex vivo multi-patch-clamp recordings together with specific pharmacology, optogenetics, genetically-modified mice and the 6-OHDA-lesioned rat model for Parkinson's disease. The results showed dependency of eCB-tLTP on presynaptic D2 receptors. Since enriched environment was shown to have beneficial effects against 6-OHDA toxicity and improves motor behavior. We further examined its effect on the STDP in the dopamine-deprived rats. In
this study, we demonstrated that enriched environment *per se* was able to significantly restore the cortico-striatal eCB-tLTP following unilateral injection of 6-OHDA.

We organized our results into two main parts:

**Result part (I): The role of Type-1 cannabinoid receptor (CB1R) and endocannabinoids (eCBs) in corticostriatal plasticity (Articles 1,2)**

First, in the collaborative work with Zsolt LENKEI (ESPCI, Paris, France), we reported that inhibiting either contractile non-muscle myosin II directly, by its specific ATPase blocker Blebbistatin, or indirectly through its upstream activator Rho-associated kinase (ROCK), inhibit the LFS-induced and eCB-dependent LTD (Figure 33), but not the short-term form of eCB-induced synaptic depression at excitatory corticostriatal synapses. Thus, we reported a novel molecular mechanism whereby cannabinoid-induced presynaptic actomyosin contraction would induce the retraction of vesicles from the presynaptic release sites, and would explain the synaptic depression. (*Article 1. Presynaptic actomyosin contraction mediates long-term synaptic plasticity.* Maureen H. McFadden#, Hao Xu#, Yihui Cui, Rebecca A. Piskorowski, Laurent Venance*, Vivien Chevaleyre4* and Zsolt Lenkei*. #:co-first authors, *:co-senior authors)
Second, we focused on endoannabinoid-mediated STDP at corticostriatal synapse and found that a very low number of spikes (~5-10 pairings at 1 Hz) are sufficient to induce a potent spike-timing dependent LTP (tLTP). This form of STDP involves the endocannabinoid system (eCB-tLTP). This result demonstrated that eCBs are not restricted to depression but are also capable of potentiation. Then, we uncovered that the eCB-tLTP depends on postsynaptic eCBs (2-AG and anandamide) synthesis and release, on the activation of CB1R and transient receptor potential vaniloid type-1 (TRPV1). Such findings expand our view of distinct coincidence detectors involved in STDP induction at corticostriatal synapses (Figure 34). Furthermore, we showed that eCB-tLTP occurs in both striatopallidal and striatonigral MSNs and is dopamine dependent. (Article 2. Endocannabinoids mediate bidirectional striatal spike-timing...
dependent plasticity. Yihui Cui, Vincent Paille&, Hao Xu&, Stéphane Genet, Bruno Delord, Elodie Fino, Hugues Berry# and Laurent Venancé#. &: co-second authors #: co-last authors. J Physiol. 2015)

Figure 34. Schematic representation of the different pathways involved in the induction of the corticostral NMAD-dependent tLTP (red) and eCB-dependent tLTD or tLTP (blue). Separate coincidence detectors for tLTP and tLTD are represented. tLTP induced by 100 post-pre pairings relies on postsynaptic NMDARs and VSCCs activation, while tLTD induced by 100 pre-post pairings and tLTP induced by 10 post-pre pairings involves group-I mGluRs, M1Rs, VSCCs, PLCβ, IP3Rs and retrograde endocannabinoid signalling. Modified from (Fino et al., 2010)
Result part (II): The role of dopamine in corticostriatal endocannabinoid-mediated STDP (Articles 3 and preliminary results)

We then demonstrated the role of dopamine in the induction phase of eCB-tLTP by an opto-inhibition of dopamine release during the STDP pairings. Based on pharmacological and genetical demonstration, we identified the involvement of the presynaptic D2 dopamine receptors in eCB-tLTP. More precisely, these D2 receptors are located onto glutamatergic cortical afferents and co-localized with CB1R. Given the relationship between dopamine level and Parkinson’s disease, we asked whether this form of plasticity would be altered in a rat model of Parkinson’s disease (dopamine depletion by the neurotoxic lesion with 6-OHDA injection in SNc). We observed that eCB-tLTP was impaired in dopamine depleted rat and could be rescued with a chronic treatment with L-DOPA or an acute application of D2 receptor agonist. (Article 3. Endocannabinoid-dopamine interactions mediate striatal tLTP that is impaired in Parkinson’s disease rodent model. Hao Xu, Sylvie Perez, Yihui Cui, Bérangère Detraux, Bertrand Degos, Alban de Kerchove d’Exaerde, Hugues Berry# and Laurent Venance#). #: corresponding author)

Finally, preliminary results show that enriched environment rescues eCB-tLTP, but not eCB-tLTD in DA-deprived rats. Moreover, this effect is prominent and long-lasting though the mechanism is not clear and the different effects onto the bidirectional eCB-dependent plasticity remain to be fully elucidated. (preliminary results. Enriched environment impacts on bidirectional endocannabinoid-dependent plasticity at corticostriatal synapse. Hao Xu, Sylvie Perez, Bertrand Degos, Laurent Venance)

In summary, the results presented in this thesis manuscript confirm and further extend a new form of synaptic plasticity, i.e. the eCB-tLTP, demonstrate that this eCB-tLTP is dopamine-mediated and showed this plasticity is impaired in Parkinson’s disease rat model and can be rescued by the chronic L-DOPA treatment. It considerably extends their spectrum of action in physiological and pathophysiological plasticity processes. Furthermore, the new
finding of enriched environment's therapeutical effect on long-term plasticity could provide a potential benefit for the symptomatic treatment of Parkinson’s disease.

Annex: Short-term synaptic plasticity at cortico-striatal and cortical synapses in a new transgenic mice model of Huntington’s disease (Article 4)

Huntington’s disease is a dominant inherited neurodegenerative disorder tightly related to the basal ganglia, which leads to progressive cell death in the striatum and cortex. In collaboration with Sandrine Humbert’s team (Institut des Neurosciences de Grenoble), we observed that the spontaneous postsynaptic current and paired-pulse ratio of neurons in cortex and striatum were markedly changed in huntingtin (HTT) protein depleted mutant mice. Therefore, the cortical and cortico-striatal activities were deeply modified in this new animal model for Huntington’s disease.

Result part (I):

The role of type-1 cannabinoid receptor (CB1R) and endocannabinoids (eCBs) in corticostriatal plasticity

(Articles 1,2)
Actomyosin contraction mediates presynaptic long-term synaptic plasticity

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Abstract:
Synaptic plasticity is critical for brain function, but presynaptic mechanisms of functional plasticity remain poorly-known. Here, by building on our recent results, which showed rapid cannabinoid-induced remodeling of developing neurons, we first show that activation of cannabinoid type 1 receptors (CB1R) on synaptic vesicle release at individual axonal boutons of cultured rat hippocampal neurons can be prevented by pharmacological inhibition of either non-muscular myosin II (NMII) or of its major activating kinase, Rho-associated protein kinase (ROCK). Similarly, both NMII and ROCK inhibition prevented long-term forms of endocannabinoid-mediated synaptic depression (eCB-LTD) in two archetypal model synapses (inhibitory/hippocampal and excitatory/corticostratal), while short-term forms, such as depolarization-induced suppression of inhibition or of excitation, i.e. DSI or DSE, remained unaffected. Collectively, these results show that the long-term, but not short-term, decrease in neurotransmitter release under CB1R activation relies on presynaptic ROCK-mediated actomyosin contraction, providing a novel mechanistic link in synaptic regulation.

Impact statement: Building on our recent results, which showed rapid cannabinoid-induced contractility of developing neurons, we report that a similar molecular mechanism regulates widespread forms of synaptic plasticity in the adult brain.

Competing interests: No competing interests declared

Author contributions:
Maureen McFadden: ; Conception and design; Acquisition of data; Analysis and interpretation of data; Drafting or revising the article Hao Xu: ; Acquisition of data; Analysis and interpretation of data Yihui Cui: ; Acquisition of data; Analysis and interpretation of data Rebecca Piskorowski: ; Acquisition of data; Analysis and interpretation of data Laurence Venance: ; Conception and design; Analysis and interpretation of data; Drafting or revising the article Vivien Chevaleyre: ; Conception and design; Acquisition of data; Analysis and interpretation of data; Drafting or revising the article Zsolt Lenkei: ; Conception and design; Analysis and interpretation of data; Drafting or revising the article

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Actomyosin contraction mediates cannabinoid-induced long-term presynaptic plasticity

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Abstract

Synaptic plasticity is critical for brain function, but presynaptic mechanisms of functional plasticity remain poorly-known. Here, by building on our recent results, which showed rapid cannabinoid-induced remodeling of developing neurons, we first show that activation of cannabinoid type 1 receptors (CB1R) on synaptic vesicle release at individual axonal boutons of cultured rat hippocampal neurons can be prevented by pharmacological inhibition of either non-muscular myosin II (NMII) or of its major activating kinase, Rho-associated protein kinase (ROCK). Similarly, both NMII and ROCK inhibition prevented long-term forms of endocannabinoid-mediated synaptic depression (eCB-LTD) in two archetypal model synapses (inhibitory/hippocampal and excitatory/corticostriatal), while short-term forms, such as depolarization-induced suppression of inhibition or of excitation, i.e. DSI or DSE, remained unaffected. Collectively, these results show that the long-term, but not short-term, decrease in neurotransmitter release under CB1R activation relies on presynaptic ROCK-mediated actomyosin contraction, providing a novel mechanistic link in synaptic regulation.

Introduction

Recently, we have described a novel molecular mechanism implicating actin dynamics in the effects of cannabinoid type 1 receptors (CB1R) in neural development (Roland et al., 2014). Here, we investigated the possible involvement of this mechanism in classical functions of CB1R in synaptic plasticity.

Synaptic elements, both presynaptic and postsynaptic, are precisely specified structures, particular both in their functional roles as well as in their morphology, the latter of which is dependent on cytoskeletal elements, namely F-actin. Synaptogenesis and synaptic elimination, therefore, greatly rely on the recruitment and reorganization of the actin cytoskeleton (Dillon and Goda, 2005). Concerning the underlying mechanisms of synaptic strength, however, models typically propose functional modifications of the synapse, such as changes in receptor numbers at the postsynaptic density (e.g. Opazo et al., 2012) and/or changes in vesicle release machinery and Ca\(^{2+}\) flux at the presynaptic site (Castillo, 2012b; Yang and Calakos, 2013). Only recently have models of synaptic plasticity started to integrate cytoskeletal modifications as substrates for synaptic strengthening and weakening. Particularly, it is now established that long-term potentiation (LTP) and long-term depression (LTD) of synapses are accompanied by respective swelling and thinning of dendritic spines (Okamoto et al., 2004; Meyer et al., 2014), which harbor the majority of post-synaptic sites, and that these changes are greatly dependent on actin dynamics within the spines, namely polymerization / depolymerization of actin filaments.

The role of the cytoskeleton in the plasticity of the presynaptic site has not been properly established, however, and models persist in describing only functional changes underlying changes in presynaptic release probability. Nonetheless, presynaptic structures are enriched in cytoskeletal elements, particularly in F-actin (Wilhelm et al., 2014), the structural organization of which, within these sites, implies an important role in the modulation of release probability (recent review in Rust and Maritzen, 2015), but exact molecular mechanisms remain to be discovered.

Several most established and widespread forms of presynaptic plasticity throughout the mammalian brain has been found to be endocannabinoid-mediated (Castillo, 2012a) and retrograde (Wilson and Nicoll, 2001; Ohno-Shosaku et al., 2001). Activity-dependent release of eCBs by the postsynaptic cell,
through activation of presynaptic CB1Rs, mediates either a short-term depression (STD) of transmitter release, such as depolarization induced suppression of inhibition (DSI) or excitation (DSE), or a long-term plasticity, such as endocannabinoid-mediated long-term depression (eCB-LTD) (Castillo et al., 2012a). Although the mechanisms underlying DSI and DSE have been properly established, the mechanisms mediating eCB-LTD, much like other forms of presynaptic plasticity, remain poorly understood.

Here first we tested whether activation of endogenous CB1Rs can affect vesicle release via modulation of ROCK-mediated actomyosin contraction at individual axonal boutons. We then tested whether activity-dependent long-term plasticity, mediated by endogenous cannabinoids, could engage similar mechanisms, by investigating two well-described eCB-mediated forms of plasticity both at inhibitory synapses in the hippocampus and at corticostriatal excitatory synapses.

Results

Actomyosin contractility mediates cannabinoid-induced suppression of vesicle release

In order to directly evaluate the involvement of presynaptic actomyosin cytoskeleton in synaptic efficacy, we first measured the effect of CB1R activation on synaptic vesicle release at individual axonal boutons by expressing synaptophysin-phluorin (SpH, Miesenböck et al., 1998) in cultured rat hippocampal neurons. Stimulation of neurons with KCl (2min; 50mM) induced an average release of 38.9% of total bouton vesicle pool under control conditions (±1.2%; n=339 over 4 independent experiments; Fig. 1a-c), as estimated through maximal bouton fluorescence upon alkaline incubation (NH₄Cl; 2min, 50mM). Vesicle pool fraction released in boutons exposed to the synthetic CB1R agonist WIN55,212-2 (1μM; 10min) was significantly lower as compared to control (27.4%±1.0%; n=357 over 4 independent experiments; P<0.001; Fig. 1a-c), indicating a suppression of vesicle release under CB1R activation, as has been previously shown in similar paradigms (Ramirez-Franco et al., 2014). Strikingly, this effect was blocked by pretreating neurons with nitroblebbstatin (Lucas-Lopez et al., 2005), a selective NMI ATPase inhibitor (Kovacs et al., 2004) (25μM; 20min; 43.2%±1.5%; n=176 over 3 independent experiments; P<0.0001; Fig. 1b,c). Furthermore, the effect of CB1R was similarly blocked by exposure of neurons to the selective ROCK inhibitor Y-27632 (10μM; 20min; 39.9%±1.5%; n=128 over 3 independent experiments; P<0.0001; Fig. 1c). CB1R activation is also known to induce synaptic silencing (Ramirez-Franco et al., 2014; Losonczy et al., 2004). Silent boutons were identified as puncta that manifested a fluorescent vesicle pool under NH₄Cl administration but whose response to KCl was lower than one standard deviation above the average baseline noise level (4%±10%; Fig. 1d). As such, the number of silent boutons in samples treated with the CB1R agonist alone was significantly greater than in the control samples (p=0.0113). This silencing was also prevented by both nitroblebbstatin (p=0.0367) and Y-27632 (p=0.0424) pretreatment (Fig. 1d).
Figure 1. CB₁R-induced suppression of vesicle release at individual axonal boutons is mediated by actomyosin contractility, as shown by pretreatment with the NMII inhibitor nitroblebbistatin (nBlebb), or the ROCK inhibitor Y-27632. (a) SpH fluorescence in control (Veh) and WIN55,212-2-treated (WIN) axons before stimulation (Baseline), after stimulation (KCl, 50mM, 2min), and after superfusion with NH₄Cl (50mM, 2min). Fluorescence intensity (arbitrary units) increases during stimulation in control conditions while WIN suppresses this effect. (b) Experimental paradigm and illustrative traces of normalized axonal bouton SpH fluorescence (c) Cumulative probability distributions of the released vesicle pool fractions. (d) Relative frequency distributions of the samples shown in c. *: p < 0.05; **: p < 0.001; ns: not significant as compared to vehicle. Kruskal-Wallis test. Scale bar: 5μm

Actomyosin contractility mediates eCB-LTD at inhibitory hippocampal synapses

In the hippocampus, depolarization of pyramidal cells triggers DSI, a short-term depression of GABA release, which was not affected by blebbistatin (Fig.2b In control: 65.2±6.5% of baseline, n=7. In blebbistatin: 69.4±5.4% of baseline, n=6, p=0.63). However blebbistatin, but not its inactive enantiomer, strikingly abolished eCB-LTD, the long-term depression mediated by eCBs at GABAergic synapses (Fig. 2c. control: 75.0±2.6% of baseline, n=9; blebbistatin: 96.9±1.9%, n=8, p=0.00002, 79.2±1.7%, n=5, p=0.27), as well as the change in the paired-pulse ratio (PPR: an indirect measure of release probability) following eCB-LTD induction (data not shown. In control: 1.22±0.03 of baseline, n=9; blebbistatin: 1.05±0.01%, n=8, p=0.00087, inactive blebbistatin enantiomer: 1.21±0.04 p=0.86). Induction of eCB-LTD requires not only CB₁R activation, but also spontaneous firing of GABAergic interneurons. In control experiments we found no change in either IPSC amplitude or frequency in the presence of blebbistatin (amplitude: baseline: 22.8 ±0.9pA, after blebbistatin: 21.9±1.0pA, n=7, p=0.18; frequency: baseline: 11.1±1.4Hz, after blebbistatin: 10.7±1.6, p=0.59). Furthermore, by using a protocol capable of rescuing eCB-LTD even when interneuron firing is completely blocked, using trains of stimulation following tetanus in order to compensate for a potential decrease in interneuron firing (Heifets et al., 2008), eCB-LTD could not be induced in the presence of blebbistatin (103.6±5.6% of baseline, n=5). These data indicate that the lack of eCB-LTD is not a side effect of blebbistatin influencing interneuron firing. Finally, because eCB-LTD induction requires glutamate release, mGluR activation and eCB release, we bypassed all these steps by looking at miniature IPSCs following direct activation of CB₁R with WIN55,212-2 (Chevaleyre et al., 2007). In control slices, WIN55,212-2 induced a significant decrease in mIPSC frequency without affecting their amplitude (Fig. 2d. Frequency: from 6.2 ±1.4 to 4.5±1.1Hz, p=0.03; Amplitude: from 28.1±1.4 to
26.5±1.6pA, p=0.46, n=5). However, in the presence of blebbistatin, activation of CB1R had no effect on mIPSC frequency (Fig. 2d. Frequency: from 5.2±0.5 to 5.6±0.6Hz, p=0.6). Finally, the specific ROCK inhibitor Y27632 also fully blocked eCB-LTD induction (Fig. 2e. 98.7±1.4% of baseline, n=5, p=0.37 with baseline and p=0.0004 with control LTD, n=6).

**Actomyosin contractility mediates eCB-LTD at excitatory corticostriatal synapses**

We then investigated the molecular mechanism of STD and LTD at an excitatory glutamatergic synapse: the corticostriatal synapse at the striatal medium-sized spiny neurons (MSNs) (Fig. 2f). Corticostriatal long-term plasticity, which mainly involves the endocannabinoid system, provides a fundamental mechanism for the function of the basal ganglia in action selection and in procedural learning (Yin and Knowlton, 2006; Koralek et al., 2012). MSNs express endocannabinoid-mediated and CB1R-dependent STD, namely a DSE, and LTD (Di Filippo et al., 2009; Puente et al., 2011; Mathur et al., 2012). A sustained depolarization of MSNs induced DSE (81±4% of baseline, n=13, p=0.0003) (Fig. 2g), which was not significantly affected with blebbistatin treatment (10μM) (79±3 of baseline, n=11, p=0.0001 with baseline; p=0.8039 with control DSE, n=13) (Fig. 2g). However, the potent eCB-LTD induced after LFS (58±2, of baseline, n=7, p<0.0001) was prevented by blebbistatin treatment (97±7% of baseline, n=7, p=0.3693 with baseline, p<0.0001 with control LTD, n=7). We ensured that the inactive enantiomer of blebbistatin (10μM) had no significant effect on LTD expression (50±6% of baseline, n=6, p<0.0001 with baseline, p=0.2041 with control LTD, n=7) (Fig. 2h). The specific ROCK inhibitor Y-27632 (10μM) also impaired eCB-LTD induction (98±11% of baseline, n=7, p=0.7204 with baseline, p=0.0038 with control LTD, n=6) (Fig. 2l).
Figure 2: Presynaptic long-term depression mediated by eCBs at both inhibitory synapses in the CA1 area of the hippocampus (a-g) and at excitatory corticostratial synapses (f-i) depends on RhoA/ROCK-induced actomyosin contraction. (a) CA1: whole-cell recorded pyramidal neuron; int: inhibitory interneuron fibers, activated by a stimulating electrode in the stratum radiatum. Fast excitatory transmission from Schaffer collateral inputs (s.c.) was blocked by the AMPA/NMDA/KA receptor antagonists D-APV and NBQX. (b) The transient depression of inhibitory transmission following a 5 second depolarization at 0 mV (white circles) was unaffected by blebbistatin (Blebb, black circles). (c) The long-term depression following high frequency stimulation (white circles) was completely abolished in the presence of blebbistatin (black circles) but not by the inactive blebbistatin enantiomer (grey circles). Average sample traces are shown on top for time points (1) and (2). (d) The decrease in miniature IPSC frequency mediated by WIN55,212-2 (WIN, white circles) was abolished by blebbistatin (black circles). Sample traces are shown on top. Right: Average miniIPSC frequencies and amplitudes. (e) The LTD evoked by high frequency stimulation (white circles) was abolished in the presence of the ROCK inhibitor Y-27632 (black circles). Average sample traces are shown on top for time points (1) and (2). (f) Whole-cell recording of a striatal output neurons and stimulation in the somatosensory cortical
layer V. cc: corpus callosum, MSN: medium-sized spiny neurons (g) The transient depression of excitatory transmission following a 10 second depolarization at 0mV (white circles) was unaffected by blebbistatin (black circles). Average sample traces before and 10sec after the depolarization are shown on the right. (h) The LTD following a low frequency stimulation (white circles) was completely abolished in the presence of blebbistatin (black circles) but was unaffected by the inactive enantiomer of blebbistatin (grey circles). Average sample traces are shown on top at the time point before (1) and after the stimulation protocol (2). (i) The ROCK inhibitor Y-27632 abolishes the LTD evoked by low frequency stimulation (black circles). Average sample traces are shown on top for time points (1) and (2).

**Discussion**

We show that activation of endogenous CB1Rs can affect vesicle release via modulation of ROCK-mediated actomyosin contraction at individual axonal boutons. Similar CB1R activation by endocannabinoids induces LTD at GABA-ergic inhibitory synapses and at corticostriatal excitatory synapses through ROCK-mediated presynaptic actomyosin contraction. These results describe a novel mechanism of presynaptic plasticity whereby dynamics of the actin cytoskeleton, led by the actin-bound motor NMII, leads to reduced vesicle release from the presynaptic site as well as long-term depression of synaptic strength under one of the most widespread forms of presynaptic plasticity, eCB-LTD. Our results are the first to demonstrate, to our knowledge, a role for actomyosin contraction on presynaptic-dependent forms of synaptic plasticity.

Interestingly, we did not find an effect of either NMII or ROCK inhibition on short-term forms of endocannabinoid-induced synaptic plasticity, neither on DSI nor DSE. The mechanisms of both DSE and DSI have been relatively well characterized and are thought to be due to the direct coupling of CB1R to G<sub>Y6</sub> proteins and their subsequent inhibition of P/Q type Ca<sup>2+</sup> channels and inwardly rectifying K<sup>+</sup> channels (GIRK) (Castillo et al., 2012a). Thus, for vesicles already docked at the active zone, release is mainly dependent on Ca<sup>2+</sup> influx, explaining the lack of effect of NMII and ROCK inhibition.

In conclusion, our study provides a novel mechanistic link - presynaptic actomyosin contractility - to relate changes in synaptic efficacy with one of the most widespread forms of synaptic plasticity in the brain, endocannabinoid-mediated LTD. These results open novel perspectives in the study of presynaptic forms of synaptic plasticity, as well as the understanding of both cognitive function and the pathogenesis of psychiatric disease.

**Materials and Methods**

**Animals**

All experiments were performed in accordance with local animal welfare committee (Center for Interdisciplinary Research in Biology and EU guidelines [directive 2010/63/EU]). Every precaution was taken to minimize stress and the number of animals used in each series of experiments. Sprague-Dawley rats (Charles River, L'Arbresle, France) were housed in standard 12 hours light/dark cycles and food and water were available ad libitum.

**Chemicals**

WIN55,212-2 and (RS)-3,5-DHPG were purchased from Tocris. Carbchol, Y-27632, active (S)-(-)-blebbistatin and inactive (R)-(+)-blebbistatin enantiomers and nitroblebbistatin were purchased from Calbiochem, Sigma and Santa Cruz Biotechnology, respectively. Note that none of the bath-applied drugs had a significant effect on basal EPSC amplitudes.
Hippocampal neuronal cultures

Cultures were performed as described previously (Carrel et al., 2011). Briefly, hippocampi were dissected from embryonic day 17-18 Sprague-Dawley rat embryos, trypsinized and further dissociated using a fire-polished Pasteur pipette. Cells were plated on Poly-D-Lysine-coated 18mm coverslips at a density of approximately 100,000 cells per coverslip and subsequently cultivated at 37°C, 5% CO2 in NeurobasalTM (LifeTech) medium supplemented with 2% B27 (LifeTech), 0.5mM L-glutamine, 10U/mL penicillin G and 10mM streptomycin containing conditioned medium obtained by incubation with glial cultures (70-80% confluence) for 24 h.

Neuron transfection

Neurons were transfected either with Synaptophysin-pHluorin (SpH), a kind gift from Dr. Stefan Krueger (Dalhousie University, Halifax, NS, Canada), and LifeAct-mCherry (Roland et al., 2014), or with SpH alone. SpH is a pH-sensitive and synaptic-vesicle specific membrane protein, whose fluorescence is quenched in the acidic vesicle lumen, only emitting a fluorescent signal under blue-light excitation when exposed to the extracellular medium during vesicle release (Miesenböck et al., 1998). Transfections were performed 7-9 days after plating, as described previously (Roland et al., 2014).

Time-lapse microscopy

Time-lapse microscopy was performed on a Nikon Eclipse Ti-E/B inverted microscope with Perfect Focus System (PFS) equipped with a Polychrome V monochromator (Till Photonics). Transfected cultures were placed in a Ludin chamber (Life Imaging Services) with preheated medium composed of 12 mM NaCl, 0.3 mM KCl, 1mM HEPES, 0.2 mM MgCl, 1mM D-Glucose and 2% B27 (pH 7.40). Chambers were mounted on a motorized microscope stage heated at 37°C.

Images were acquired at a rate of 1 image/channel/min using a CoolSnap HQ2 camera (Photometrics) piloted by Metamorph 7.7 software (Molecular Devices). During experiments, pharmacological treatments were applied directly to the culture medium. Neuronal depolarization was elicited through the application of 50mM KCL for 2min. Pretreatments were applied 30min before KCL application, with treatments applied 10min before. The treatment solvent dimethylsulfoxide was used as a control (vehicle), concentrations ranging from 0.02% to 0.1% of the chamber medium. NH4Cl was applied 2min after KCL application.

Time-lapse image analysis

For each position, images were stacked and realigned using Image J software. Round ROIs of approximately 3x3μm were placed manually around visually identified axonal boutons and the fluorescence intensity of SpH at each ROI was measured.

Measurement analysis was carried out in Excel (Microsoft). For each timepoint, mean basal axonal fluorescence intensity was subtracted from each ROI. Selection of axonal boutons for analysis was based on two criteria: axonal bouton SpH response to NH4Cl had to be superior to that of KCL and baseline fluorescence per ROI had to be contained within 2x the standard deviation around the baseline population mean. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software). Unless otherwise noted, statistical analyses of treatment effect were performed using a Kruskal-Wallis test with Dunn’s post-hoc Multiple Comparison Test. Unless otherwise noted, n indicates the number of axonal boutons analyzed and effects are represented as mean±/ SEM.

Electrophysiological recordings and analysis from hippocampal slices

400 μM transverse hippocampal slices were prepared from 6- to 8-week-old C57BL6 male mice. Animals were euthanized in accordance with institutional regulations under anesthesia with isoflurane. Hippocampi were removed and placed upright into an agar mold and cut with a vibratome (Leica VT1200S, Germany) in ice-cold extracellular solution containing (in mM): 10 NaCl,
195 sucrose, 2.5 KCl, 15 glucose, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 CaCl₂ and 2 MgCl₂). The slices were then transferred to 30°C ACSF (in mM: 125 NaCl, 2.5 KCl, 10 glucose, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 Na Pyruvate, 2 CaCl₂ and 1 MgCl₂) for 30min and kept at room temperature for at least 1.5 hr before recording. All experiments were performed at 33°C. Cutting and recording solutions were both saturated with 95% O₂ and 5% CO₂ (pH 7.4).

Whole-cell recordings were obtained from CA1 PNs in voltage clamp mode. Inhibitory synaptic transmission was monitored in the continuous presence of the NMDA receptor antagonist d(-)-2-amino-5-phosphonopentanoic acid (d-APV; 50 μM) and the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfonylbenzoflquinoxaline (NBQX; 10 μM) inhibitory currents were recorded at +10 mV with a patch pipette (3–5 MQ) containing (in mM): 135 CsMethylSulfate, 5 KCl, 0.1 EGTA-Na, 10 HEPES, 2 NaCl, 5 ATP, 0.4 GTP, 10 phosphocreatine (pH 7.2; 280–290 mOsm). Series resistance (typically 12–18 MQ) was monitored throughout each experiment; cells with more than 15% change in series resistance were excluded from analysis. Synaptic potentials were evoked by monopolar stimulation with a patch pipette filled with ACSF and positioned in the middle of CA1 SR. A HFS (100 pulses at 100Hz repeated twice) was applied following 15 – 20min of stable baseline. The amplitudes of the IPSCs were normalized to the baseline amplitude. The magnitude of LTD was estimated by comparing averaged responses at 30-40min after the induction protocol with baseline-averaged responses 0–10min before the induction protocol. All drugs were bath-applied following dilution in the external solution from concentrated stock solutions. We used Axograph X software for data acquisition and Origin Pro for data analysis. Student’s t-test was performed for statistical comparisons and results are reported as mean±SEM.

Electrophysiological recordings and analysis from corticostriatal slices

Horizontal brain slices containing the somatosensory cortex and the corresponding corticostriatal projection field were prepared according to the methods previously published (Fino et al., 2005; Pailie et al., 2013). Corticostriatal connections (between somatosensory cortex layer 5 and dorsal striatum) are preserved in a horizontal plane. Briefly, horizontal brain slices with a thickness of 330 or 300μm were prepared, from rats (males, P20–25) using a vibrating blade microtome (VT1200S, Leica Micosystems, Nussloch, Germany). Brains were sliced in a 95% CO₂/5% O₂-bubbled, ice-cold cutting solution containing (in mM) 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 1 pyruvic acid, and then transferred into the same solution at 34°C for one hour and then moved to room temperature.

Patch-clamp recordings were performed as previously (Fino et al., 2005; Pailie et al., 2013). Briefly, borosilicate glass pipettes of 4-6MW resistance contained for whole-cell recordings (in mM): 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA (adjusted to pH 7.35 with KOH). The composition of the extracellular solution was (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 10mM pyruvic acid bubbled with 95% O₂ and 5% CO₂. Signals were amplified using EPC10-2 amplifier (HEKA Elektronik, Lambrecht, Germany). All recordings were performed at 34°C using a temperature control system (Bath-controller V, Luigs&Neumann, Ratingen, Germany) and slices were continuously superfused at 2-3 ml/min with the extracellular solution. Slices were visualized on an Olympus BX51WI microscope (Olympus, Rungis, France) using a 4x/0.13 objective for the placement of the stimulating electrode and a 40x/0.80 water-immersion objective for localizing cells for whole-cell recordings. Series resistance was not compensated. Current-clamp recordings were filtered at 2.5 kHz and sampled at 5 kHz and voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz using the Patchmaster v2x32 program (HEKA Elektronik).

Plasticity induction protocols

Electrical stimulations of the cerebral cortex were performed with a bipolar electrode (Phymep) placed in the layer 5 of the somatosensory cortex (Fino et al., 2005). Electrical stimulations were monophasic at constant current (ISO-Flex stimulator). Currents were adjusted to evoke striatal EPSCs
ranging in amplitude from 50 to 200 pA. Repetitive control stimuli were applied at 0.1 Hz, a frequency for which neither short- nor long-term synaptic efficacy changes in EPSC amplitudes were induced. Series resistance was monitored and calculated from the response to a hyperpolarizing potential (5 mV) step during each sweep throughout the experiments and a variation above 20% led to the rejection of the experiment. Repetitive control stimuli were applied at a frequency of 0.1 Hz for 60 min. Drugs were applied in the bath, after recording 10 min of baseline and 10 min before cellular conditioning protocol, and were present continuously until the end of the recording. Off-line analysis was performed using Igor-Pro 6.0.3 (Wavemetrics). LTD was induced with low frequency stimulation protocol consisting in 900 cortical stimulations at 1 Hz paired with postsynaptic concomitant depolarization of the MSN during 50 ms. For DSE induction, MSN was depolarized from RMP to 0 mV during 10 sec (with bath-applied carbachol, 10 mM, and DHPG, 50 mM) (Puente et al., 2011).

**Electrophysiological data analysis**

Off-line analysis was performed using Fitmaster (Heka Elektronik) and Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA). Statistical analysis was performed using Prism 5.0 software (San Diego, CA, USA). In all cases “n” refers to a single cell experiment from single slice. All results were expressed as mean±s.e.m, and statistical significance was assessed using two-sided Student’s t test or the one sample t test when appropriate at the significance level (p) indicated.

**Author Contributions**

MHM, LV, VC and LZ designed research, MHM, HX, VC, RAP and VC realized the experiments, MHM, RAP, LV, VC and LZ wrote the article.

**References**


Endocannabinoids mediate bidirectional striatal spike-timing-dependent plasticity

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Key points

• Although learning can arise from few or even a single trial, synaptic plasticity is commonly assessed under prolonged activation. Here, we explored the existence of rapid responsiveness of synaptic plasticity at corticostrial synapses in a major synaptic learning rule, spike-timing-dependent plasticity (STDP).
• We found that spike-timing-dependent depression (tLTD) progressively disappears when the number of paired stimulations (below 50 pairings) is decreased whereas spike-timing-dependent potentiation (tLTP) displays a biphasic profile: tLTP is observed for 75–100 pairings, is absent for 25–50 pairings and re-emerges for 5–10 pairings.
• This tLTP induced by low numbers of pairings (5–10) depends on activation of the endocannabinoid system, type-1 cannabinoid receptor and the transient receptor potential vanilloid type-1.
• Endocannabinoid-tLTP may represent a physiological mechanism operating during the rapid learning of new associative memories and behavioural rules characterizing the flexible behaviour of mammals or during the initial stages of habit learning.

Abstract Synaptic plasticity, a main substrate for learning and memory, is commonly assessed with prolonged stimulations. Since learning can arise from few or even a single trial, synaptic strength is expected to adapt rapidly. However, whether synaptic plasticity occurs in response to limited event occurrences remains elusive. To answer this question, we investigated whether a low number of paired stimulations can induce plasticity in a major synaptic learning rule, spike-timing-dependent plasticity (STDP). It is known that 100 pairings induce bidirectional STDP, i.e. spike-timing-dependent potentiation (tLTP) and depression (tLTD) at most central synapses. In rodent striatum, we found that tLTD progressively disappears when the number of paired stimulations is decreased (below 50 pairings) whereas tLTP displays a biphasic profile: tLTP is observed for 75–100 pairings, absent for 25–50 pairings and re-emerges for 5–10 pairings. This tLTP, induced by very few pairings (~5–10) depends on the endocannabinoid (eCB) system. This eCB-dependent tLTP (eCB-tLTP) involves postsynaptic endocannabinoid synthesis, requires paired activity (post- and presynaptic) and the activation of type-1 cannabinoid receptor (CB1R) and transient receptor potential vanilloid type-1 (TRPV1). eCB-tLTP occurs in both striatopallidal and striatonigral medium-sized spiny neurons (MSNs) and is dopamine dependent. Lastly, we
show that eCB-LTP and eCB-LTD can be induced sequentially in the same neuron, depending on the cellular conditioning protocol. Thus, while endocannabinoids are usually thought simply to depress synaptic function, they also constitute a versatile system underlying bidirectional plasticity. Our results reveal a novel form of synaptic plasticity, eCB-tLTP, which may underlie rapid learning capabilities characterizing behavioural flexibility.

Introduction

Cardinal cognitive abilities can display rapid learning dynamics. Forming new associative memories and behavioural rules can be learned within a few (5–10) or even a single trial (Schultz et al. 2003; Pasupathy & Miller, 2005; Tse et al. 2007; Quilodran et al. 2008; Ito & Doya, 2009). In cortex and striatum, neurons that respond to behaviourally relevant events (cues, actions or rewards) fire very few spikes (one to a dozen) upon each trial (i.e. they typically discharge at a frequency of 5–25 Hz during a 0.1–0.5 s period; Schultz et al. 2003; Pasupathy & Miller, 2005; Quilodran et al. 2008). This evidence suggests that the discharge of a small number (2–50) of spikes should be sufficient to induce synaptic plasticity, a substrate for learning and memory (Martin & Morris, 2002). However, typical cell conditioning protocols for initiating long-term plasticity, such as high- or low-frequency stimulations, rely on the repetition of hundreds of pre- or postsynaptic spikes. Noticeable exceptions are reports showing the existence of single-shot LTD in visual cortex or single-burst LTP in hippocampus (Holthoff et al. 2004; Remy & Spruston, 2007). Besides these reports introducing the possibility of bidirectional plasticity induced by limited stimulation, the possible existence of rapid responsiveness of synaptic plasticity still needs to be extended to other synapses and cell conditioning protocols.

Here, we tested the hypothesis that a low number of spikes could lead to long-term synaptic plasticity. For this purpose, we chose spike-timing-dependent plasticity (STDP) as a synaptic Hebbian learning paradigm. Indeed, STDP (tLTP and tLTD) depends on the relative timing between pre- and postsynaptic spikes, and relies on much fewer events (around 100 paired stimulations) than the high- or low-frequency stimulation protocols (hundreds of stimulations) (Sjöström et al. 2008; Feldman, 2012). We first investigated if limited occurrences of paired stimulations, from 2 to 100 pre–post or post–pre pairings, could induce STDP at corticostrial synapses. In the striatum, bidirectional STDP with NMDA receptor (NMDAR)-mediated tLTP and endocannabinoid (eCB)-mediated tLTD has previously been reported with 100 paired stimulations (Fino et al. 2005, 2010; Shen et al. 2008; Pawlak & Kerr, 2008; Fino & Venacce, 2010; Paille et al. 2013). In the present study we report that these forms of plasticity disappear when the number of paired stimulations decreases. However, a reliable and robust tLTP re-emerges for a low number of paired stimulations (~5–10). We show that this tLTP is not dependent on NMDAR but is eCB-mediated. This eCB-tLTP depends on the activation of type-1 cannabinoid receptor (CB1R) and transient receptor potential vanilloid type-1 (TRPV1). eCB-tLTP can be induced in both striatopallidal and striatonigral MSNs and is dopamine dependent. Finally, we observe that eCB-tLTP and eCB-tLTD can be sequentially induced at the same synapse, thus demonstrating that eCBs serve as a generic signalling system able to encode for bidirectional plasticity. eCBs have emerged as a major signalling system in learning and memory because of their powerful influence on synaptic plasticity which has been associated with the depression of neuronal communication on short or long timescales (Kano et al. 2009; Katona & Freund, 2012; Castillo et al. 2012; Mathur & Lovinger, 2012; Melis et al. 2014). The eCB-tLTP reported here shows that eCBs in fact...
support bidirectional plasticity. This new form of plasticity may underlie the quick reactivity necessary for synaptic weight adaptation during rapid learning.

Methods

Animals

All experiments were performed in accordance with local animal welfare committee (Centre for Interdisciplinary Research in Biology and EU guidelines, directive 2010/63/EU). Every precaution was taken to minimize stress and the number of animals used in each series of experiments. Sprague–Dawley rats (Charles River, L’Arbresle, France) and C57BL/6 mice, CB1R−/− and CB1R+/+ littermates (Ledent et al. 1999), and D1-eGFP mice were used for brain slice electrophysiology. Animals were housed in standard 12 h light–dark cycles and food and water were available ad libitum.

Brain slice preparation

Horizontal brain slices containing the somatosensory cortex and the corresponding corticostriatal projection field were prepared according to methods previously published (Fino et al. 2005; Paillé et al. 2013). Corticostriatal connections (between somatosensory cortex layer 5 and dorsal striatum) are preserved in a horizontal plane. Briefly, horizontal brain slices with a thickness of 330 or 300 μm were prepared, respectively, from rats (males and females, postnatal days 17–25) or mice (males and females, postnatal days 17–25 and 60–90) using a vibrating blade microtome (VT1200S, Leica Microsystems, Nussloch, Germany). Brains were sliced in a 95% CO2–5% O2-bubbled, ice-cold cutting solution containing (in mM) 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 1 pyruvic acid, and then transferred into the same solution at 34°C for 1 h and then moved to room temperature.

Electrophysiology recordings

Patch-clamp recordings were performed as previously described (Fino et al. 2010; Paillé et al. 2013). Briefly, for whole-cell recordings borosilicate glass pipettes of 4–6 MΩ resistance contained (in mM): 105 potassium gluconate, 30 KCl, 10 Heps, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA (adjusted to pH 7.35 with KOH). The composition of the extracellular solution was (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, with 10 μM pyruvic acid, bubbled with 95% O2 and 5% CO2. Signals were amplified using EPC10-2 amplifier (HEKA Elektronik, Lambrecht, Germany). All recordings were performed at 34°C using a temperature control system (Bath-controller V, Luigs & Neumann, Ratingen, Germany) and slices were continuously superfused at 2–3 ml min−1 with the extracellular solution. Slices were visualized on an Olympus BX51WI microscope (Olympus, Rungis, France) using a ×4/0.13 objective for the placement of the stimulating electrode and a ×40/0.80 water-immersion objective for localizing cells for whole-cell recordings. Medium-sized spiny neurons (MSNs), the striatal output neurons, were distinguished from interneurons based on passive and active membrane properties (Fino et al. 2008). Series resistance was not compensated. Current-clamp recordings were filtered at 2.5 kHz and sampled at 5 kHz and voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz using the Patchmaster v2×32 program (HEKA Elektronik).

Chemicals

Chemicals were bath applied or injected only in the recorded postsynaptic neuron through the patch-clamp pipette. DL-2-amino-5-phosphono-pentanoic acid (0-AP5, 50 μM; Tocris, Ellisville, MO, USA), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP hydrochloride, 10 μM; Tocris), 5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one dihydrochloride (pirenzepine dihydrochloride, 1 μM; Sigma) were dissolved directly in the extracellular solution and bath applied. N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, 3 μM; Tocris), picrotoxin (50 μM; Sigma), 1,4-dihydropyrido-2,6-dimethyl-4-(3-nitropheryl)-3,5-pyridinedicarboxylic acid 2-methoxyethyl 1-methylthyl ester (nimodipine, 1 μM; Tocris) and (2E)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylphényl)phenyl]-2-propenamide (AMG9810, 1 μM; Tocris), R-(-)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390, 4 μM; Sigma) and (S–)-5-aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide (sulpiride, 10 μM; Tocris) were dissolved in ethanol and then added in the external solution at a final concentration of ethanol of 0.01–0.1%. BAPTA (1 mM; Sigma) and GDP-B-S (2 mM) were dissolved directly into the intracellular solution and applied via the patch-clamp pipette. U73122 (5 μM; Sigma) was dissolved in ethanol and then added to the intracellular solution at a final concentration of ethanol of 0.033%. Tetrahydroflupentixol (THI, 10 μM; Sigma) was dissolved in DMSO and applied internally via the patch-clamp pipette at a final DMSO concentration of 0.1%. (S)-α-methyl-4-carboxyphenylglycine (MCPG, 500 μM; Tocris) was dissolved in 1.1 equiv NaOH, and then added in the external solution. N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H
Table 1. Effects of bath-applied drugs on corticostriatal EPSC baseline amplitude

<table>
<thead>
<tr>
<th>Bath-applied drugs</th>
<th>Normalized EPSC (baseline drugs/baseline control) amplitude</th>
<th>n, P (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-APS (50 μM)</td>
<td>98 ± 4%</td>
<td>n = 6, P = 0.6076</td>
</tr>
<tr>
<td>AM251 (3 μM)</td>
<td>99 ± 5%</td>
<td>n = 8, P = 0.7988</td>
</tr>
<tr>
<td>MCPG (500 μM)</td>
<td>94 ± 6%</td>
<td>n = 5, P = 0.4029</td>
</tr>
<tr>
<td>MPEP (10 μM)</td>
<td>100 ± 4%</td>
<td>n = 4, P = 0.9243</td>
</tr>
<tr>
<td>Pirenzepine (1 μM)</td>
<td>92 ± 7%</td>
<td>n = 6, P = 0.3739</td>
</tr>
<tr>
<td>Nimodipine (1 μM)</td>
<td>96 ± 4%</td>
<td>n = 4, P = 0.3790</td>
</tr>
<tr>
<td>Capsazepine (10 μM)</td>
<td>100 ± 4%</td>
<td>n = 5, P = 0.9778</td>
</tr>
<tr>
<td>AMG9810 (1 μM)</td>
<td>97 ± 8%</td>
<td>n = 5, P = 0.6800</td>
</tr>
<tr>
<td>Picrotoxin (50 μM)</td>
<td>96 ± 3%</td>
<td>n = 6, P = 0.1493</td>
</tr>
<tr>
<td>SCH23390 (4 μM) + sulpiride (10 μM)</td>
<td>102 ± 2%</td>
<td>n = 7, P = 0.4458</td>
</tr>
</tbody>
</table>

*None of the bath-applied drugs had a significant effect on EPSC baseline amplitude. In all cases, only the baselines with the bath-applied drugs were compared to EPSC baselines measured after 60 min recording.

-2-benzazepine-2-carbothioamide (capsazepine, 10 μM; Tocris) and 2-arachidonoylglycerol (2-AG, 100 μM; Tocris) were dissolved in DMSO and then added to the external solution at final DMSO concentrations of 0.0025 and 0.1%, respectively. We verified that DMSO (0.1%) or ethanol (0.033%) when applied intracellulary did not alter the tLTP observed in control conditions (165 ± 11%, \(P < 0.0001, n = 27\)). DMSO (0.1%), the vehicle used to dilute iTHL, did not prevent the tLTP induced with 10 post–pre pairings (213 ± 22%, \(P = 0.0153, n = 4\); \(P = 0.0656\) when compared with control). Likewise, ethanol (0.033%), the vehicle used to dilute iU73122, did not prevent tLTP (176 ± 20%, \(P = 0.0139, n = 6\); \(P = 0.0120\) when compared with control).

Note that none of the bath-applied drugs had a significant effect on basal EPSC amplitudes (Table 1).

**Spike-timing-dependent plasticity induction protocols**

Electrical stimulations were performed with a bipolar electrode (Phymep, Paris, France) placed in layer 5 of the somatosensory cortex (Fino et al. 2005, 2010). Electrical stimulations were monophasic at constant current (ISO-Flex stimulator, AMPI, Jerusalem, Israel). Currents were adjusted to evoke 50–200 pA EPSCs. Repetitive control stimuli were applied at 0.1 Hz. STDP protocols consisted in pairings of pre- and postsynaptic stimulations (at 1 Hz) with the two events separated by a specific temporal interval (\(\Delta_{STDP}\)). Presynaptic stimulations corresponded to cortical stimulations and the postsynaptic stimulation of an action potential evoked by a depolarizing current step (30 ms duration) in MSNs. Suprathreshold depolarization for 30 ms was chosen to mimic corticostriatal summation of EPSPs induced by cortical activity as observed in in vivo studies (Mahon et al. 2006). MSNs were maintained throughout the STDP experiments at a constant holding membrane potential which corresponds to their initial resting membrane potential (−75 ± 1 mV, \(n = 103\)). Thus, EPSCs during baseline recording or after the STDP protocol were measured at the same membrane potential (in voltage-clamp mode); note that the STDP pairings (performed in current-clamp mode) were also conducted at the same holding membrane potential. Neurons were recorded for 10 min during baseline amplitude and for at least 60 min after the STDP protocol; long-term synaptic efficacy changes were measured after 50 min. Thirty successive EPSCs (at 0.1 Hz) were individually measured and then averaged. Variation of series resistance beyond 20% led to the rejection of the experiment. After recording of 10 min control baseline amplitude, drugs were applied in the bath. A new baseline with drugs was recorded after a time lapse of 10 min (to allow the drug to be fully perfused) for 10 min before the STDP protocol (see effects of the bath-applied drugs on baseline amplitude in Table 1). Drugs were present until the end of the recording (except when specified). In a subset of experiments (for U73122, THL, BAPTA and GDP-B-S) drugs were applied intracellularly through the patch-clamp pipette. Once the cell patched, drugs were allowed to diffuse into the cell for at least 15 min before starting recording of the baseline amplitude. Local applications of 2-AG were performed through a patch-clamp pipette placed in the vicinity (50 μm) of the recorded neuron and linked to a Picospritzer II system (Parker, USA), which supplies repeatable pressure pulses.

It should be noted that STDP protocols consisting of 5–10 post–pre pairings (with a single postsynaptic spike) were sufficient to induce potent tLTP in rats while in C57BL/6 mice 15 pairings (with 2–3 postsynaptic spikes per postsynaptic discharge) were necessary to trigger tLTP.
**Electrophysiological data analysis**

Off-line analysis was performed using Fitmaster (Heka Elektronik) and Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA). Statistical analysis was performed using Prism 5.0 software (San Diego, CA, USA). In all cases

\( n \) refers to the number of repetitions of an experiment (each experiment being performed on different brain slices) from single slice. Experimenters were blind to the genotype of \( CB_1R^{-/-} \) and \( CB_1R^{+/+} \) littermate mice during electrophysiological recordings and analysis. All results were expressed as means ± SEM in the text and, for visualization purposes, as means ± SD in the figures, and statistical significance was assessed using two-sided.

![Diagram](image)

**Figure 1.** A low number of paired stimulations induces spike-timing-dependent potentiation. A, left: scheme of the recording and stimulating sites in corticostriatal slices. A, right: characteristic voltage responses of a MSN to a series of 500 ms current pulses from -150 to +180 pA with current steps increasing by 30 pA (black traces) and to +60 pA above spike threshold (grey trace). B, STDP protocol: a spike evoked in striatal neuron was paired with cortical stimulation \( N \) times at 1 Hz. \( \Delta t \) indicates the time delay between pre- and post-synaptic stimulations. \(-30 < \Delta t < 0\) ms and \(0 < \Delta t < 30\) ms refers to post-pre and pre-post pairings, respectively. C, 100 post-pre and pre-post pairings \((n = 10)\) induced bidirectional plasticity, i.e. tLTP and tLTD, respectively. D, 75 post-pre and pre-post pairings \((n = 8)\) induced tLTP and tLTD, respectively. E, 50 post-pre and pre-post pairings \((n = 7)\) induced unidirectional plasticity, i.e. no plasticity and tLTD, respectively. F, 25 post-pre and pre-post pairings \((n = 11)\) did not induce significant plasticity. G, 10 post-pre and pre-post pairings \((n = 16)\) induced unidirectional plasticity, i.e. tLTP and no plasticity, respectively. H, 5 post-pre and pre-post pairings \((n = 16)\) induced tLTP and no plasticity, respectively. I, summary graph showing the effect of different numbers of pairings (from 100 to 2) on long-term plasticity induction. There is an absence of corticostriatal tLTP with 50, 25 or 2 post-pre pairings while 75–100 or 5–10 post-pre pairings induced significant tLTP. Bidirectional (tLTD and tLTP) STDP is observed for 75–100 pairings, unidirectional (tLTD) STDP for 50 pairings and unidirectional (tLTP) STDP for 5–10 pairings. Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 50 min after STDP protocol (grey traces). Error bars represent SD. * \( P < 0.05 \).
Student’s t test or the one sample t test when appropriate at the significance level (P) indicated.

Results
We investigated whether a low number of post- and presynaptic paired stimulations induced plasticity in a major synaptic learning rule such as STDP. As previously described (Fino et al. 2005; Shen et al. 2008; Pawlik & Kerr, 2008; reviewed in Kreitzer & Malenka, 2008; Fino & Venance, 2010; Feldman, 2012), 100 pairings induced bidirectional STDP in MSNs: post–pre pairings (−30 < ΔtSTDP < 0 ms) induced tLTP (mean value of the EPSC amplitude recorded 50 min after STDP protocol: 142 ± 16%, P = 0.0262, n = 10), while pre–post pairings (0 < ΔtSTDP < +30 ms) induced tLTD (66 ± 10%, P = 0.0124, n = 7) (Fig. 1A–C and I). We observed a similar bidirectional STDP with 75 pairings; post–pre and pre–post pairings induced tLTP (167 ± 26%, P = 0.0378, n = 8) and tLTD (64 ± 5%, P = 0.0010, n = 6), respectively (Fig. 1D and I). We then decreased the number of pairings to 50 and observed contrasting effects on synaptic plasticity: while a potent tLTD persisted with pre–post pairings (66 ± 6%, P = 0.0013, n = 7), the tLTP usually associated with post–pre pairings disappeared (103 ± 12%, P = 0.7902, n = 7) (Fig. 1E and I). In turn tLTD disappeared for 25 pre–post pairings (94 ± 3%, P = 0.0801, n = 8) (Fig. 1F and I). On the post–pre side there was still no significant plasticity (132 ± 24%, P = 0.1985, n = 11) following 25 pairings, although half of the cells displayed tLTP and the other half no plasticity (Fig. 1F and I). Unexpectedly, decreasing the number of paired stimulations further unveiled another trend: whereas 10 pre–post pairings failed to induce significant plasticity (99 ± 10%, P = 0.9267, n = 9), 10 post–pre pairings were sufficient to induce a potent tLTP (165 ± 11%, P < 0.0001, n = 27) (Fig. 1G and I). A similar picture was obtained even with 5 pairings: post–pre pairings induced tLTP (139 ± 13%, P = 0.0087, n = 16) while pre–post pairings induced no significant plasticity (93 ± 6%, P = 0.2417, n = 6) (Fig. 1H and I). Finally, no significant plasticity was detected with 2 post–pre pairings (108 ± 9%, P = 0.4013, n = 6) (Fig. 1I). Note that a
In conclusion, tLTD disappears with decreasing numbers of paired stimulations whereas tLTP displays a biphasic profile since tLTP is observed for 75–100 pairings and 5–10 pairings (with similar amplitudes) and is absent for 25–50 pairings.

Figure 3. Induction of tLTP by 10 pairings involves postsynaptic 2-AG signalling
A and B, summary graphs of pharmacological experiments delineating the intracellular signalling pathways involved in 10 pairings-induced tLTP. A, tLTP was prevented by inhibition of group-I mGluR with MCPG (500 μM, n = 5) or of DAGLα by i-THL (10 μM, n = 5); the prefix ‘i’ indicates that the drug was applied in the recorded postsynaptic neuron through the patch-clamp pipette). B, summary bar graphs showing that tLTP was mediated by mGluR5 and M1R. Indeed, tLTP was prevented by inhibition of group-I mGluR with MCPG (500 μM, n = 5) and more specifically by inhibition of mGluR5 with MPEP (10 μM, n = 6); inhibition of M1R also prevented tLTP (1 μM pirenzepine, n = 6). Downstream of these receptors, inhibition of postsynaptic G-protein-coupled receptors (with 2 mM i-GDP-β-S, n = 10), PLCβ (with 5 mM i-U73122, n = 5), DAGLα (with 10 μM i-THL, n = 5) shows the involvement of PLCβ and 2-AG synthesis. In addition, bar graphs show the involvement of postsynaptic intracellular calcium (inhibited by 10 mM i-BAPTA, n = 5) and VSCCs (inhibited by 1 μM nimodipine, n = 5), since their blockade prevented the expression of tLTP. C, repeated brief application of 2-AG induces LTD. A series of 100 2-AG puffs (100 μM, 300 ms duration each) delivered at 1 Hz at the vicinity (50–100 μm) of the recorded striatal neuron, induced LTD in the absence of any STDP protocol (n = 8). This 2-AG-mediated LTD was prevented by AM251 (3 μM, n = 6). D, limited brief application of 2-AG induces LTD. Application of 10 2-AG puffs (100 μM, 300 ms duration each) delivered at 1 Hz was able to induce LTD in the absence of any STDP paired stimulation (n = 8). Inhibition of CB1R with bath-applied AM251 (3 μM, n = 5) prevented the induction of LTD by 2-AG puffs. Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 50 min after STDP protocol (grey traces). Error bars represent SD. *P < 0.05.
tlTP induced by 10 pairings is NMDAR independent

We then questioned the mechanism of tLTD and tLTP induced by these different numbers of pairings. We observed that the corticostriatal 100 pairings-induced tLTD was CB1R dependent, as previously demonstrated (Shen et al. 2008; Pawlak & Kerr, 2008; Fino et al. 2010; Paillé et al. 2013). Indeed, pharmacological inhibition of CB1R with AM251 (3 μM) prevented the expression of 100 pairings-induced tLTD (106 ± 6%, P = 0.4153, n = 5; Fig. 2A). Note that AM251 alone (without electrical stimulation) had no effect on basal synaptic transmission (99 ± 5%, P = 0.7988, n = 8; Table 1), indicating that CB1R had no constitutive activity at corticostriatal synapses. Similarly to the 100 pairings-induced tLTD, the 50 pre–post pairings-induced tLTD was prevented with AM251 (3 μM) (114 ± 17%, P = 0.4541, n = 7; Fig. 2B). Thus, the pre–post corticostriatal tLTD was CB1R mediated.

Concerning the post–pre pairing-induced tLTP, we confirmed that the 100 post–pre pairings-induced tLTP was NMDAR mediated since it was prevented with the selective NMDAR blocker d-AP5 (50 μM) (104 ± 5%, P = 0.4310, n = 5; Fig. 2C), as previously reported (Shen et al. 2008; Pawlak & Kerr, 2008; Fino et al. 2010; Paillé et al. 2013). We then explored the mechanism of the tLTP induced by 10 post–pre pairings and found that it does not rely on the same signalling pathway (i.e. NMDAR). Indeed, the tLTP induced by 10 pairings was not significantly affected by d-AP5 (169 ± 22%, P = 0.0098, n = 13; Fig. 2C), questioning the identity of the signalling pathways underlying this new form of tLTP.

tLTP induced by 10 pairings involves postsynaptic 2-AG signalling

The corticostriatal synapse is glutamatergic and we first tested whether glutamatergic G-protein-coupled receptors were required for the expression of tLTP induced by 10 pairings. MSNs express group-I metabotropic glutamate receptors (mGluRs) (Testa et al. 1994) belonging to the class of Gq/11-coupled receptors. tLTP induced by 10 pairings was prevented by the inhibition of group-I mGluR with MCPG (500 μM) (100 ± 8%, P = 0.9636, n = 5; Fig. 3A and B). More specifically, among group-I mGluRs, MSNs express prominently the mGluR5 isoform (Uchigashima et al. 2007). MPEP (10 μM), a blocker of mGluR5, prevented the induction of 10 pairings-induced tLTP and a slight depression was observed (68 ± 8%, P = 0.0108, n = 6; Fig. 3B). Besides glutamate, acetylcholine is also released following cortical activation of the corticostriatal synapses, since striatal cholinergic interneurons are tonically active and directly contacted by cortical pyramidal cells. Interestingly, MSNs express the M1 muscarinic receptors which are also another class of Gq/11-coupled receptors (Hersch et al. 1994; Yamasaki et al. 2010). We thus tested whether these receptors could be involved in the 10 pairings-induced tLTP. We found that the inhibition of M1 muscarinic receptors with pirenzepine (1 μM) prevented tLTP (95 ± 20%, P = 0.8037, n = 6; Fig. 3B). Altogether, these results show that the tLTP triggered by 10 pairings requires the concomitant activation of mGluR5 and M1 muscarinic receptors. We investigated the localization of these receptors with the application in the recorded postsynaptic neuron of a non-hydrolysable nucleotide GDPβS which prevents G-protein activation (2 mm i-GDPβS applied intracellularly through the patch-clamp pipette). i-GDPβS precluded tLTP (90 ± 10%, P = 0.3249, n = 10) indicating that mGluR5 and/or M1 receptors were postsynaptically located (Fig. 3B). Group-I mGluRs and M1 receptors are Gq/11-coupled receptors and thus activate phospholipase Cβ (PLCβ; Rebecchi & Pentyala, 2000). We then tested if PLCβ activation was involved in the 10 pairings-induced tLTP. In the presence of a PLCβ inhibitor applied intracellularly through the patch-clamp pipette (i-U73122, 5 μM) 10 post–pre pairings failed to induce any significant plasticity (94 ± 7%, P = 0.4255, n = 5; Fig. 3B), confirming the implication of postsynaptic PLCβ.

After activation by Gq/11-coupled receptors, PLCβ increases the levels of calcium ions (Rebecchi & Pentyala, 2000). We therefore tested the involvement of calcium in the expression of 10 pairings-induced tLTP. We first showed that postsynaptic calcium elevation, in the recorded striatal neuron, was mandatory for tLTP induction. Indeed, specific loading of the recorded postsynaptic neuron with the fast calcium buffer BAPTA (10 mm i-BAPTA applied intracellularly) prevented tLTP induction (108 ± 10%, P = 0.4774, n = 5; Fig. 3B). We next demonstrated that calcium entry via L-type voltage-sensitive calcium channels (VSCCs), the main type of activated VSCCs in MSNs (Carter & Sabatini, 2004), is responsible since their blockade (1 μM nimbodipine, 95 ± 9%, P = 0.6092, n = 5) precluded tLTP (Fig. 3B). Further downstream in the signalling pathway, these concomitant activations are expected to promote diacylglycerol lipase-α (DAGLα) activity and therefore 2-arachidonoylglycerol (2-AG) synthesis (Hashimotodani et al. 2005; Piomelli et al. 2007; Di Marzo, 2008; Kano et al. 2009; Tanimura et al. 2010; Alger & Kim, 2011). 2-AG is produced from the PLCβ product diacylglycerol by calcium-activated DAGLα and is the principal eCB involved in modulating synaptic strength by selectively activating CB1R (Piomelli et al. 2007). We found that a DAGLα inhibitor, tetrahydrolipstatin (10 μM i-THI applied intracellularly), prevented tLTP (91 ± 7%, P = 0.2607, n = 5; Fig. 3A and B). Importantly, since i-THI application was restricted to the recorded neuron, this result indicates that the production of 2-AG needed to activate CB1R arises from the postsynaptic striatal neuron.
engaged in the paired stimulations. In summary, tLTP induced with 10 pairings involves the 2-AG synthesis pathway.

To further demonstrate the key role of 2-AG in bidirectional STDP (tLTD and tLTP with 100 pre–post pairings and 10 post–pre pairings, respectively), we applied local puffs of 2-AG of different duration in the vicinity (50–100 μm) of the recorded striatal neuron. We first applied 100 brief (300 ms) puffs of 2-AG (100 μM) at 1 Hz, i.e. the same total duration as a 100 pairings STDP protocol at 1 Hz. In these conditions (i.e. in the absence of STDP protocols), we observed that local application of 2-AG was able to induce a significant LTD (68 ± 10%, P = 0.0156, n = 8; Fig. 3C) with a magnitude similar to the tLTD induced by 100 pre–post electrical pairings (P = 0.8624). This form of LTD involves CB1R activation since 2-AG puffs did not induce any more plasticity when AM251 (3 μM) was bath applied (96 ± 5%, P = 0.5000, n = 6; Fig. 3C).

We then aimed at mimicking the 10 pairing-induced LTP by applying brief puffs (300 ms) of 2-AG (100 μM) 10 times at 1 Hz, thus with the same total duration as a 10 pairings STDP protocol at 1 Hz. We observed that even in the absence of STDP protocol, local application of 2-AG was able to induce a significant LTP (139 ± 24%, P = 0.0391, n = 8; Fig. 3D) with a magnitude similar to the tLTP induced by 10 post–pre pairings (P = 0.3705). This LTP involved CB1R activation since 2-AG puffs did not induce any more plasticity in the presence of AM251 (3 μM) (92 ± 4%, P = 0.1542, n = 5; Fig. 3D).

tLTP induced by 10 pairings is CB1R activation mediated and presynaptic

Since 2-AG is a specific ligand of CB1Rs (Piomelli et al. 2007; Di Marzo et al. 2008; Alger & Kim, 2011; Katona & Freund, 2012), we then asked whether tLTP induced by 10 pairings was indeed CB1R mediated. Pharmacological inhibition of CB1R with AM251 (3 μM) prevented the expression of 10 pairings-induced LTP (80 ± 11%, P = 0.1424, n = 6; Fig. 4A).

This pharmacological result was further confirmed by experiments with CB1R-knockout (CB1R−/−) mice (Ledent et al. 1999), where no significant plasticity was observed following 15 pairings (93 ± 4%, P = 0.0882, n = 16) whereas tLTP could be induced in the littermate wild-type CB1R+/+ mice (135 ± 5%, P = 0.0001, n = 10; Fig. 4B and C). Note that in C57BL/6 mice 15 pairings with 2–3 postsynaptic APs were required to efficiently induced tLTP while 5–10 pairings with a single postsynaptic AP per pairing were sufficient to trigger tLTP in rats. This CB1R-mediated tLTP was expressed up to adulthood since we observed a reliable tLTP in postnatal days 60–90 CB1R+/+ mice (137 ± 8%, P = 0.0109, n = 5), which was absent in postnatal days 60–90 CB1R−/− mice (93 ± 6%, P = 0.3048, n = 5; Fig. 4C). Pharmacological and genetic evidence demonstrated that tLTP induced by 10 pairings is eCB mediated. We thus refer to this new form of LTP as eCB-tLTP.

The results described above show that synthesis and release of eCBs in eCB-tLTP relies on signalling pathways that are located in the postsynaptic neuron. We then searched for the locus of eCB-tLTP maintenance. As shown above, eCB-tLTP is CB1R activation dependent and since CB1Rs are expected to be located on presynaptic corticostriatal terminals (Katona & Freund, 2012), the locus of eCB-tLTP maintenance is likely to be presynaptic. We used paired-pulse stimulations to test this hypothesis. Paired-pulse intervals of 50 ms induced a significant EPSC paired-pulse facilitation (PPF) in striatal output neurons (Goubard et al. 2011). Thus, we applied paired pulses with 50 ms interpulse interval before and after STDP protocol. We observed a significantly decreased PPF (PPF, plasticity/baseline = 0.912 ± 0.037, P = 0.4366, n = 9), indicating a presynaptic origin of the plasticity (Fig. 2D). This was further confirmed by the mean variance analysis of EPSCs: we obtained a CV2 value of 3.1 ± 0.6 (P = 0.0014, n = 20), which also indicated a presynaptic maintenance of eCB-tLTP (Fig. 2E). In conclusion, our results indicate that eCB-tLTP is induced postsynaptically through the synthesis and release of eCBs, and maintained presynaptically downstream of CB1Rs.

eCB-LTP induction involves TRPV1

Besides 2-AG, the production of another eCB, anandamide, can also be increased upon cellular activity (Piomelli et al. 2007; Alger & Kim, 2011). Whereas 2-AG is a specific ligand of CB1R, anandamide activates both CB1R (albeit less potently than 2-AG) and TRPV1. TRPV1 is a non-selective cationic channel (Ross, 2003; Starowicz et al. 2007; Di Marzo, 2008) involved in eCB-mediated short- and long-term depression (Gibson et al. 2008; Maione et al. 2009; Chávez et al. 2010; Grueter et al. 2010; Puente et al. 2011). We therefore tested whether TRPV1 was implicated in eCB-tLTP. Note that in the absence of paired stimulation, application of capsazepine (10 μM), a TRPV1 antagonist, had no significant effect on basal EPSC (100 ± 4%, P = 0.9778, n = 5; Table 1), indicating that TRPV1 has no constitutive activity at corticostriatal synapses. We then found that the application of capsazepine (10 μM) during the STDP stimulation protocol (10 post–pre pairings) efficiently blocked eCB-tLTP (83 ± 11%, n = 6, P = 0.1133; Fig. 5). To confirm this result, we used AMG9810, another competitive TRPV1 antagonist, structurally distinct from capsazepine, and observed that AMG9810 (1 μM) also blocked eCB-tLTP (93 ± 6%, P = 0.3046, n = 5; Fig. 5B).
Altogether, our results demonstrate that 10 pairings-induced tLTP is mediated by eCB (2-AG and anandamide), acting on both CB₁R and TRPV1.

**eCB-tLTP occurs in both striatopallidal and striatonigral MSNs and is dopamine dependent**

Pyramidal cells from cortex layer 5 contact two MSN subpopulations belonging to the direct (striatonigral) or indirect (striato-pallido-subthalamo-nigral) trans-striatal pathways (Gerfen & Surmeier, 2011; Calabresi et al. 2014). We investigated whether eCB-tLTP is induced in both striatopallidal and striatonigral MSNs. The two MN subtypes express different dopaminergic receptors (D₁R-like and D₂R-like for the direct and indirect pathways, respectively) allowing us to identify them with transgenic D₁-eGFP mice and to investigate eCB-tLTP occurrence in D₁⁺ and non-D₁⁺ MSNs (Fig. 6A and B). We observed that 15 post–pre pairings (see Methods) induced tLTP in both D₁⁺ (165 ± 18%, \( P = 0.0166, n = 7 \)) and non-D₁⁺ 

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**Figure 4. tLTP induced by 10 pairings is CB₁R mediated**

A, tLTP induced by 10 pairings is prevented by a specific CB₁R inhibitor, AM251 (3 μM, \( n = 6 \)). B, 10 post–pre pairings induced tLTP in wild-type CB₁R⁺/⁺ mice (\( n = 10 \)) while no plasticity was observed in CB₁R⁻/⁻ mice (\( n = 16 \)). C, summary bar graphs with CB₁R⁻/⁻ and CB₁R⁺/⁺ mice illustrate that eCB-tLTP is CB₁R mediated both in juvenile (postnatal days 18–25) and adult (postnatal days 60–90) animals. D, representative traces and summary bar graphs (\( n = 9 \)) of paired-pulse cortical stimulation with 50 ms interstimulus interval illustrate a decrease of facilitation after STDP. This indicates a presynaptic locus of the eCB-tLTP. E, mean variance analysis (CV², \( n = 20 \)) indicates a presynaptic locus of eCB-tLTP maintenance. Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 50 min after STDP protocol (grey traces). Error bars represent SD. *\( P < 0.05 \).

**Figure 5. eCB-LTP is also TRPV1 mediated**

A, tLTP induced by 10 pairings was prevented when TRPV1 was inhibited by capsazepine (10 μM, \( n = 6 \)). B, summary bar graphs show that capsazepine (10 μM, \( n = 6 \)) or AMG9810 (1 μM, \( n = 5 \)) prevented the 10 pairings-induced tLTP. Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 50 min after STDP protocol (grey traces). Error bars represent SD. *\( P < 0.05 \), ns, non-significant.
Endocannabinoids mediate bidirectional striatal STDP

(156 ± 20%, P = 0.0215, n = 9) MSNs (Fig. 6A and B). This indicates that tLTP can be induced with few pairings in both striatopallidal and striatonigral MSNs.

Striatum receives excitatory afferents from the cortex as well as a dense innervation from midbrain dopaminergic neurons. Dopamine, a key regulator of action selection and associative learning (Yin & Knowlton, 2006; Redgrave & Gurney, 2006; Schultz, 2007), efficiently modulates corticostriatal synaptic plasticity and particularly the ‘classical’ eCB-LTD (Kreitzer & Malenka, 2008; Di Filippo et al. 2009; Gerfen & Surmeier, 2011). We then asked whether dopamine was involved in eCB-tLTP induction. We examined which dopaminergic receptor subtype is involved in eCB-tLTP. For this purpose we first bath applied a mixture of D₁R and D₂R antagonists, SCH23390 (4 μM) and sulpiride (10 μM), respectively. We found that this cocktail prevented the induction of eCB-tLTP and a depression was observed (75 ± 29%, P < 0.0001, n = 7; Fig. 6B). Thus, eCB-tLTP is dopamine dependent. We then selectively inhibited either D₁R or D₂R. When we applied the D₁R antagonist SCH23390 (4 μM) alone, we observed a potent tLTP (149 ± 16%, P = 0.0211, n = 7) while tLTD could be elicited when the D₂R antagonist sulpiride (10 μM) was bath applied alone (68 ± 10%, P = 0.0209, n = 6; Fig. 6C). This indicates that eCB-tLTP is D₂R mediated and not dependent on D₁R.

Corticostratial eCB-tLTP is paired-activity dependent

To confirm that eCB-tLTP requires both pre- and postsynaptic activity, we performed paired recordings of two neighbouring MSNs (perisomatic distance < 50 μm) in which one neuron was subject to 10 post–pre pairings (STDP protocol) while the second received only 10 presynaptic stimulations (n = 6 pairs; Fig. 7A). We observed a potent tLTP (150 ± 13%, P = 0.0143, n = 6) only in the neuron subject to post–pre pairings, while the neighbouring neuron, which received only the presynaptic stimulation, did not show any significant plasticity (106 ± 3%, P = 0.0833, n = 6), indicating that corticostriatal eCB-tLTP is paired-activity dependent.

In the hippocampus, facilitation of LTP via eCB-induced presynaptic depression of GABAergic transmission has been reported (Carlson et al. 2002; Chevaleyre & Castillo, 2004; Zhu & Lovinger, 2007). Here, the observed eCB-tLTP could arguably arise from a decrease of GABA release, through an activation of CB₁Rs located on GABA

![Figure 6](https://example.com/figure6.png)

Figure 6. eCB-tLTP is induced in both striatopallidal and striatonigral MSNs and is dopamine dependent. A, tLTP induced by 15 pairings is observed in both striato-pallidal (D₁-eGFP positive neurons, D₁⁺, n = 7) and striato-nigral (D₁-eGFP negative neurons, D₁⁻, n = 9) MSNs. B, co-application of antagonists of D₁R and D₂R, SCH23390 (4 μM) and sulpiride (10 μM), prevents eCB-tLTP (n = 7). C, tLTP was induced in presence of the D₂R antagonist SCH23390 (4 μM, n = 7), while no plasticity was observed with the D₂R antagonist sulpiride (10 μM, n = 6). Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 40 (A) and 50 (B and C) min after STDP protocol (grey traces). Error bars represent SD.
terminals, thus decreasing the inhibitory tonus during the pairing protocol. To test this hypothesis, we blocked the GABA<sub>A</sub> receptors with picrotoxin (50 μM). A significant tLTP was still observed for 10 pairings (135 ± 12, n = 6; P = 0.0300; Fig. 7B and C). The magnitude of 10 pairings-induced tLTP was not affected by the blockade of GABA<sub>A</sub> transmission since there was no significant difference compared to control conditions (P = 0.1200). In agreement with our recent report of STDp induced by 100 pairings (Paille et al. 2013), GABA controls the polarity of the timing dependence of STDp: with picrotoxin, tLTP was observed with 10 pre–post pairings (n = 6; Fig. 7C) while 10 post–pre pairings did not induce plasticity (96 ± 7, P = 0.5865, n = 7; Fig. 7B). We confirmed that the tLTP observed in the presence of picrotoxin was still eCB-mediated: the co-application of picrotoxin (50 μM) and AM251 (3 μM) prevented the induction of tLTP (103 ± 79, P = 0.7454, n = 5; Fig. 7C). In conclusion, GABAergic microcircuits are not involved in the synaptic efficacy changes induced by 10 pairings but control the polarity of the timing dependence of the eCB-tLTP.

**Bidirectional eCB-STDP in the same neuron**

eCB-tLTP and eCB-tLTD could represent functional inverses of each other. This could be demonstrated if both phenomena could be sequentially triggered in the same neuron to modify the synaptic weight and then bring it back to its baseline. We tested this hypothesis by applying successively two protocols leading to unidirectional plasticity, which exclusively imply eCBs: 10 post–pre pairings (eCB-tLTP; Figs 1 and 4) and 50 pre–post pairings (eCB-tLTD; Fig. 2B). We found that eCB-tLTP and eCB-tLTD can indeed be induced sequentially in the very same neuron independently of the order of induction protocols (P < 0.0001 for both forms of plasticity, comparing the 30 EPSCs preceding the STDP protocols to the last 30 EPSCs 25–30 min after the STDP.

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**Figure 7. Corticostriatal eCB-tLTP is paired activity dependent**

*Figure* scheme and raw traces illustrating paired recordings of MSNs (perisomatic distance < 50 μm): one neuron received 10 pairings STDP protocol (pre- and postsynaptic stimulations) while the other one only received 10 presynaptic stimulations. tLTP was induced exclusively in the neuron with the STDP protocol (10 post–pre pairings, black circles, n = 6) while no significant plasticity was observed in the neighboring neuron (presynaptic stimulations only, n = 6). B: Inhibition of the GABA<sub>A</sub> transmission with picrotoxin did not affect eCB-tLTP magnitude but controlled the time dependence of eCB-tLTP. With bath-applied picrotoxin (50 μM), a potent tLTP was induced by 10 pre–post pairings (n = 6; blue symbols) while no significant plasticity was observed with 10 post–pairings (n = 7; black). The occurrence and magnitude of tLTP were not affected by a blockade of GABA<sub>A</sub> transmission since induced plasticities were not significantly different from the ones observed in control conditions. This tLTP induced with 10 pre–post pairings under GABA<sub>A</sub> receptor blockade is mediated by eCB since it was prevented with bath application of AM251 (3 μM). GABAergic microcircuits are not involved in the synaptic efficacy changes themselves but control the time dependence of the eCB-tLTP. Representative traces are the average of 15 EPSCs during baseline recording (black traces) and 50 min after STDP protocol (grey traces). Error bars represent SD.

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protocols, \( n = 5 \); Fig. 8). Following eCB-LTP induction by 10 post–pre pairings, the potentiated synaptic weight can be decreased back to its basal level by applying 50 pre–post pairings (Fig. 8A). Conversely, the synaptic weight depressed by an eCB-tLTD could be increased again by eCB-tLTP induction in the same neuron (Fig. 8B). These results demonstrate that eCB-tLTP and eCB-tLTD can be induced sequentially in the same neuron.

**Discussion**

Corticostratial long-term plasticity provides a fundamental mechanism for the function of the basal ganglia in action selection and in procedural learning (Yin & Knowlton, 2006; Yin et al. 2009; Koralek et al. 2012). Thus, characterizing the striatal plasticity repertoire in physiological conditions is crucial. The striatum receives a wide range of patterns of cortical activities from isolated trains of few spikes to prolonged bursting events. While corticostriatal plasticity under prolonged activation is well elucidated, its occurrence in response to few spikes has remained unexplored. Here, we report the existence of an eCB-tLTP induced by a low number of pairings in the striatum of both juvenile and adult rodents. Indeed, a few coincident pre- and postsynaptic spikes (5–10) were found to increase synaptic efficacy through a signalling pathway that relies on the eCB system. eCB-tLTP induction relies on activation of CB1R and TRPV1 and on 2-AG elevations triggered by coupled rise of calcium and DAGLα activity (mediated by mGluR5, muscarinic M1 receptors and VSCCs) in MSNs. Both activation of glutamatergic afferents from cerebral cortex and striatal cholinergic interneurons (which are monosynaptically contacted by cortical pyramidal cells (Fino et al. 2008)) promote the induction of eCB-tLTP.

We also found that eCB-tLTP is dopamine dependent. More precisely, eCB-tLTP is D2R mediated and not dependent on D1R. We then investigated the localization (pre- or postsynaptic) of the D2R involved in eCB-tLTP. The postsynaptic localization at MSNs was a priori less likely. Indeed, due to the segregation of expression of D1R and D2R among MSNs (Kreitzer & Malenka, 2008; Gerfen & Surmeier, 2011; Calabresi et al. 2014), roughly half of MSNs are expected to be D2R-expressing neurons. If eCB-tLTP was supported by the postsynaptic D2R MSNs, one would expect to induce eCB-tLTP in ~50% of the (randomly chosen) MSNs. In our experiments, eCB-tLTP was successfully induced in 83% of the (randomly chosen) MSNs in rats, thus suggesting a presynaptic localization of the D2R. This was confirmed with experiments performed in D1R-eGFP mice, which show that eCB-tLTP can be induced in both striatopallidal and striatonigral MSNs. This suggests that the D2Rs involved in eCB-tLTP are not postsynaptically located. Presynaptic D2Rs are expressed at three different locations: the nigrostriatal dopaminergic afferents (De Mei et al. 2009), the cholinergic interneurons (Hersch et al. 1995) and the corticostriatal glutamatergic afferents (Bamford et al. 2004). The precise locus of presynaptic D2R involved in eCB-tLTP remains to be determined.

We describe here a paired-activity-dependent tLTP in mammals, wherein eCB signalling directly underlies both the induction and the long-term maintenance of synaptic weight increase. eCB signalling exhibits bidirectional plasticity with both eCB-tLTP and eCB-tLTD. Bidirectionality is of paramount functional importance since it allows LTP and LTD to reverse each other at a single synapse.

eCBs have emerged as a major actor in learning and memory because of their powerful influence on synaptic

![Figure 8. Bidirectional eCB synaptic plasticity in a single neuron](image)
plasticity (Katona & Freund, 2012; Castillo et al. 2012; Melis et al. 2014). The eCB system is mainly composed of biolipids synthesized and released on demand, acting as retrograde neurotransmitters on presynaptic CB;R (one of the most abundant G protein-coupled receptors in the brain) and postsynaptic TRPV1. eCBs have been reported to depress synaptic weight, i.e. short- or long-term depression, through the activation of CB;R (Kano et al. 2009; Katona & Freund, 2012; Castillo et al. 2012; Melis et al. 2014) or TRPV1 (Gibson et al. 2008; Maione et al. 2009; Chávez et al. 2010; Grueter et al. 2010; Puente et al. 2011). Noticeable exceptions are reports of an indirect role of eCBs in promoting LTP at mixed (chemical and electrical) synapses of the goldfish Mauthner cell via intermediary dopaminergic neurons (Cachope et al. 2007) or at hippocampal CA1 synapses via a GABA_A receptor-mediated mechanism (Lin et al. 2011; Xu et al. 2012). Likewise, facilitation of LTP in the hippocampus via eCB-induced presynaptic depression of GABAergic transmission (Carlson et al. 2002; Chevaleyre & Castillo, 2004; Zhu & Lovinger, 2007), and mediation of heterosynaptic short-term potentiation via intermediary astrocytes (Navarrete & Araque, 2010) have been reported. However, to our knowledge, the present study is the first report of a paired-activity eCB-dependent LTP in mammals, with direct implication of eCBs in the induction and long-term maintenance of spike-timing-dependent potentiation of the stimulated synapse itself.

mGluR5 and M1R need to be simultaneously activated to elicit eCB-tLTP. Both receptors (mGluR5 and M1) are G_q/11-coupled receptors and positively coupled to PLC_B, thus leading to DAG production, and favouring the synthesis of 2-AG. Whereas cholinergic activation is not sufficient to trigger eCB-LTP, it remains necessary for eCB-LTP induction. Assuming that mGluR5 and M1 are localized postsynaptically (which remains to be demonstrated), our hypothesis is that eCB-LTP is induced only when large levels of 2-AG are produced. Our results from M1, mGluR5, VSCC and TRPV1 blocking experiments indicate that it is mandatory to activate all possible cumulative contributions to 2-AG production (PLC_B activation for DAG production, VSCC and TRPV1 to increase calcium surge, thus activating DAGLR) in order to reach high levels of 2-AG, which would promote eCB-tLTP.

Just as in hippocampal pyramidal cells (Shouval et al. 2002; Graupner & Brunel, 2012), postsynaptic calcium levels (or time course) could be crucial in the induction of eCB-STDP in the striatum. Since many of the steps along the eCB pathways are Ca^{2+} dependent (including 2-AG and anandamide synthesis), the 'Ca^{2+} hypothesis' would translate to the CB;R pathway. This would lead to a scenario where low to moderate peak levels of eCB would lead to LTD whereas high eCB levels would yield LTP. According to this scenario, our results could be reconciled if the first 5–20 post–pre pairings produce very large peak levels of 2-AG, and thus LTP. If the amplitude of the 2-AG peaks decreases for subsequent post–pre pairings, this initial LTP would be de-potentiated by the subsequent pairings, thus restricting the expression of eCB-tLTP to the first 5–20 pairings. On the other hand, the LTP observed with 100 post–pre pairings entirely results from an increase of the postsynaptic weight through activation of the CaMKII pathway by NMDARs, with no additional contribution of the eCB-LTP; indeed, when CB;R was inhibited, the NMDAR-tLTP induced with 100 post–pre pairings was not significantly affected (Fino et al. 2010). Thus, according to this scenario, eCB-LTP would start to be expressed after 5–20 post–pre pairings and subsequent pairings would then erase this potentiation while, independently, triggering the expression of NMDA-LTP.

There is a large diversity of the STDP rules at play in the brain and even within the same structure, variety seems to be the rule (Feldman, 2012). Indeed, in striatum the main neuronal population, the MSNs, express NMDAR-tLTP and eCB-tLTD (Shen et al. 2008; Pawlak & Kerr, 2008; Fino et al. 2010; Paille et al. 2013) and eCB-tLTP (the present report). Whereas neighbouring striatal fast-spiking GABAergic interneurons express solely NMDAR-dependent STD, both for LTP and LTD, for 100 pairings (Fino et al. 2008) but lack plasticity at low numbers of pairings (data not shown). In addition, our results provide evidence that eCB-tLTP is anti-Hebbian at corticostriatal synapses and tightly controlled by GABAergic interneurons, similar to the bidirectional corticostriatal STD (i.e. NMDAR-tLTP and eCB-tLTD; Paille et al. 2013). It has been reported that the endocannabinoid-mediated LTP at hippocampal CA1 synapses induced with high-frequency (Lin et al. 2011), low-frequency (Zhu & Lovinger, 2007) or paired stimulations (Xu et al. 2012) were prevented not only by inhibition of CB;R but also by inhibition of GABA_A receptors. Here, we show that GABA is not involved in eCB-tLTP induction or magnitude at corticostriatal synapses but controls the polarity of eCB-tLTP.

Due to their on-demand intercellular signalling modus operandi (Alger & Kim, 2011), eCB biosynthesis and release are evoked by precisely timed and positioned physiological stimuli (Katona & Freund, 2008). However, evidence for TRPV1 activation by physiological neuronal activity patterns was lacking. As previously described, our study confirms that STDP indeed efficiently triggers eCB signalling and we demonstrate that STDP is able to engage the TRPV1 signalling pathway. Being a cationic channel highly permeable to calcium (Ross, 2003; Starowicz et al. 2007; Di Marzo, 2008), TRPV1 may contribute to eCB-tLTP induction by boosting the calcium transients in the postsynaptic element. As recently described for short- and long-term depression (Puente et al. 2011), our results illustrate the versatility of eCB signalling as
a system displaying polymodal activation through CB$_1$R and TRPV1, to trigger LTP.

eCB-LTP is promoted by very low numbers of pairings ($\sim$5–10), therefore providing a mechanism whereby synapses react to the very first occurrences of incoming activity. This ability contrasts strongly with NMDAR-dependent LTP which requires the iteration of at least 75–100 paired stimulations to be expressed in the classical (1 Hz) STDP context. In mammals, associative memories and behavioural rules can be learned within a few trials (5–10) or even sometimes within a single trial (Schultz et al. 2003; Pasupathy & Miller, 2005; Tse et al. 2007; Quilodran et al. 2008; Ito & Doya, 2009). In the cortex or striatum, neurons with behaviour-related activities fire a few spikes upon behaviourally relevant events during each trial (i.e. at a frequency of 5–25 Hz and during 0.1–0.5 s, typically < 10 spikes; Schultz et al. 2003; Pasupathy & Miller, 2005; Quilodran et al. 2008), suggesting that a few trials should be sufficient to induce synaptic plasticity. eCB-LTP may be used for learning and memorizing salient events from a few spikes. Hence, eCB-LTP may represent a molecular substrate operating during rapid learning of new arbitrary associative memories and behavioural rules characterizing the flexible behaviour of mammals or during the initial stages of slower habit learning (Barnes et al. 2011). Considering a specific context, the impairment of working memory by marijuana intoxication can be re-evaluated in light of our results. This impairment was hitherto interpreted solely as the effect of cannabinoids on the promotion of synaptic depression. Our results, together with recent reports (Lin et al. 2011; Xu et al. 2012), open new perspectives since they suggest that synaptic potentiation may as well be implied in the effects of marijuana.

References


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Endocannabinoids mediate bidirectional striatal STDP

Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

Y.C., H.B. and L.V. conceived and designed the experiments; Y.C., V.P., H.X. and E.F. performed electrophysiological experiments and analysis; H.B., B.D. and S.G. contributed to analytical tools; Y.C., H.B. and L.V. wrote the manuscript; all authors have edited and corrected the manuscript.

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Result part (II):

The role of dopamine in corticostriatal endocannabinoid-mediated STDP

(Articles 3 and preliminary results)
Endocannabinoid-dopamine interactions mediate striatal tLTP that is impaired in Parkinson’s disease rodent model

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ABSTRACT

Corticostriatal synaptic plasticity, a key substrate for action selection and procedural learning, is impaired in Parkinson’s disease. Thus, characterizing striatal plasticity repertoire in physiological and pathophysiological conditions is crucial. The striatum receives a wide range of patterns of cortical activities from isolated trains of few spikes to prolonged bursting events. While plasticity under prolonged activation is well elucidated, its expression to few spikes remains less documented. In rodent striatum, we recently unraveled a spike-timing-dependent potentiation (tLTP) mediated by endocannabinoid (eCB) system induced with very few spikes (~10-15). Here, we found that this eCB-tLTP is dopamine dependent using pharmacological tools and optogenetics. eCB-tLTP involves activation of D2R, and not dependent on D1R, but occurs in both striatopallidal and striatonigral MSNs. eCB-tLTP relies on the activation of presynaptic D2 receptors expressed in corticostriatal glutamatergic afferents. When we genetically-ablated selectively D2R-expressing MSN or ChAT interneurons using Cre-mediated expression of the diphteria toxin receptor and stereotaxic diphteria toxin in striatum, eCB-tLTP was expression was not prevented. Similarly, the selective ablation of dopaminergic cells with 6-OHDA did not prevent eCB-tLTP expression when supplemented with a D2R agonist. Lastly, we observed that eCB-tLTP was impaired in a rodent model of Parkinson’s disease and was rescued by chronic L-DOPA treatment. Thus, endocannabinoids constitute a versatile system underlying bidirectional plasticity, implicated in the pathophysiology of basal ganglia.
INTRODUCTION

Endocannabinoids (eCBs) have emerged as a major signaling system in learning and memory because of their powerful influence on synaptic plasticity, mainly as depressing synaptic function (Kano et al., 2009; Castillo et al., 2012; Katona and Freund, 2012; Mathur and Lovinger, 2012; Melis et al., 2014). Indeed, LTD mediated by eCBs is one of the most widespread plasticity in various structures in the brain. The eCB system is mainly composed of biolipids synthesized and released on-demand acting as retrograde neurotransmitters on presynaptic CB₁R (one of the most abundant G protein-coupled receptors in the brain) and postsynaptic TRPV1. eCBs have been mainly reported to depress synaptic weight, i.e. short- or long-term depression, through the activation of CB₁R (Kano et al., 2009; Castillo et al., 2012; Katona and Freund, 2012; Melis et al., 2014) or TRPV1 (Gibson et al., 2008; Maione et al., 2009; Chávez et al., 2010; Grueter et al., 2010; Puente et al., 2011).

Noticeable exceptions to the endocannabinoid-mediated synaptic depression, are reports of an indirect role of eCBs in promoting LTP at mixed (chemical and electrical) synapses of the goldfish Mauthner cell via intermediary dopaminergic neurons (Cachope et al., 2007) or at hippocampal CA1 synapses via a GABA₄ receptor-mediated mechanism (Lin et al., 2011; Xu et al., 2012). Likewise, facilitation of LTP in the hippocampus via eCB-induced presynaptic depression of GABAergic transmission (Carlson et al., 2002; Chevaleyre and Castillo, 2004; Zhu and Lovinger, 2007), and mediation of heterosynaptic short-term potentiation via intermediary astrocytes (Navarrete and Araque, 2010) have been reported.

In the striatum, using spike-timing dependent plasticity, a synaptic Hebbian learning rule which depends on the activity on either side of the synapse (Feldman, 2012), we previously reported STDP with NMDAR-mediated tLTP and endocannabinoid(eCB)-mediated tLTD, induced respectively by 100 post-pre and 100 pre-post paired stimulations (Fino et al., 2005, 2010; Fino and Venance, 2010). More recently we reported a spike-timing dependent
potentiation (tLTP) induced by a very low numbers of pairings (5-15 pairings), mediated by endocannabinoids (eCB-tLTP) and involving activation of CB1R and TRPV1 (Cui et al., 2015). To our knowledge, this eCB-tLTP is the first report of a paired-activity eCB-dependent LTP in mammals, with direct implication of eCBs in the induction and long-term maintenance of tLTP of the stimulated synapse itself. Knowing the crucial role of dopamine in striatal physiology and pathophysiology (Albin et al., 1989; DeLong, 1990; Schultz, 2007; Cools, 2011) and in particular in synaptic plasticity (Calabresi et al., 2007; Cerovic et al., 2013), we questioned the role of dopamine onto eCB-tLTP. We found that opto-inhibition of dopaminergic neurons prevent eCB-tLTP induction and that dopaminergic type 2 (D2R) receptors located at corticostriatal glutamatergic afferents are required for eCB-tLTP expression. We show that eCB-tLTP is impaired in a rodent model of Parkinson’s disease and is rescued by chronic L-DOPA treatment. Thus, eCB-tLTP relies on tight interactions between endocannabinoid and dopaminergic systems: eCB-tLTP is dependent on CB1R and TRPV1 activation as well as on D2 receptor activation located at corticostriatal glutamatergic afferents. eCB-tLTP is a new form of plasticity that may underlie the quick reactivity necessary for adapting the response of the synaptic weight during rapid learning and is altered in Parkinson’s disease.
METHODS

Animals

All experiments were performed in accordance with local animal welfare committee (Center for Interdisciplinary Research in Biology and the Université Libre de Bruxelles School of Medicine Ethical Committee) and EU guidelines (directive 2010/63/EU). Every precaution was taken to minimize stress and the number of animals used in each series of experiments. Sprague-Dawley rats (Charles River, L’Arbresle, France) and C57BL/6 mice (DAT-Cre (Turiault et al., 2007), Archeorhodopsin3-GFP, Adora2a-Cre, inducible-DTR, Drd1a-GFP and ChAT-cre) were used for brain slice electrophysiology. Animals were housed in standard 12 hours light/dark cycles and food and water were available ad libitum.

Brain slice preparation and patch-clamp recordings

Horizontal brain slices containing the somatosensory cortical area and the corresponding corticostriatal projection field were prepared according to the methods previously published (Paille et al., 2013; Cui et al., 2015). Corticostriatal connections (between somatosensory cortex layer 5 and dorsal striatum) are preserved in a horizontal plane. Briefly, horizontal brain slices with thickness of 330 or 300 μm were prepared, respectively, from rats (P(20-25) for most experiments, P50 for 6-OHDA lesion and P(60-65) for Parkinson’s disease animal model) or mice (P(17-25)) using a vibrating blade microtome (VT1200S, Leica Microsystems, Nussloch, Germany). Brains were sliced in a 95% CO2/5% O2-bubbled, ice-cold cutting solution containing (in mM) 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 1 pyruvic acid, and then transferred into the same solution at 34°C for one hour and then moved to room temperature.

Patch-clamp recordings were performed as previously described (Fino et al., 2005, 2010; Paille et al., 2013). Briefly, borosilicate glass pipettes of 4-6MΩ resistance contained for
whole-cell recordings (in mM): 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA (adjusted to pH 7.35 with KOH). The composition of the extracellular solution was (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 10μM pyruvic acid bubbled with 95% O₂ and 5% CO₂. Signals were amplified using EPC10-2 amplifiers (HEKA Elektronik, Lambrecht, Germany). All recordings were performed at 34°C using a temperature control system (Bath-controller V, Luigs&Neumann, Ratingen, Germany) and slices were continuously superfused at 2-3 ml/min with the extracellular solution. Slices were visualized on an Olympus BX51WI microscope (Olympus, Rungis, France) using a 4x/0.13 objective for the placement of the stimulating electrode and a 40x/0.80 water-immersion objective for localizing cells for whole-cell recordings. Current-clamp recordings were filtered at 2.5 kHz and sampled at 5 kHz and voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz using the Patchmaster v2x32 program (HEKA Elektronik). The series resistance was compensated at 75-80%.

Chemicals
N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, 3 μM) (Tocris) and (S)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide (sulpiride, 10 μM, Tocris) were dissolved in ethanol and then added in the external solution at a final concentration of ethanol of 0.015 and 0.1%, respectively. R(+-)7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390, 4 μM, Sigma) and (4aR-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride (Quinpirole, 10 μM, Tocris) were dissolved in water and then added in the external solution.
Note that none of the bath-applied drugs had a significant effect on basal EPSC amplitudes: AM251 (3 μM): 101±5% (p=0.8610, n=7), co-application of SCH23390 (4 μM) and sulpiride (10μM): 102±3% (p=0.5056, n=9) and quinpirole (10μM): 98±2% (p=0.3449, n=8).

**Spike-timing-dependent plasticity induction protocols**

Electrical stimulations were performed with a bipolar electrode (Phymep, Paris, France) placed in the layer 5 of the somatosensory cortex (Fino et al., 2005; Cui et al., 2015). Electrical stimulations were monophasic at constant current (ISO-Flex stimulator, AMPI, Jerusalem, Israel). Currents were adjusted to evoke 50-200 pA EPSCs. Repetitive control stimuli were applied at 0.1Hz. STDP protocols consisted in pairings of pre- and postsynaptic stimulations (at 1 Hz) with the two events separated by a specific temporal interval ($\Delta t_{STDP}$). Presynaptic stimulations corresponded to cortical stimulations and the postsynaptic stimulation of an action potential evoked by a depolarizing current step (30 ms duration) in MSNs. 30ms suprathreshold depolarization was chosen to mimic corticostriatal summation of EPSPs induced by cortical activity as observed in *in vivo* studies (Mahon et al., 2006). MSNs were maintained all along the STDP experiments at a constant holding membrane potential which corresponds to their initial resting membrane potential (-81.6±0.4 mV, n=61). Thus, EPSCs during baseline or after STDP protocol were measured at the same membrane potential (in voltage-clamp mode); STDP pairings (performed in current-clamp mode) were conducted also at this very same holding membrane potential. Neurons were recorded for 10 min during baseline and for at least 60 min after STDP protocol; long-term synaptic efficacy changes were measured from 50 min. 30 successive EPSCs (at 0.1 Hz) were individually measured and then averaged. Variation of series resistance beyond 20% led to the rejection of the experiment. After recording of 10 min control baseline, drugs were applied in the bath. A new baseline with drugs was recorded after a time lapse of 10 min (to allow the drug to be fully
perfused) for 10 min before the STDP protocol. Drugs were present until the end of the recording (except when specified). It should be noted that STDP protocol consisting in 5-10 post-pre pairings (with a single postsynaptic spike) were sufficient to induce potent tLTP in rat while in C57BL/6 mice 15 pairings (with 2-3 postsynaptic spikes) were necessary to trigger tLTP.

Electrophysiological data analysis

Off-line analysis was performed using Fitmaster (Heka Elektronik) and Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA). Statistical analysis was performed using Prism 5.0 software (San Diego, CA, USA). In all cases “n” refers to the number of repetitions of an experiment (each experiment being performed on different brain slices) from single slice. Experimenters were blind to the genotype of Adora2a-Cre+/−: iDTR+/−: Drd1a-GFP+/−, Adora2a-Cre−/−: iDTR+/−: Drd1a-GFP+/−, ChAT-cre+/−: iDTR+/−, ChAT-cre−/−: iDTR+/−, DAT-Cre−/−: Arch3-GFP+/− and DAT-Cre+/: Arch3-GFP+/− mice during electrophysiological recordings and analysis. All results were expressed as mean±s.e.m, and statistical significance was assessed using Mann-Whitney test or the one sample t test when appropriate at the significance level (p) indicated.

Optogenetic experiments

We bred C57BL/6 DAT-Cre+/− and C57BL/6 Arch3-GFP+/− mice (Jackson laboratory) leading to mice that selectively expressed Archeo-rhodopsin in DAT-expressing neurons (DAT-Cre+/−: Arch3-GFP+/− mice); DAT-Cre−/−: Arch3-GFP+/− mice were used as a control. 585 nm yellow-green light was delivered via field illumination using a high-power LED source (pE excitation System, CoolLED, Andover, UK).
Double immunostaining for GFP and TH. Brain slices (300µm) were fixed overnight in 2% paraformaldehyde at 4°C. Non-specific binding was blocked by incubated the slices for two hour at room temperature in 10% normal goat serum (Merk-Millipore, Molsheim, France) in 2% BSA and 1% Triton X-100 solution. After PBS washings, brain slices were incubated with a pair of primary antibodies, chicken anti-GFP (1:2000, AB13970, Abcam, Paris, France) and mouse anti-TH (1:500, MAB318, Merk-Millipore), in 0.5% Triton X-100, 1% BSA and 2.5% normal goat serum on a shaker overnight at 4°C. After PBS washings, brain slices were incubated with the secondary antibodies, goat anti-chicken Alexa (1:1000, A11039, Life Technology-Invitrogen, Villebon-sur-Yvette, France) and goat anti-mouse IgG1 (1:200, 1070-03, Southern Biotech-Clinisciences, Montrouge, France) in 0.5% Triton X-100, 1% BSA and 2.5% normal goat serum on a shaker overnight at 4°C. Brain slices were mounted using Fluoromount-G (Southern Biotech) and stored at 4°C. Photomicrographs were acquired using a Leica TCS SP5 inverted confocal laser-scanning microscope (Leica Microsystems) with a 40x oil objective and the 488 and 561nm channels.

Selective ablation of D2R-MSNs

We bred C57BL/6 Adora2a-Cre<sup>+/−</sup> and inducible C57BL/6 DTR<sup>+++</sup> (iDTR<sup>+++</sup>) mice leading to double heterozygous: mice that selectively expressed the DTR in D<sub>2</sub>R striatal output neurons (Adora2a-Cre<sup>+/−</sup> : iDTR<sup>+/−</sup>, A<sub>2A</sub>-DTR<sup>+/−</sup> mice) and Adora2a- Cre<sup>+/−</sup> : iDTR<sup>+/−</sup> (A<sub>2A</sub>-DTR<sup>+/−</sup> mice) used as the corresponding control as previously described (Durieux et al., 2012). A<sub>2A</sub> receptor promoter was chosen because specifically expressed in striatum only by D<sub>2</sub>R-expressing striatal output neurons (Durieux et al., 2012). Inducible ablation allows a high spatial resolution (achieved with stereotaxic injections of toxin) and prevents developmental adaptations. We then bred Adora2a-Cre<sup>+/−</sup> : iDTR<sup>+/−</sup> mice with Drd1a-GFP mice (Gong et al., 2003) to ensure recording D1R-expressing striatal output neurons. Adora2a-Cre<sup>+/−</sup> : iDTR<sup>+/−</sup> :
Drd1a-GFP<sup>+/+</sup> mice (and their control Adora2a-Cre<sup>−/−</sup>: iDTR<sup>−/−</sup>: Drd1a-GFP<sup>+/−</sup>) were deeply anesthetized at the age of 4 weeks, and placed on a stereotaxic apparatus. 1μl of diphtheria toxin (Sigma-Aldrich) by side (diluted in PBS 0.01M to a concentration of 100pg/μl) was slowly stereotaxically injected at 0.25μl/min with a blunt needle in four sides of the dorsal striatum with the following coordinates (atlas of Franklin and Paxinos, with bregma as references): anterior +1.2mm, lateral ±1.5mm, ventral +3.6mm; or anterior +0.5mm, lateral ±1.8mm, ventral +3.6mm. We reported an almost complete loss of striatal A<sub>2A</sub> receptor binding from 14 days after toxin injections (Durieux et al., 2009, 2012). Therefore, electrophysiological recordings were performed from 15-20 days after injection of the diphtheria toxin.

**In situ hybridization.** Striatal 20μm coronal sections were cut from fresh-frozen brain and mounted onto glass slides. In situ hybridization was performed as described previously (Schiffmann and Vanderhaeghen, 1993; Blum et al., 2004). After the hybridization procedure, sections were exposed to Kodak Biomax MR film for 1-2 week(s) depending on the marker studied and digitalized images were generated from the autoradiograms. For quantification of in situ hybridization and binding (ImageJ software), an averaged optical density (OD) in different areas of interest was measured and background level was subtracted to obtain corrected values. Two to three sections were used for each animal to calculate the mean OD.

**Selective ablation of ChAT interneurons.**

We bred C57BL/6 ChAT-Cre<sup>+/−</sup> (Rossi et al., 2011) and inducible C57BL/6 DTR<sup>−/−</sup> (iDTR<sup>+/+</sup>) (Buch et al., 2005) mice leading to double heterozygous: mice that selectively expressed the DTR in cholinergic interneurons (ChAT-Cre<sup>+/−</sup>: iDTR<sup>−/−</sup>, ChAT-DTR<sup>+/−</sup> mice) and ChAT-Cre<sup>−/−</sup>: iDTR<sup>+/+</sup> (ChAT-DTR<sup>+/−</sup> mice) used as the corresponding control. The ablation is performed in the same condition than D<sub>2</sub>R-MSNs. 1μl of diphtheria toxin (Sigma-Aldrich) by
side (diluted in PBS 0.01M to a concentration of 100pg/μl) was slowly stereotaxically
injected at 0.25μl/min with a blunt needle in four sides of the dorsal striatum with the
following coordinates (atlas of Franklin and Paxinos, with bregma as references): anterior
+1.2mm, lateral ±1.5mm, ventral +3.6mm; or anterior +0.5mm, lateral ±1.8mm, ventral
+3.6mm. We reported an almost complete loss of ChAT immunostaining in the striatum from
14 days after toxin injections. Therefore, electrophysiological recordings were performed
from 15-20 days after injection of the diphtheria toxin.

**ChAT immunostaining.** Mice were deeply anesthetized and perfused transcardially with
0.01M PBS, followed by 4% paraformaldehyde for tissue fixation. Brains were dissected,
dehydrated in 20%-30% sucrose solutions and frozen. Coronal free-floating sections of 30μm
thickness were cut through the striatum with a freezing microtome (Leica). Sections were
treated with 0.3% H2O2 in PBS (pH 7.4) 0.1% Triton X-100 for 45 min and non specific
antibody binding was blocked with normal horse serum in PBS (pH 7.4) 0.1% Triton X-100
for 60 min. Sections were then incubated 48 hours at 4°C with the rabbit primary antibody:
anti-ChAT (1/1000) (Chemicon). The sections were then incubated with donkey anti-rabbit
biotinylated antibody (1/200, Jackson ImmunoResearch) and visualized with the ABC
diaminobenzidine reaction.

**Unilaterally substantia nigra pars compacta (SNC) or medial forebrain bundle (MFB) 6-
hydroxydopamine (6-OHDA)-lesioned rats and L-DOPA treatment**

6-OHDA-lesioned animals were performed as previously described (Fino et al., 2005, 2010;
Paille et al., 2013). Sprague-Dawley rats weighing 125-150g (Charles River Laboratories,
L’Arbresle, France) were anesthetized with sodium pentobarbital (30 mg/kg ip; Ceva Sante
Animale, Libourne, France) supplemented by injections of ketamine (27.5 mg/kg, im;
Imalgène, Merial, Lyon, France) repeated as needed. Thirty minutes before the injection of 6-
OHDA (or vehicle in sham-operated animals), all animals received a bolus of desipramine dissolved in saline (25 mg/kg, ip; Sigma-Aldrich) to prevent neurotoxin-induced damage of noradrenergic neurons. Animals were fixed in a stereotaxic head frame (Kopf Instruments, Tujunga, CA, USA). Body temperature was maintained at 36.5°C with a homeothermic blanket (Harvard Apparatus, Kent, UK). A small craniotomy was made unilaterally (left side) over the SNC or MFB and the overlying dura mater was removed. For the Parkinson’s disease rat model, an unilateral 6-OHDA lesion of the substantia nigra pars compacta was performed. For the removal of the nigrostriatal dopaminergic cells, an unilateral 6-OHDA of the MFB was performed. A single stereotaxic injection of 6-OHDA (or of vehicle in the sham-operated animals) was delivered into the substantia nigra pars compacta on the left side (stereotaxic coordinates anteriority from the interaural line (A): 3.7 mm, laterality from the midline (L): 2.1 mm, depth from the cortical surface (H): -7.55 mm, according to the coordinates of Paxinos and Watson (2007). Concerning the MFB lesion, 6-OHDA was injected into the left MFB (stereotaxic coordinates anteriority from the bregma (A): 4.5 mm, laterality from the midline (L): 1.2 mm, depth from the cortical surface (H): -7.9 mm. The neurotoxin 6-OHDA (hydrochloride salt; Sigma) was dissolved immediately prior use in ice-cold 0.9% w/v NaCl solution containing 0.01% w/v ascorbic acid to a final concentration of 2.5 mg/ml. Then 4.0 μl of this 6-OHDA solution (or vehicle in sham-operated animals) was injected at a rate of 16 μl/h via a steel canula (0.25 mm outside diameter) attached to a 10μl Hamilton microsyringe (Cole-Parmer, London, UK) controlled by an electrical pump (KDS100; KD Scientific, Holliston, MA). A delay of 5 min was observed between the time the canula was inserted into the substantia nigra pars compacta and the onset of the 6-OHDA injection, and the canula was left in place 10 min following the end of injection before removal. After surgery, rats received an intramuscular injection of gentamicin to prevent bacterial infection (3 mg/kg, im; Gentalline, Schering-Plough, Levallois-Perret, France). Sham and 6-OHDA experiments were
performed on animals of similar ages.

**Chronic L-DOPA treatment.** Two weeks after the 6-OHDA injection, rats were splitted into two groups which received i.p. injection twice-daily for 10 days of either L-DOPA (10 mg/kg) and benzerazide (7.5 mg/kg) or saline. The sham-operated animals were also subjected to L-DOPA or saline injections with the same schedule. Animals received the last injection of L-DOPA or saline 30-60 minutes before being sacrificed for *ex vivo* experiments.

**Tyrosine hydroxylase (TH) immunostaining.** The severity of the 6-OHDA lesions was quantified after electrophysiological whole-cell recordings by striatal TH immunostaining. Brain sections were incubated in 0.1% hydrogen peroxide and 10% methanol in PBS for 15 min at room temperature prior to incubation in primary antibody. TH immunostaining was performed by first incubation of the slices in 0.1% hydrogen peroxide and 10% methanol in PBS (15 min at room temperature) and then in a 1/500 dilution of mouse anti-TH monoclonal antibody (MAB318; Merk-Millipore, Molsheim, France) overnight at 4°C. Biotin-goat anti-mouse secondary antibody (Life Technology-Invitrogen, Villebon-sur-Yvette, France) was incubated at a dilution of 1/500 for 2.4 h at room temperature and visualized using with avidin-biotin complex (ABC Elite standard, PK-4000, Vector Laboratories, Burlingame, CA, USA) before visualization with DAB detection kit (Vector Laboratories) according to the instructions of the manufacturer.
RESULTS

Endocannabinoid-tLTP and -tLTD are differently D₁R/D₂R-mediated

It has been widely reported that at corticostriatal synapses one of the main form of plasticity is an endocannabinoid-mediated LTD (eCB-LTD) which is induced with 100 to many hundreds of stimulations upon various cell conditioning paradigms (HFS, LFS, theta-burst or STDP protocols) (reviewed in: Kreitzer and Malenka, 2008; Di Filippo et al., 2009; Fino and Venance, 2010; Feldman, 2012; Mathur and Lovinger, 2012). We recently found a LTP endocannabinoid-mediated (eCB-tLTP), dependent on type-1 cannabinoid receptor (CB1R) and transient receptor potential vanilloid type-1 (TRPV1) activation and induced with a very low number of pairings in MSNs of the dorsal striatum (Cui et al., 2015). Namely, 10 post-pre STDP pairings (-30<ΔSTDP<0 ms) induced potent tLTP (mean value of the EPSC amplitude recorded 50 min after STDP protocol: 158±11%, p<0.0006, n=9), which was prevented with AM251 (3 μM), a CB₁R specific inhibitor (80±14%, p=0.2223, n=5) (Fig. 1a-b1). In our experimental conditions, we also confirm the expression of an eCB-tLTD, induced with 100 pairings as previously described (Fino et al., 2005, 2010; Pawlak and Kerr, 2008; Shen et al., 2008; Paille et al., 2013). Indeed, 100 pre-post pairings (0<ΔSTDP<+30 ms) induced tLTD (71±10%, p=0.0253, n=7), which was prevented with AM251 (3 μM) (103±7%, p=0.7500, n=7) (Fig. 1c1).

Striatum receives excitatory afferents from the cortex as well as a dense innervation from midbrain dopaminergic neurons. Dopamine, a key regulator of action selection and associative learning (Redgrave and Gurney, 2006; Yin and Knowlton, 2006; Schultz, 2007), efficiently modulates corticostriatal synaptic plasticity and particularly the “classical” eCB-LTD (Kreitzer and Malenka, 2008; Di Filippo et al., 2009; Gerfen and Surmeier, 2011; Mathur and Lovinger, 2012). We first confirmed these observations using STDP protocol: we observed that eCB-tLTD was impaired by a mixture of D₁R and D₂R antagonists, SCH23390
(4 µM) and sulpiride (10 µM), respectively, (106±14%, p=0.6714, n=7) (Fig. 1c2). We then selectively inhibited either D1R or D2R. We did not observe significant plasticity (105±16%, p=0.7405, n=8) when we inhibited D1R with SCH23390 (4 µM), while we found LTP with sulpiride (10 µM) (199±36%, p=0.0335, n=7) (Fig. 2c3). Thus, eCB-tLTD is mediated by D1R and D2R. We thus questioned whether dopamine is involved in eCB-tLTP as well as in eCB-tLTD. For this purpose we first bath-applied SCH23390 (4 µM) and sulpiride (10 µM), and found that this cocktail prevented the induction of eCB-tLTP and a slight depression was observed (77±4%, p=0.0002, n=9) (Fig. 3b2). We then selectively inhibited either D1R or D2R. When we applied a D1R antagonist, (SCH23390, 4 µM), we still observed a potent tLTP (169±21%, p=0.0113, n=9) while no plasticity could be elicited when a D2R antagonist (sulpiride, 10 µM), was bath-applied (73±8%, p=0.0117, n=8) (Fig. 1b3). This indicates that eCB-tLTP is D2R-mediated and not dependent on D1R while eCB-LTD is mediated by both D1- and D2Rs.

eCB-LTP requires dopaminergic receptors activation during the paired-activity on either side of the synapse.

We then asked if dopamine was important for the induction phase of eCB-tLTP, and more precisely just during the few pairings needed for its expression. Indeed, upon cortical stimulation, dopamine is released concomitantly because of glutamatergic afferents onto dopaminergic terminals (Taber and Fibiger, 1993; Taber and Fibiger, 1995; Morari et al., 1998). For this purpose, we chose an opto-inhibition of dopaminergic neurons just during the paired activity to test if dopamine was required for eCB-tLTP induction. To do so we bred DAT-Cre with Arch3-GFP<sup>++</sup> mice to obtain archeorhodopsin-3 (Arch3) expression in dopaminergic neurons. We confirmed the Arch3-GFP expression in dopaminergic neurons by performing tyrosine hydroxylase (TH) and GFP immunostainings (n=10) (Fig. 2a). We ensure
the efficiency of the opto-inhibition by recording the spontaneous spiking activity of the
dopaminergic neurons from DAT-Cre$^{+/+}$: Arch3-GFP$^{+/+}$ and observed an absence of spikes
upon photostimulation (with 0pA current injection: 2.1±0.5 Hz without light and 0 Hz with
light, n=5) (Fig. 2b). In addition, without spontaneous firing with -100 pA current injection,
we observed a hyperpolarization of the RMP upon photostimulation (-19±5 mV, n=5). We
first verified that we could successfully induce tLTP with 15 post-pre pairings (see Methods)
in DAT-Cre$^{+/+}$: Arch3-GFP$^{+/+}$ mice without photostimulation (143±12%, p=0.0091, n=8) (Fig.
2d). In DAT-Cre$^{+/+}$: Arch3-GFP$^{+/+}$ mice, a photostimulation of dopaminergic neuron terminals
concomitantly with the STDP protocol (16 sec duration, starting 1sec before STDP protocol
and ending with the last pairing of the protocol) prevented the induction of tLTP (102±8%,
p=0.7943, n=6) (Fig. 2d). As a control we verified that photostimulation itself in DAT-Cre$^{-/-}$:
Arch3-GFP$^{+/+}$ mice does not impair tLTP induction: tLTP was successfully induced with 15
post-pre pairings applied concomitantly with a pulse photostimulation (140±12%, p=0.0151,
n=7) (Fig. 2e). Thus, the activation of D$_2$R by dopamine is required during the pairings time-
lapse to elicit eCB-tLTP.

Where are located the D$_2$R required for eCB-tLTP induction?

We then asked the question of the location of the D$_2$R involved in the eCB-tLTP induction.
Indeed, D$_2$R can be located at different locations in the striatum: postsynaptically in D$_2$R-
expressing MSNs (Gerfen and Surmeier, 2011; Calabresi et al., 2014) and presynaptically in
cholinergic interneurons (ChAT interneurons) (Hersch et al., 1995), nigrostriatal
dopaminergic neurons (De Mei et al., 2009) and/or glutamatergic cortical afferents (Bamford
et al., 2004). To solve this question, we chose the following strategy: we genetically-ablated
selectively D$_2$R-expressing MSN or ChAT interneurons using Cre-mediated expression of the
diphtheria toxin receptor (DTR) and stereotoxic diphtheria toxin (Durieux et al., 2009; 2012)
and 6-ODHA-ablated selectively dopaminergic cells specifically in the substantia nigra pars compacta and examined if eCB-tLTP could still be expressed.

eCB-tLTP is expressed in both D1R- and D2R-expressing MSNs and does not depend on D2R expressed by MSNs belonging to the indirect pathway

We first questioned the postsynaptic localization of the D2R involved in eCB-tLTP at the level of MSNs. Due to the segregation of expression of D1- and D2Rs among MSNs (D1R-like and D2R-like for the direct and indirect pathways, respectively) (Gerfen and Surmeier, 2011; Calabresi et al., 2014), roughly half of the MSNs are expected to be D2R-expressing neurons. If eCB-tLTP was supported by the postsynaptic D2R MSNs, one would expect to induce eCB-tLTP in ~50% of the (randomly chosen) MSNs. In our experiments, eCB-tLTP was successfully induced in 83% (n=27) of the (randomly chosen) tested MSNs, which does not favor the hypothesis of involvement of D2R expressed by MSNs in eCB-tLTP. To confirm this, we first used transgenic D1-eGFP mice and to investigate eCB-tLTP occurrence in D1+ and non-D1+ MSNs (Fig. 3a-b). We observed tLTP in both D1+ (148±8%, p=0.0033, n=6) and non-D1+ (150±7%, p=0.0006, n=7) MSNs (Fig. 3b), indicating that tLTP can be induced in both striatopallidal and striatonigral MSNs and that a postsynaptic D2R in MSNs was a priori less likely involved in eCB-tLTP.

To further test the involvement of the D2R-expressing MSNs, we selectively ablated these neurons by Cre-mediated expression of DTR and diptheria toxin injection (Durieux et al., 2009). Cre recombinase was under the control of A2AR promoter because in the striatum A2AR expression is restricted to D2R-expressing MSNs (Fig. 3c1). The diptheria toxin was stereotaxically injected into the dorsal striatum to produce unilateral ablation of D2R MSNs (see Methods). Indeed, in A2A-Cre+/−: iDTR+/−: Drd1a-GFP+/− triple transgenic mice, there was no detectable D2+ cells but only D1-GFP MSNs after stereotaxic of the diptheria toxin.
while in A2A-Cre\(\text{+/-}\) : iDTR\(\text{+/-}\) : Drd1a-GFP\(\text{+/-}\) both populations of MSNs (D1\(^+\) and D2\(^+\)) were still observed (Fig. 3c). Whole-cell recordings of D1R-GFP striatal neurons, performed in brain slices obtained from A2A-Cre\(\text{+/-}\) : iDTR\(\text{+/-}\) : Drd1a-GFP\(\text{+/-}\) mice injected with diphteria toxin, showed that 15 post-pairings were still able to cause LTP (146±7%, p=0.0007, n=7) (Fig. 3d). As a control, we similarly injected diphteria toxin in mice lacking DTR (A2A-Cre\(\text{-/-}\) : iDTR\(\text{+/-}\) : Drd1a-GFP\(\text{+/-}\)) and also observed tLTP (137±10%, p=0.0174, n=6) that was not significantly different from tLTP obtained with ablation of D2R-expressing output neurons (p=0.3566) (Fig. 3d). These results indicate that eCB-tLTP does not depend on postsynaptic D2R-activation. These results indicate that eCB-tLTP depends on the activation of presynaptically located D2R.

**eCB-tLTP is not dependent on D2R expressed by striatal cholinergic interneurons**

We then selectively ablated striatal cholinergic interneurons by Cre-mediated expression of DTR and diphteria toxin injection to test the participation of the D2R express by striatal cholinergic interneurons in eCB-tLTP (Fig. 4a). Cre recombinase was under the control of ChAT promoter because in the striatum ChAT expression is restricted to cholinergic interneurons (Fig. 4b). Unilateral ablation of cholinergic interneurons was induced by the diphteria toxin, which was stereotaxically injected into the dorsal striatum (see Methods). Indeed, in ChAT-Cre\(\text{+/-}\) : iDTR\(\text{+/-}\) double transgenic mice, there was no detectable ChAT cells (with ChAT immunostaining) after stereotaxic of the diphteria toxin while in ChAT-Cre\(\text{+/-}\) : iDTR\(\text{+/-}\) positive ChAT staining was observed (Fig. 4b). Whole-cell recordings of MSNs from ChAT-Cre\(\text{+/-}\) : iDTR\(\text{+/-}\) mice injected with diphteria toxin, showed that 15 post-pairings induced tLTP (161±20%, p=0.0182, n=8) (Fig. 4d). We verified that this tLTP was D2R-mediated by applying sulpiride (10 µM). In these conditions, we did not observed significant plasticity (99±10%, p=0.9160, n=6) (Fig. 4d). As a control, we similarly injected diphteria
toxin in mice lacking DTR expression (ChAT-Cre$^{+/-}$; iDTR$^{+/+}$) and observed tLTP (150±13%, p=0.0171, n=5) that was similar to tLTP obtained with ablation of cholinergic interneurons (p=0.8221) (Fig. 4c). These results indicate that eCB-tLTP does not depend on D$_2$R-activation located in cholinergic interneurons.

eCB-tLTP is not dependent on D$_2$R located in nigro-striatal dopaminergic neurons

We then selectively lesioned dopaminergic neurons with 6-hydroxy-dopamine (6-OHDA), a neurotoxic synthetic organic compound which leads, when associated with desipramine, to the selective degeneration of dopaminergic neurons. We performed stereotaxic injection within the MFB of P35 rats to test the involvement of the D$_2$R of nigrostriatal dopaminergic neurons in eCB-tLTP two weeks after lesion at P50 (Fig. 5a-b). For these experiments we only used animals for which the loss of striatal tyrosine hydroxylase (TH) staining reached at least 65% at P50 (two weeks after the 6-OHDA lesions) (-67.1±3.5%, p<0.0001, n=8) (Fig. 5b-c). In parallel, we also used control (without surgery aged of P50) and sham-operated (with saline injection instead of 6-OHDA) animals (Fig. 5b-c). We first verified that tLTP was induced with 10 post-pre pairings in P50 control rats (158±16%, p=0.0168, n=6) and in sham-operated rats (147±10%, p=0.0032, n=7) (Fig. 5d,f); these tLTP were not significantly different (p=0.7133). As expected, in 6-OHDA animals we did not observed any significant plasticity with 10 post-pre pairings (96±4%, p=0.3565, n=7) (Fig. 5d). We then bath-applied quinpirole (10 µM), an agonist of D$_2$R, and a significant tLTP was then observed (141±9%, p=0.0076, n=6) (Fig. 5e); this latter tLTP was not significantly different form those observed in control or sham-operated rats (p=0.3052 and p=0.7984, respectively). These results indicate that presynaptic D$_2$R on nigrostriatal dopaminergic afferents are not required for eCB-tLTP since tLTP was observed in 6-OHDA-lesioned animals with their D$_2$R activated with quinpirole, characterized by the loss of these dopaminergic afferents (and thus of their D$_2$R).
eCB-tLTP is impaired in a rodent model of Parkinson’s disease and rescued by chronic L-DOPA treatment.

Because eCB-tLTP depends on dopamine, we asked whether this form of plasticity could be altered in Parkinson’s disease. For this purpose, we used a 6-OHDA-lesioned rat model of Parkinson’s disease in which dopaminergic transmission is impaired. We performed unilateral lesion of substantia nigra pars compacta by injecting 6-OHDA (see schematic protocol in Fig. 6a). Here, we waited two weeks after 6-OHDA injection before saline or L-Dopa treatment for 10 additional days, i.e. the *ex vivo* recordings were performed at P(60-65). We also used sham-operated animals with saline injection for comparison with the 6-OHDA-lesioned rats and we verified that saline injection in sham-operated animals left the TH staining unaffected (data not shown). We first verified that tLTP was successfully induced with 10 post-pre pairings in adult control rats (i.e. without any surgery and recorded at similar age than the sham-operated and 6-OHDA-lesioned rats, i.e. P(60-65)) (153±22%, p=0.0505, n=7) and in sham-operated rats (147±7%, p=0.0002, n=9) (Fig. 6b,d). tLTP induced in adult control animals and in sham-operated rats were not significantly different (p=0.5848). In contrast, in 6-OHDA-lesioned animals, we did not observed significant plasticity with 10 post-pre pairings (94±5%, p=0.2741, n=9) showing that the degeneration of dopaminergic neurons was deleterious for the induction of eCB-tLTP (Fig. 6b).

L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor to dopamine, is a mainstay for symptomatic treatment of Parkinson’s disease. We tested whether a chronic treatment with L-DOPA (two injections per day of L-DOPA 10mg/kg with benzerazide 7.5mg/kg for 10 days starting two weeks after the surgery, see Fig. 6a) could rescue the induction of eCB-tLTP in 6-OHDA-lesioned rats. In slices obtained from 6-OHDA-lesioned animals treated with L-DOPA, whole-cell recordings showed that 10 post-pre pairings caused tLTP (171±19%,
p=0.0096, n=7) (Fig. 6c); this tLTP was not significantly different from tLTP obtained in sham-operated rats (p=0.3481). Note that tLTP was observed in the sham-operated animals L-DOPA-treated (164±20%, p=0.0201, n=7) (Fig. 6c) and this tLTP was not significantly different from those obtained in sham-operated rats (p=0.4038) or in 6-OHDA-lesioned animals treated with L-DOPA (p=0.7791). In 6-OHDA-lesioned animals treated with L-DOPA, t-LTP induced with 10 post-pre pairings was CB₁R-mediated since prevented with AM251 (3µM) (81±6%, p=0.0244, n=6) (Fig. 6c). Therefore, eCB-tLTP was impaired in a rodent model of Parkinson’s disease and was rescued by chronic L-DOPA treatment.
DISCUSSION

Corticostriatal long-term plasticity provides a fundamental mechanism for the function of the basal ganglia in procedural learning (Yin and Knowlton, 2006; Yin et al., 2009). We uncovered the existence of eCB-tLTP induced by a low number of pairings in the striatum of both juvenile and adult rodents (Cui et al., 2015; the present report). Indeed, few coincident pre- and postsynaptic spikes were found to strengthen synaptic efficacy through a signaling pathway that is not NMDAR-dependent but that relies on eCB activation of CB1R. eCBs have been reported to depress synaptic weight, i.e. short- or long-term depression, through the activation of CB1R (Kano et al., 2009; Puente et al., 2011; Castillo et al., 2012; Katona and Freund, 2012; Melis et al., 2014). Noticeable exceptions are reports of an indirect role of eCBs in promoting LTP at mixed (chemical and electrical) synapses of the goldfish Mauthner cell via intermediary dopaminergic neurons (Cachope et al., 2007), facilitation of LTP induction in the hippocampus (Carlson et al., 2002), and mediation of heterosynaptic short-term potentiation via intermediary astrocytes (Navarrete and Araque, 2010). We describe here a long-term spike-timing dependent potentiation in mammals, wherein eCB signaling directly underlies both the induction and the long-term maintenance of synaptic weight increase.

Dopamine is a well-known key actor of action selection and associative learning (Redgrave and Gurney, 2006; Yin and Knowlton, 2006; Schultz, 2007) and for the modulation of striatal projection (Kreitzer and Malenka, 2008; Di Filippo et al., 2009; Gerfen and Surmeier, 2011; Mathur and Lovinger, 2012). Our results show that dopamine is also a key element for eCB-tLTP expression through the activation of D2R. eCB-tLTP does not require the postsynaptic D2R, expressed by striatopallidal neurons. Indeed, the selective ablation of those D2R-striatal neurons or the selective pharmacological targeting of postsynaptic adenylate cyclase activity both failed to prevent eCB-tLTP expression. Moreover, we observed eCB-tLTP among 83% of the recorded neurons, which exceed from far the 50-50 distribution expected for the D1R...
and D$_2$R expression among MSNs. Lastly, we observed eCB-tLTP in D$_1$R-expressing MSNs as well as D$_1$R-non-expressing cells. This demonstrates that D$_2$Rs involved in eCB-tLTP are presynaptically located. The presynaptic D$_2$R are expressed at three different locations: the nigrostriatal dopaminergic afferents (De Mei et al., 2009), the cholinergic interneurons (Hersch et al., 1995) and the corticostriatal glutamatergic afferents (Bamford et al., 2004).

Presynaptic D$_2$R on nigrostriatal dopaminergic afferents are not required for eCB-tLTP since eCB-tLTP was observed in L-DOPA-treated 6-OHDA-lesioned animals characterized by the loss of these dopaminergic afferents (and thus of their D$_2$R); Note that similar results were obtained with injection of 6-OHDA in the MFB or in the SNc. We exclude the involvement of D$_2$R located in cholinergic interneurons since tLTP was observed in mice with the cholinergic interneurons ablated. Moreover, involvement of D$_2$R expressed by cholinergic interneurons is unlikely since the activation of those D$_2$R would decrease acetylcholine release, which would promote LTD, and not LTP (Wang et al., 2006). Thus, our results indicate that D$_2$R expressed by the corticostriatal glutamatergic afferents are the best candidates to account for the involvement of presynaptic D$_2$R in eCB-tLTP. Moreover, colocalization of D$_2$R and CB$_1$R on the same presynaptic terminals would act synergistically to inhibit adenylate cyclase activity and to promote either eCB-LTP or eCB-tLTD.

Our results strongly suggest a CB$_1$R- and D$_2$R-control of STDP whereby the same signal, adenylate cyclase inhibition (caused by e.g. CB$_1$R and D$_2$R activation), can trigger LTP or LTD depending on its dynamics. A similar hypothesis has been proposed for NMDAR-dependent STDP where the outcome of plasticity (LTP or LTD) depends on the dynamics of intracellular calcium (Shouval et al., 2010; Graupner and Brunel, 2012). The presynaptic compartment is difficult to manipulate pharmacologically so we are mostly limited to indirect conclusions. To explain the molecular mechanism, we propose the following hypothesis. The presynaptic weight $W_{pre}$ is determined by the antagonistic action of PKA and CaN on a target
molecule T that sets $W_{\text{pre}}$. As suggested by our experimental results, we assume that PKA-phosphorylated T increases the $W_{\text{pre}}$ whereas CaN-dephosphorylated T decreases it. The outcome of plasticity (LTP or LTD) depends on the relative values of PKA activity compared to that of CaN. Inhibition of presynaptic adenylate cyclase via CB$_1$R and D$_2$R might decrease PKA activity (Stephens and Mochida, 2005; Castillo et al., 2012). On the other hand, adenylate cyclase inhibition also inhibits presynaptic VSCCs (Mato et al., 2008), which is expected to hamper presynaptic calcium. A reasonable assumption is that such a decrease in the presynaptic calcium levels would diminish the activity of (calcium-activated) CaN. As a result, in parallel to its inhibitory effect on PKA, adenylate cyclase inhibition would reduce CaN activity as well. Depending on the exact shape of the decay of PKA and CaN activity with increasing adenylate cyclase inhibition, PKA activity could dominate CaN for some range of adenylate cyclase inhibition (thus yielding LTP), whereas CaN dominates in others (yielding LTD). This situation leads to the expression of tLTD for intermediate adenylate cyclase inhibition and a potent tLTP for adenylate cyclase inhibition. In this case, the level of adenylate cyclase inhibition would set the STDP polarity. Our experimental results are consistent with a mechanism in which eCB-tLTP is triggered when adenylate cyclase inhibition is large enough to ensure the dominance of PKA over CaN in the presynaptic compartment. Conversely, moderate adenylate cyclase inhibition would lead to the dominance of CaN over PKA and thus to LTD.

Disruptions in the eCB system seem to contribute to action-learning defects in Parkinson’s disease (Hilário and Costa, 2008; Mathur and Lovinger, 2012; Zhuang et al., 2013). So far, this alteration was thought to rely mainly on disruption of the well-characterized eCB-LTD, which is D$_2$R dependent (Kreitzer and Malenka, 2008; Di Filippo et al., 2009; Mathur and Lovinger, 2012). Here, we uncovered a new form of plasticity, eCB-tLTP that is also D$_2$R-dependent and is disrupted in a rodent model of Parkinson’s disease as well and can be
rescued with a chronic L-DOPA treatment. It remains to analyze the effects of a loss of eCB-tLTP in vivo to address the roles of eCB-tLTP in physiological and pathophysiological states. eCB-LTP may represent a molecular substrate operating in rapid learnings of new arbitrary associative memories and behavioral rules characterizing the flexible behavior of mammals or during initial stages of slower habit learnings (Barnes et al., 2011), which may possibly be disrupted in Parkinson’s disease (Hilário and Costa, 2008; Mathur and Lovinger, 2012; Zhuang et al., 2013).
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Author contribution:

H.X., H.B. and L.V. designed the study; H.X., S.P. and Y.C. performed electrophysiological experiments and analysis; B.D and A.de K d’E generated the Adora2a-Cre and ChAT-cre mice and produced the A2A-Cre+/+: iDTR+/+: Drd1a-GFP+/+ and ChAT-cre+/+: iDTR+/+: Drd1a-GFP+/+ triple transgenic mice, the ChAT-Cre+/-: iDTR+/- double transgenic mice and performed stereotaxic injection of diphtheric toxin; B.D. performed 6-OHDA lesions; H.B. contributed to analytic tools; H.X, H.B. and L.V. wrote the manuscript and all authors have edited and corrected the manuscript. L.V. supervised the whole study.
FIGURE LEGENDS

Figure 1. Endocannabinoid-tLTP is D2R-activation dependent (and not D1R) while endocannabinoid-tLTD relies on both D1R and D2R.

(a) Scheme of the recording and stimulating sites in corticostriatal slices. Characteristic voltage responses of a striatal output neuron to a series of 500ms current pulses from -150 to +180pA with current steps increasing by 30pA (black traces) and to +60pA above spike threshold (grey trace). Right panels: STDP protocol: a spike evoked in one MSN was paired with a cortical stimulation repeated N times at 1Hz. Δt indicates the time shift between pre- and postsynaptic stimulations. -30<Δt<0ms and 0<Δt<+30ms refers to post-pre and pre-post pairings, respectively. (b) A low number of paired stimulations, 10 post-pre pairings (-30<Δt<0ms), induced tLTP which is CB1R- and D2R-activation dependent. (b1) eCB-tLTP induced with 10 post-pre pairings (n=9) is prevented (b1) with a specific CB1R inhibitor, AM251 (3µm, n=5), with (b2) the co-application of antagonists of D1R and D2R, SCH23390 (4µM) and sulpiride (10µM) (n=9), and (b3) with the D2R antagonist, sulpiride (10µM, n=8) but tLTP was left unaffected by the with the D1R antagonist, SCH23390 (4µM, n=9). (c) tLTD induced with 100 pre-post pairings (0<Δt<+30ms) is CB2R-, and both D1R- and D2R-activation dependent. (c1) tLTD induced with 100 pre-post pairings is endocannabinoid-mediated since prevented with AM251 (3µm, n=7), (c2) prevented with a mixture of D1R and D2R antagonists, SCH23390 (4µM) and sulpiride (10µM) (n=7). (c3) eCB-tLTD was impaired with either SCH23390 (4µM, n=8) or sulpiride (10µM, n=7).

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem. *: p<0.05. ns: not significant.
Figure 2. Induction of eCB-LTP requires dopamine release during the STDP paired-activity paradigm.

(a) Double immunostaining for tyrosine hydroxylase (indicating dopaminergic neurons; upper panel) and GFP (indicating Arch3-expression; middle panel) in SNc slices; merge images are shown in the lower panel. (b) Spontaneous firing of dopaminergic neurons recorded in DAT-Cre\textsuperscript{+/−}: Arch3-GFP\textsuperscript{+/−} without light (upper traces) or with light (lower traces). (c) Double immunostaining for tyrosine hydroxylase (indicating dopaminergic afferences; upper panel) and GFP (indicating Arch3-expression; lower panel) in corticostratal slices; merge images are shown in the lower panel. Scale bar: 50\,\mu m. (d) 15 post-pre pairings induced tLTP (n=8) in DAT-Cre\textsuperscript{+/−}: Arch3-GFP\textsuperscript{+/−} mice without photostimulation, while photostimulation during STDP pairings prevents tLTP induction (n=6). This illustrates that eCB-tLTP require DA release during STDP pairings. (e) 15 post-pre pairings induced tLTP with concomitant photostimulation (16sec duration) during STDP protocol in DAT-Cre\textsuperscript{−/−}: Arch3\textsuperscript{+/−} mice (n=7).

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem. *: p<0.05. ns: not significant.

Figure 3. eCB-tLTP is expressed in both D\textsubscript{1}R- and D\textsubscript{2}R-expressing MSNs and does not depend on D\textsubscript{2}R expressed by MSNs belonging to the indirect pathway

(a) Shematic view of the location of the D\textsubscript{2}Rs in striatum. Experiments described in (b) and (c-d) aim at investigating the expression of tLTP in MSNs expressing D1R or not, and in remaining MSNs when D2R-expressing MSNs were genetically ablated (indicated by the red cross), respectively. (b) tLTP induced with 15 post-pre pairings is observed in both striato-pallidal (D1-eGFP positive neurons, D1-eGFP\textsuperscript{+}, n=6) and striato-nigral (D1-eGFP negative neurons, non-D1-eGFP\textsuperscript{+}, n=7) MSNs. (c) Microphotographs of in situ hybridization in A\textsubscript{2A}-
Cre$^{+/-}$ : iDTR$^{+/-}$ : Drd1a-GFP$^{+/-}$ (right panels) and A$_{2A}$-Cre$^{+/-}$ : iDTR$^{+/-}$ : Drd1a-GFP$^{+/-}$ mice (left panels) injected with dipherteria toxin stereotaxically in the dorsal striatum. (d) In mice in which D$_2$R-expressing striatal neurons were ablated (A$_{2A}$-Cre$^{+/-}$ : iDTR$^{+/-}$ : Drd1a-GFP$^{+/-}$ mice injected with dipherteria toxin stereotaxically in the dorsal striatum) tLTP was induced with 15 post-pre pairings (n=7). As a control, tLTP was also induced in mice lacking DTR with injection of dipherteria toxin (A$_{2A}$-Cre$^{+/-}$ : iDTR$^{+/-}$ : Drd1a-GFP$^{+/-}$) (n=6).

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem. *: p<0.05. ns: not significant.

Figure 4. eCB-tLTP is not dependent on D$_2$R expressed by striatal cholinergic interneurons

(a) Shematic view of the location of the D$_2$Rs in striatum. Experiments described in (b-d) investigate tLTP in MSNs when ChAT-expressing neurons (i.e. cholinergic interneurons) were genetically ablated (indicated by the red cross). (b) ChAT immunostaining in ChAT-Cre$^{+/-}$ : iDTR$^{+/-}$ (upper panels) and ChAT-Cre$^{+/-}$ : iDTR$^{+/-}$ mice injected with dipherteria toxin stereotaxically in the dorsal striatum, show the absence of ChAT immunostaining only in ChAT-Cre$^{+/-}$ : iDTR$^{+/-}$ as quantified in the histogram. (c-d) tLTP was induced with 15 post-pre pairings in ChAT-Cre$^{+/-}$ : iDTR$^{+/-}$ (n=5) (c) as well in ChAT-Cre$^{+/-}$ : iDTR$^{+/-}$ (n=8) (d) mice; in these latter, ChAT-expressing striatal neurons were ablated with dipherteria toxin stereotaxically in the dorsal striatum. tLTP observed in ChAT-ablated mice was D$_2$R-mediated because prevented with sulpiride (10 µM, n=6).

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem. *: p<0.05. ns: not significant.
Figure 5. eCB-tLTP is not dependent on D_{2}R located in nigro-striatal dopaminergic neurons

(a) Schematic view of the location of the D_{2}Rs in striatum. Experiments described in (b-f) investigate tLTP in MSNs when dopaminergic neurons were lesioned with 6-OHDA (indicated by the red cross). (b) Time course of the 6-OHDA lesions and recordings. (c) Unilateral 6-OHDA injection in medial forebrain bundle led to degeneration of DA nigral neurons and a loss of their striatal afferences as illustrating by tyrosine hydroxylase (TH) immunostaining. Sham-operated rats display equivalent TH staining in both striata. Scale bars: 1mm and 200µm. Right panel: summary bar graph of TH staining quantification. (d) 10 post-pre pairings induced LTP in sham-operated (n=7) rats and no plasticity in 6-OHDA-lesioned rats (n=7). (e) A D_{2}R agonist, quinpirole (10 µM, n=6) rescued tLTP in 6-OHDA-lesioned rats. (f) P_{50} control (n=6) displayed tLTP with similar magnitudes as sham-operated group.

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem. *: p<0.05, ns: not significant.

Figure 6. eCB-tLTP is impaired in a rat model of Parkinson’s disease and rescued by chronic L-DOPA treatment.

(a) Protocols of the 6-OHDA lesion (or sham) in P_{35} rats followed two weeks after by chronic L-DOPA treatment (or saline) for 10 days. (b) 10 post-pre pairings induced eCB-tLTP in sham-operated rats (n=9) while no plasticity was observed in 6-OHDA-lesioned rats (n=9). (c) Chronic L-DOPA treatment consisting in twice daily injection of L-DOPA (10mg/kg) for 10 days two weeks after 6-OHDA lesion allowed to recover tLTP (n=7) induced with 10 post-pre pairings. This tLTP was CB_{1}R-mediated since prevented by AM251 (3µm, n=6) in 6-OHDA-lesioned rats treated with L-DOPA. (d) 10 post-pre pairings induced eCB-tLTP in control
adult rats ($P_{60-65}$) (n=7). (e) tLTP was induced with 10 post-pre pairings in sham-operated rats which were treated with L-DOPA (n=7).

Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent SD. *: p<0.05. ns: not significant.
Figure 3

(a) Glutamatergic terminal

(b) Cholinergic interneuron

(c) Dopaminergic terminal

(c) A2A-Cre+/−: iDTR+/−: Drd1a-GFP+/- mice

(d) Normalized EPSC (%) vs. Time (min)

- non-D1-eGFP+ (n=7)
- D1-eGFP+ (n=6)

(e) DTR+ (DTR+/−)

(f) DTR− (DTR−/−)

(g) Normalized EPSC (%) vs. Time (min)

- A2A-Cre+/−:iDTR+/−+DT (n=7)
- A2A-Cre−/−:iDTR+/−+DT (n=6)
Figure 6

(a) Schematic overview of the experimental setup. 

- SNC 6-OHDA-lesioned or Sham-operated rats
- L-DOPA or Saline
- ex vivo whole-cell recordings, TH staining
- - 6-OHDA + L-DOPA
- - 6-OHDA + saline
- - Sham + L-DOPA
- - Sham + saline

(b) Normalized EPSC (% vs. Time (min)) for Sham (n=9) and 6-OHDA (n=9).

(c) Normalized EPSC (% vs. Time (min)) for 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + AM251 (n=6).

(d) Normalized EPSC (% vs. Time (min)) for Control (P60-65) (n=7) and Sham + L-DOPA (n=7).

(e) Normalized EPSC (% vs. Time (min)) for 6-OHDA + L-DOPA (n=7) and Sham + L-DOPA (n=7).
Preliminary results:

Enriched environment impacts on bidirectional endocannabinoid-dependent plasticity at corticostriatal synapse

Introduction

Behavioral experience such as enriched environment (EE) has profound long-lasting effects on learning and memory performance (Van Praag et al., 2000; Nithianantharajah and Hannan, 2006; Sale et al., 2009; Ruediger et al., 2011). EE combining complex inanimate and social interactions (Rosenzweig et al., 1978) provides sensory, motor and cognitive stimulations that normally are lacking in standard environment housing. Rearing animals in environmental enrichment aims at mimicking the circumstances of a stimulating and interesting living environment that is conducive to cognitive functions. Cognitive functions are mainly underlain by synaptic plasticity that scales in both direction the synaptic weight as widely observed with the long-term potentiation (LTP) and long-term depression (LTD) (Martin and Morris, 2002; Malenka and Bear, 2004). EE has been reported to display a neuroprotective effect (from very subtle to significant) in various animal models of neurodegenerative diseases, including Huntington’s, Alzheimer’s and Parkinson’s disease (Nithianantharajah and Hannan, 2006; Laviola et al., 2008; Hannan, 2014). Compared to extensively investigations in hippocampus for Alzheimer’s disease, the effect of EE studies in animal models of Parkinson’s disease remains in its infancy.

Parkinson’s disease is an age-related neurodegenerative disorder mainly characterized by a progressive loss of dopaminergic neurons in the nigrostriatal system (Dauer and Przedborski, 2003). Among the neurotoxins used to induce dopaminergic neurodegeneration, the administration of 6-hydroxydopamine (6-OHDA) and MPTP are the most commonly used by experimentalists, and allow observing rapidly a parkinsonian syndrome. EE was first shown to have beneficial effects against MPTP toxicity in mice, showing a lesser extent of the loss of dopaminergic neurons after MPTP treatment (Bezard et al., 2003). The mice housed 2 months
in enriched conditions after weaning exhibit an increase in the expression of brain-derived neurotrophic factor (BDNF) and a decrease of dopamine transporter (DAT) in the striatum (Bezard et al., 2003). These findings have been confirmed in the adult mice exposed to EE for 3 weeks (Goldberg et al., 2011) or 3 months (Faherty et al., 2005). Similarly, EE also induces a neuroprotection of the remaining DAergic neurons after unilateral 6-OHDA injection in rats (Anastasia et al., 2009). The behavioral tests showed an improvement of skilled motor function and a decrease of turning behavior in response to amphetamine (Jadavji et al., 2006). Furthermore, physical exercise interventions (e.g. moderate treadmill running), which is one component of the EE paradigm, have also been demonstrated to be responsible for a resistance to 6-OHDA or MPTP insult; it allows reducing behavioral impairment involving forelimb use and movement (Tillerson et al., 2003; Petzinger et al., 2007).

The mechanism by which EE improves motor behavior appears to be quite complex. Nevertheless, such an effect involves synthesis and release of trophic factors (Nithianantharajah and Hannan 2006). Surprisingly, the synaptic plasticity, classically admitted to be the biological substrate underlying learning and memory function, has not been studied yet on the EE-housed animals in a Parkinson's disease model. Therefore, we examined whether EE has a significant impact on the endocannabinoids/dopamine mediated bidirectional plasticity (i.e. tLTP and t LTD) since both of them are impaired in the 6-OHDA rats.

**Experimental procedures**

Sprague–Dawley rats (only males, postnatal days 35) received unilateral injections of 6-OHDA in the right substantia nigra pars compacta (SNC). Animals were randomly distributed into four groups: standard environment (SE) or enriched environment (EE) housing (Figure 1) for 10 days or 2 months. From the group in 10 days-EE, we assigned another two groups of rats, which were individually caged 2 weeks or 2 months after EE (i.e. EE+SE) to check the persistence of influence of EE. *Ex vivo* whole cell recordings were
applied to assess the corticostriatal synaptic plasticity and tyrosine hydroxylase (TH) immunostaining was used to evaluate the dopamine depletion in striatum and SNC.

*Standard environment.* Cages for standard housing environment were standard laboratory cages (47x35x21cm) that contained only bedding without complex inanimate stimulation inside the cages. Animals were housed in 12 hours light/dark cycles and litter, food and water were available ad libitum in both housing conditions.

*Enriched environment.* Based on the standard definition of an enriched environment “a combination of complex inanimate and social interactions” (Rosenzweig et al., 1978), for enriched environment housing, 3 rats at a time (from 125-150g, postnatal 35 days) were housed in large cages (80x57x21cm) containing each one running wheel to allow voluntary physical exercise and at least 2-3 differently shaped objects (tunnels, shelters, boxes, movable balls, plastic and glass toys, pieces of tissue) for 8 weeks. The objects were fully substituted every 2 days during the full 8 weeks or 10 days to ensure continued novelty and complexity. Beside the running wheel, caution was taken to provide at least one object aiming at decreasing the stress (tunnel or shelters with various shapes and textures with increasing size and diameter according to the rodent development) and another object promoting voluntary physical activity and exploration (toys, balls, tanks of hidden food); We used toys and balls that displayed various shapes/diameters, textures, colors. Running wheels were randomly removed from the cage one day every 2 weeks to promote novelty. We used a protocol of enriched environment in which only male rats from the same litter were reared together (3 per cage). The rats were exposed continuously to the enriched environment for 8 weeks or 10 days before its influence on synaptic plasticity was evaluated.
**Figure 1. Enriched environment protocol.** a: the standard environment (SE) consisted of middle-sized cages (L x W x H: 47x35x21 cm) with one rat per cage without objects. b: three rats raised in an enriched environment (EE) were placed in larger cages (L x W x H: 80x57x21 cm) equipped with a running wheel, plastic toys and a set of tunnels, shelter and nesting material. Objects were randomly changed every two days.

**Results**

Based on our previous results (Fino et al., 2005; Cui et al., 2015), we first confirmed that 10 post-pre pairings and 100 pre-post pairings induced corticostriatal eCB-tLTP (mean value of the EPSC amplitude recorded 45-50 min after STDP protocol: 165±11%, p<0.0001, n=27) and eCB-tLTD (66±10%, p=0.0124, n=7), respectively. Because these both endocannabinoid-mediated plasticity are dopamine dependent (see article 3), it is thus expected that they would be abolished in 6-OHDA-lesioned rats. Indeed, we did not observed significant changes of the synaptic efficacy with either 10 post-pre pairings (95±4%, p=0.1665, n=9) or 100 pre-post pairings (115±9%, p=0.1363, n=8) (Figure 2). Strikingly, 2 months EE restored the expression of tLTP in 6-OHDA-lesioned rats. Indeed, a robust tLTP (159±22%, p=0.0376, n=7) was observed for 10 post-pre pairings. In contrast, 2 months EE did not rescue the tLTD observed in control conditions, since no significant plasticity was observed with 100 pre-post pairings (115±10%, p=0.1674, n=11) (p=0.9374). In conclusion, 2 months EE successfully rescue eCB-tLTP, but not tLTD, in 6-OHDA-lesioned rat (Figure 2).
Figure 2. The long-term enriched environment treatment (for 2 months) rescued eCB-tLTP but not eCB-tLTD at corticostriatal synapse. (a) Time chart illustrating the order of behavioral manipulations and electrophysiological recordings. After administration of 6-OHDA, the rats were housed in EE or standard environment for 2 months. (b) 10 post-pre pairings induced tLTP in 6-OHDA-lesioned rats after 2-month-EE housing (n=7) while no plasticity was observed in standard environment housing for 6-OHDA-lesioned rats (n=9). (c) 100 pre-post pairings induced no plasticity in 6-OHDA-lesioned rats in EE (n=11) or standard environment (n=8). Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent SEM.

Furthermore, EE housing did not affect significantly the survival of dopaminergic neurons in the SNc or the loss of their striatal afferences, as shown by the dramatic decrease of TH immunoreactivity in striata of EE rats (-65±2%, p<0.0001, n=7) when compared to rats raised in standard environment (-63±2%, p<0.0001, n=6) (p=0.6556 between the two groups) (Figure 3). It should be noted that this result is in apparent contradiction with some previous
studies which reported that EE had a beneficial effect onto DAergic neurons by attenuating the loss of DAergic neurons after 6-OHDA-lesion and TH-positive fibers (Bezard et al., 2003; Faherty et al., 2005; Anastasia et al., 2009). The main difference between our present results and these studies is that animals were placed in EE at least 20 days before dopamine depletion, and in our case, we started EE conditions 2 days after the 6-OHDA lesion. Our result is consistent with the conclusion from another study using similar post-lesion EE procedure (Steiner et al., 2006) which reported no effect onto DAergic neuron survival.

Figure 3. Enriched environment (2 months post-lesion) did not prevent 6-OHDA-induced loss of tyrosine hydroxylase (TH) at terminal fibers in striatum. Unilateral 6-OHDA injection in substantia nigra pars compacta led to a massive loss of striatal TH-positive fiber as illustrating by TH immunostaining in horizontal brain slices. There was no difference in TH staining between animal raised in standard environment (n=6) and those raised in EE (n=7). ***: p<0.001. ns: not significant.
We then asked if a brief period of EE was sufficient for rescuing the induction of eCB-tLTP. For this purpose, we considerably reduced the EE housing period from 2 months to 10 days only (Figure 4a): We placed the animals in EE conditions just after 6-OHDA-lesioning. In rats that were subjected to 6-OHDA lesion, a potent tLTP was observed for 10 post-pre pairings (173±16%, p=0.0010, n=11). We verified that this tLTP was eCB-mediated since it was prevented with bath-applied AM251 (3 µM), a CB1R specific inhibitor (108±23%, p=0.7227, n=8) (Figure 4c). This indicates that tLTP rescued by EE displays a similar mechanism than tLTP observed in control conditions. We then ensured that eCB-tLTP could be still induced with 10 post-pre pairings in sham-operated (with saline injection instead of 6-OHDA) rats after 10 days-EE (157±20%, p=0.0310, n=7) (Figure 4d), which was not significantly different from those observed in sham-operated rats raised in standard environment (147±7%, p=0.0004, n=9) (p=0.6698) (Figure 4b). These results indicate that the EE has no additional effect on the non-lesioned rats, although LTP appeared to be enhanced in the hippocampus or cortex slices after EE in the normal animals (Van Praag et al., 2000).

![Diagram](image)
Figure 4. The brief enriched environment treatment (for 10 days) rescued eCB-tLTP (10 post-pre pairings). (a) Time chart illustrating the order of behavioral manipulations and electrophysiological recordings. The rats were exposed to EE for 10 days after unilateral 6-OHDA injections. (b) tLTP was successfully induced by 10 post-pre pairings in sham-operated rats while impaired in 6-OHDA rats. (c) 10-days-EE housing allowed recovering tLTP (n=11). This tLTP was CB1R-mediated since prevented by AM251 (3µm, n=8). (d) EE had no effect on tLTP in sham-operated rats (n=7). Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem.

We then ask the question of the duration of EE along time. For this purpose, to determine how long the restorative effect of EE would last, we transferred the animals exposed to 10 days-EE to standard housing conditions and then assessed the expression of eCB-tLTP after 2 weeks and 2 months, respectively (Figure 5a). First, after 2 weeks of standard environment housing (post 2 weeks EE), 10 post-pre pairings were still able to induced tLTP (3 out of 5 cells exhibited significant LTP) in 6-OHDA-lesioned animals subjected to 2 weeks of EE; however, at this day, we did not observe a significant effect after averaging all the cells (139±18%, p=0.0912, n=5) (Figure 5b). After 2 months of standard environment housing (post 2 weeks EE), we did not observed any plasticity (94±10%, p=0.5475, n=7) (Figure 5b). Therefore, only the continuous stimulation by EE treatment could reverse corticostrial tLTP impairment induced by 6-OHDA, and this strong effect would be long-lasting (around 2 weeks; but this needs to be confirm, or not, by additional experiments).
Figure 5. The sustaining enriched environment treatment is necessary for eCB-tLTP (10 post-pre pairings). (a) Time chart illustrating the order of behavioral manipulations and electrophysiological recordings. After 10 days-EE, one sub-group of rats was placed in standard environment for 2 weeks, while the other group remained in standard environment for 2 months. (b) The exposure to an EE resulted in a significant restoration in the eCB-tLTP, which progressively declined after the rats were transferred to standard environment. After 2 weeks standard environment, 10 post-pre pairings could still induce some tLTP, but in smaller proportion of neurons, (n=5). However, no plasticity was observed in 2 months standard environment (n=7). Representative traces are the average of 15 EPSCs during baseline (black traces) and 45 min after STDP protocol (grey traces). Error bars represent sem.
Discussion

In this study, we demonstrate that EE per se is able to significantly restore the cortico-striatal eCB-tLTP following unilateral injection of 6-OHDA. Our findings may imply a synaptic mechanism for the previously reported EE prevention of the motor deficit in rodent models of Parkinson's disease (Tillerson et al., 2003; Jadavji et al., 2006; Petzinger et al., 2007). We also answered an important question which is how long do the consequences of EE could last? Even only 10 days of EE after the 6-OHDA lesion are able to rescue a potent tLTP and this effect could last at least 2 weeks without EE (rats being replaced in standard environment). However, it is important to note that this latter point needs to be confirmed with additional experiments.

The major contribution of the present study is the integration of electrophysiological evidences together with former morphological and functional behavior results that helps to understand the processes occurring after EE condition in animal model of Parkinson’s disease. Our new finding of EE’s restorative effect on long-term plasticity could guide the development of a new non-pharmacological (so non-invasive strategy) to potentiate beneficial (but incomplete) effects obtained with the classical treatments for Parkinson’s disease. EE should be viewed as a significant add-on to pharmacological treatments and not as a treatment by itself.

However, the mechanism of the plasticity recovery remains pretty much unclear. In most of the studies, EE is used in a preventive way, i.e. before the lesion, rather than post-lesion EE (curative way), and resulted in a significant nigrostriatal dopaminergic system neuroprotection (preservation of dopaminergic neurons as well as their striatal connections) (Bezard et al., 2003; Faherty et al., 2005; Jadavji et al., 2006; Anastasia et al., 2009). Due to the distinct experimental protocols, our study reports a restorative effect on synaptic plasticity, but not neuroprotection of SNC neurons or striatal TH-positive fibers, which suggests that EE can result in compensatory influence through multiple factors. Indeed, several concomitant micro-environmental changes were reported during exposure to EE, such as modified...
dendritic spine morphology (Hosseiny et al., 2014), and increased expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF), together with neurogenesis and gliogenesis (Kempermann et al., 1997; Pham et al., 2002; Nithianantharajah and Hanman, 2006; Sale et al., 2014). Furthermore, extensive evidences indicate that EE feedback controls the hypothalamic-pituitary-adrenal (HPA) axis responsiveness in young animals and modulate serotonin release, which in turn promotes the synaptic plasticity (Sale et al., 2014). In striatum, beside the prominent involvement of trophic factors in EE, a down-regulation of dopamine transporter (DAT) binding was observed (Bezard et al., 2003), which potentially affect the dopamine dependent eCB-tLTP as reported in our aforementioned observation (Article 4). In addition, spine density was diminished in striatal MSNs in dopamine-denervated animals, and was found to be restored by chronic L-DOPA treatment (Suárez et al., 2014). Since we described that both L-DOPA (see article 4) and EE are able to restore eCB-tLTP, morphological modification may be another candidate involved in these processes. Further work is needed to determine the molecular mechanisms underlying the functional changes elicited by EE.

One interesting question is why EE specifically rescue eCB-tLTP but not eCB-tLTD (Fig 6)? So far, our previous studies suggest that the induction of eCB-tLTP and eCB-tLTD share similar signaling pathway, namely, both are mediated by mGluR5, muscarinic M1R, VSCCs and retrograde eCBs signaling (PLCβ, MAGL, IP3 receptors and CB1R to name a few). However, a noticeable difference is that eCB-tLTP is D2R-mediated and not dependent on D1R, while eCB-tLTD requires co-activation of both D1R and D2R. We could hypothesize that EE could specifically acts, on pathways (that remain to be deciphered), on D2R-mediated signaling for rescuing eCB-tLTP. In the close future, several lines of genetically-modified mice, including D2R-MSNs ablated mice (Adora2a-Cre+/- : iDTR+/− mice) and cholinergic interneuron ablated mice (ChAT-Cre+/- : iDTR+/− mice), would be used to identify the precise locus of D2 receptors critical to eCB-tLTD.
EE generally refers to the housing conditions with enhanced novelty and complexity. Besides the social communication, the objects used for EE vary in shape, size, material, smell, textures and colors in order to facilitate sensory, cognitive and motor stimulation (Figure 7). Considering the diurnal activity patterns and the limitations of the rodent visual system, the somatosensory and olfactory stimuli could be the most salient parameters (Nithianantharajah and Hannan, 2006). Nevertheless, whether specific components of the EE contribute to particular effects on specific brain areas and behavior remain to be elucidated. Since, we investigated STDP at the level of the somatosensory striatum, in a forthcoming step, we should design more specific composition of objects in order to stimulate somatosensory and promote the beneficial effects of EE on the cortico-striatal transmission.
Figure 7. Enriched environment and the effects of enhanced sensory, cognitive and motor stimulation on different brain areas. EE can promote neuronal activation, signaling and plasticity throughout various brain regions. Enhanced sensory stimulation, including increased somatosensory and visual input, activates the somatosensory (red) and visual (orange) cortices. Increased cognitive stimulation, for example, the encoding of information relating to spatial maps and object recognition is likely to activate the hippocampus (blue) and other cortical areas. In addition, enhanced motor activity, such as exploratory movements (including fine motor skills that differ radically from running wheel alone), stimulates areas such as the motor cortex and cerebellum (green). From (Nithianantharajah and Hannan, 2006)
Reference


GENERAL DISCUSSION AND PERSPECTIVES

The basal ganglia are involved in adaptive control of behavior and procedural learning and memory (Haber, 2003; Graybiel, 2005; Yin and Knowlton, 2006; Grillner and Robertson, 2015). The striatum plays a key role in the integration of the information from cortical and thalamic inputs, and in turn regulates the neuronal activity of the basal ganglia through mainly the direct and indirect trans-striatal pathways. Thus, the characterization of the corticostriatal synaptic plasticity is crucial to reveal its involvement in various aspects of procedural learning and memory (Costa et al., 2004; Citri and Malenka, 2008; Yin et al., 2009a; Koralek et al., 2012) and how it is impacted in related diseases such as Parkinson’s or Huntington’s disease (Calabresi et al., 1996; Mahon et al., 2004b; Shepherd, 2013). This PhD work mainly investigated the short-term (DSE, PPR) and long-term (LTP, LTD) synaptic efficacy changes at the corticostriatal synapses under the neuromodulatory effect of the endocannabinoid and dopaminergic systems.

The role of cannabinoid type-1 receptor (CB1R) and endocannabinoids (eCBs) in corticostriatal plasticity

Mechanism concerning the canonical form of striatal LTD: the eCB-LTD

We initiated this work with the widely studied eCB-LTD (Mathur and Lovinger, 2012), which is induced by a low-frequency stimulation (1Hz) at the excitatory synapses in the striatum. Here we unravel a putative novel mechanism accounting for the LTD mediated by eCBs. Indeed, pharmacological inhibition of either non-muscular myosin II (NMII) or of its major activating kinase, Rho-associated protein kinase (ROCK) prevented the eCB-LTD. These results demonstrated a new mechanism linking cannabinoid-induced presynaptic actomyosin contraction and long-term synaptic efficacy changes (Article 1). These results expanded our view of the mechanisms behind presynaptic CB1R-dependent synaptic plasticity and indicated that synapses are dynamically pruned during brain plasticity and putatively learning. Interestingly, it has been previously proposed that mediators of neuronal
development and neurite growth may retain their structural effects in mature brain functions (Figure 35) (Holtmaat et al., 2013), and our report would represent an illustration of this concept.

Figure 35. Schematic representation of the interplay between functional synaptic plasticity and structural synaptic network changes. Deprivation-mediated disinhibition (1) facilitates spike-timing-dependent LTP (2), which strengthens synapses (3). The continuous interplay between these processes may lead to synapse growth (4) and new synapse formation (5). Upon long-term sensory deprivation disinhibition may become structural (6), which continues to drive the processes above (2)–(5). Ultimately, this may lead to formation of synaptic connections with neurons that were not initially part of the network (7), and recruitment of their activity (8). From (Holtmaat et al., 2013)

*A low numbers of paired stimulation are able to induce a plasticity: the eCB-LTP; a further step to the cellular substrate for fast learning?*

The changes in synaptic strength do not always rely on low- or high-frequency stimulations (hundreds to thousands of stimulations), which are rarely recorded *in vivo*. Our lab previously reported that a bidirectional corticostriatal long-term plasticity can occur upon modest neuronal activity induced by spike-timing dependent plasticity (STDP) protocol (assessed with the classical form of STDP: 100 pairings at 1 Hz) (Fino et al., 2005, 2009, 2010; for review see Fino and Venance, 2010 and 2011).

Synapses display various forms of STDP depending on multiple factors (i.e. pairings frequency, number of pairings and temporal window) (Dan and Poo, 2006; Caporale and Dan,
2008; Sjöström et al., 2008; Feldman, 2012). To unveil the borders of expression of the corticostriatal STDP, we first lowered the number of pairings. In parallel, we built a realistic biophysical model (in collaboration with Hugues Berry, INRIA, Lyon) accounting for corticostriatal STDP to generate predictions. With this model-driven experiment strategy, we observed at the single cell level that the corticostriatal synapses are highly plastic to few pairings. Namely, we observed the existence of a unidirectional tLTD occurring at medium number of paired stimulations (50 pairings) and more importantly the re-emergence of an unidirectional tLTP upon a low number of paired stimulations (5-10 pairings) (Articles 2).

In general, the magnitudes of both tLTP and tLTD are expected to increase with the number of pairings of pre- and post-synaptic activities before reaching a saturation plateau. In addition, the tLTD induction would required a greater number of pairings than tLTP (Froemke et al., 2010). Therefore, the absence of tLTD with 10 pairings is expected. However, similar results of tLTP (or more generally of plasticity) induced by low number of STDP pairings are still rare; They have been observed in the optic tectum of the tadpole in vivo and in the visual cortex of rats ex vivo, in which tLTP reached a plateau, in term of magnitude, around 80 pairings, while 20 and 10 pairings still induced a moderate but significant potentiation (Zhang et al., 1998b; Froemke et al., 2006). Regardless of the STDP paradigm, a single-burst of spikes in the Schaffer collateral pathway induced LTP in CA1 pyramidal cells (Remy and Spruston, 2007). In addition, the individual local dendritic spike evoked in layer 5 neocortical pyramidal cells induced stable LTD due to Ca$^{2+}$ transient triggered by activation of NMDARs (Holthoff et al., 2004). The high susceptibility of synaptic efficacy changes to low number of stimulations may underlie the potentiation or depression effect at some synapses during or immediately after brief conditioning, which potentially encode for the so-called fast learning or fast mapping (Schultz et al., 2003; Pasupathy and Miller, 2005; Tse et al., 2007; Quilodran et al., 2008; Ito and Doya, 2009). Indeed, studies in monkeys during associative learning provided the evidence that in the prefrontal cortex or striatum, neurons fire a few spikes responding to visual cues or rewards within the first 5–10 trials (i.e. at a frequency of 5-25 Hz and during 0.1-0.5 s, typically < 10 spikes; Schultz et al. 2003; Pasupathy & Miller, 2005; Quilodran et al. 2008). Particularly, learning-dependent changes in the striatum appear sooner and progress more rapidly than in the
cortex (Pasupathy and Miller, 2005). Hence, our results concerning the expression of eCB-tLTP, should help bridging the gap between in vitro studies in which plasticity is usually triggered using numerous spikes and behavioral situations where learning can be obtained even within a few trials involving a very limited number of spikes.

*The eCB-tLTP features*

We shown that eCB-tLTP relied on both CB1R and TRPV1 activation. As predicted by our biophysical model (built in collaboration with Hugues Berry, INRIA Lyon; data not shown), the transient concentration of eCBs appears to be the key element controlling the polarity of STDP (i.e. eCB-tLTP or eCB-tLTD) attributable to CB1R activation; TRPV1 serving as an additional postsynaptic calcium boost for the eCB synthesis. We confirmed the crucial role of the concentration of 2-AG in selecting eCB-tLTP or eCB-tLTD, by directly applying brief puff’s of 2-AG at "high" concentration (100 μM) adjacent to the dendrites of the recorded MSN for 10 seconds (a time duration corresponding to 10 post-pre pairings at 1Hz), and we observed a robust LTP. Whereas, applying 2-AG for 100 seconds (a time duration corresponding to 100 pre-post pairings at 1Hz) resulted in a potent LTD (data from a manuscript in preparation: 2-AG levels gates bidirectional endocannabinoid plasticity. Cui Y*, Prokin I*, Xu H, Delord B, Genet S, Venance L#, Berry H#). This indicates that a specific level of eCB is required to induce a specific type of plasticity implying different learning process. On the other hand, the pharmacological inhibition of CB1R with AM251 (3 μM) or by using the CB1R knockout mice have further confirmed the essential role of CB1Rs in 10 post-pre pairings induced tLTP. Importantly, the prevalence of eCB-tLTP expressed on both juvenile and adulthood of rodent (rats and mice) indicates that this form of plasticity may be widespread to encode salient events from few spikes.

As classically admitted (and widely reported in the literature by hundreds of papers), eCBs contribute to synaptic depression (at a short- and long-term scales) in a retrograde manner (Chevaleyre et al., 2006; Heifets and Castillo, 2009; Kano et al., 2009). However, it exists noticeable exceptions to this common view, pointing out that eCBs can also mediated
LTP. Indeed it has been reported eCB-LTP at mixed (chemical and electrical) synapses of the goldfish Mauthner cell via intermediary dopaminergic neurons (Cacho et al., 2007), at hippocampal CA1 synapses induced either by low-frequency (Zhu and Lovinger, 2007), high-frequency (Lin et al., 2011), theta-burst stimulation (Chevaleyre and Castillo, 2004) or paired stimulations (Xu et al., 2012), and lastly a heterosynaptic short-term potentiation via intermediary astrocytes (Navarrete and Araque, 2010). Here, we unraveled a new form of synaptic plasticity (eCB-tLTP) and demonstrate that eCBs are not restricted to depression but are also capable of potentiation in the dorsal striatum, putting them in line with other neurotransmitter such as the glutamatergic system.

Because the presynaptic compartment is difficult to manipulate with electrophysiological techniques, some of the explanations for eCB-tLTP and tLTD expression are based on hypothesis proposed by the biophysical model that remain to be experimentally fully confirmed. The main prediction from the biophysical model is that the polarity of eCB-dependent plasticity depends on the magnitude of the inhibition of presynaptic adenylyl cyclase by dopamine (via D2 receptors) and eCBs (via CB1R). In turn, adenylyl cyclase controls its downstream signaling cascades, i.e., modulating cAMP production and CaN/PKA balance. High inhibition of adenylyl cyclase leads to tLTP while moderate inhibition triggers tLTD. Although our present results support these assumptions, e.g., the involvement of presynaptic PKA and D2 receptors in the induction of eCB-tLTP, some open questions about the inner presynaptic processes remain to be elucidated and additional experiments are needed. For example, it is not clear the signaling cascades aim at reducing PKA activation and inhibiting presynaptic VSCCs should facilitate the eCB-tLTP through reducing CaN activity. Moreover, the mechanism of tonic adenylyl cyclase activation is not clear, and including cAMP in the internal solution could be an option to check the contribution of pre- and postsynaptic adenylyl cyclase in the eCB-dependent plasticity. In addition, direct evidence for increasing adenylyl cyclase inhibition which should occurred with decreasing number of pairings are still lacking.
The temporal window of eCB-tLTP expression is in agreement with anti-Hebbian NMDA-tLTP observed in striatum (Fino et al., 2005, 2010), and is reversed to the Hebbian STDP found in cortex (Feldman, 2000; Froemke et al., 2005), in hippocampus (Bi and Poo, 1998) or even in striatum (Pawlak and Kerr, 2008). One major difference of the experimental conditions between the study by Pawlak et al. and our study is that they blocked GABAa transmission. Striatum is modulated by local microcircuits formed by GABAergic neurons (interneurons and MSNs). Namely, a strong feed-forward inhibition exerted by fast-spiking and low-threshold-spiking interneurons, together with a feed-back inhibition produced by MSN axon collaterals are capable to alter the spike timing and back-propagating APs in MSNs (Teppé and Bolam, 2004; Venance et al., 2004; Teppé et al., 2004; Wilson, 2007; Kreitzer, 2009). Such control of the spike timing could specifically modulates the STDP induction, in which occurrence of LTP or LTD relies on the precise relative timing (in milliseconds) of presynaptic and postsynaptic events. Indeed, a recent study from our lab showed that blockade of GABAa receptors completely reversed the polarity of the bidirectional corticostriatal STDP (induced with 100 pairings) (Paille et al., 2013). It means that GABA would act as a hebbian/anti-Hebbian switch. Similarly, we found that GABA controls the temporal order of the eCB-tLTP (10 pairings) as well: with picrotoxin, tLTP was observed with 10 pre-post pairings while 10 post-pre pairings did not induce plasticity. In summary, GABAergic microcircuits are neither involved in the eCB-tLTP induction nor magnitude but control the polarity of the timing dependence of eCB-tLTP.

A perspective to this project is to explore if such eCB-LTP can be triggered in the other brain regions and whether it could be observed in vivo. We have experimental evidences showing that this eCB-ILTP is not restricted to the striatum but is also expressed in the somatosensory cortex between layer 2/3 and 5 (article in preparation). Cortical synapses exhibit eCB-tLTP for 10 pairings, similarly to corticostriatal synapses, with the noticeable difference of a Hebbian versus anti-Hebbian polarity. Therefore, these results show that, similarly to corticostriatal synapses, cortical synapses can also adapt and learn from limited occurrences of coincident activity through eCB-LTP.
Dopamine is the gatekeeper for endocannabinoid-mediated STDP

Dopamine and glutamate are pivotal neurotransmitters involved in procedural learning and memory. The glutamatergic and dopaminergic systems converge on both MSNs and interneurons in the striatum. Dopamine modulation of synaptic plasticity at glutamatergic corticostriatal synapses is thought to be a teaching signal for procedural learning in the striatum (Costa et al., 2004; Kreitzer and Malenka, 2005; Tritsch and Sabatini, 2012; Surmeier et al., 2014). Thus, we investigated how dopamine regulates MSN plasticity and more specifically eCB-tLTP.

The main findings of the this project can be divided into two parts: (1) concerning eCB-tLTD, the "canonical" synaptic plasticity at corticostriatal synapse, we showed that eCB-tLTD induction was prevented by either D1 or D2 receptor antagonist, and impaired in Parkinson’s disease rat model and rescued by chronic L-DOPA treatment; (2) concerning eCB-tLTP, a new form of corticostriatal synaptic plasticity, we demonstrated that eCB-tLTP was dependent on presynaptic D2 receptor (and not D1 receptors). Thank to a genetic tool allowing ablating different striatal subpopulations (Durieux et al., 2009, 2012) we were able to exclude the participation of D2 receptors expressed in MSNs, cholinergic interneuron and in the dopaminergic terminals from SNc for its permissive role for eCB-tLTP expression (Figure 36). We thus hypothesized that D2 receptor located on the corticostriatal terminals would be the gatekeeper for eCB-tLTP (Article 3). To clearly demonstrate that D2 receptor located at cortical terminals would be responsible for eCB-tLTP expression, we are currently establishing mice lines in which D2 receptor is specifically knock-out in glutamatergic terminals.
**LTD** at excitatory inputs onto MSNs is the most commonly observed plasticity in the dorsal striatum, which can be induced by various protocols: Hebbian high-frequency stimulation (HFS) (Calabresi et al., 1992b; Wickens et al., 1996), Hebbian medium-frequency stimulation (MFS) (Kreitzer and Malenka, 2005; Ronesi and Lovinger, 2005), Hebbian and non-Hebbian low-frequency stimulation (LFS) (Fino et al., 2005), STDP protocol (Fino et al., 2005; Pawlak and Kerr, 2008; Shen et al., 2008; Fino et al., 2010; Paillé et al., 2013) or brief subthreshold depolarization-dependent plasticity (Fino et al., 2009a). Although all these studies agreed on the point that striatal LTD is eCB-dependent and requires D2 receptor activation, their conclusion differed considering the locus of D2 receptor involved in plasticity induction (Wang et al., 2006; Kreitzer and Malenka, 2007b). Indeed, Wang et al., (2006) observed LTD on both D1R- and D2R-MSNs, which was blocked by D2 receptor antagonists. They proposed that the dependence upon D2 receptor was attributed to its inhibitory action on cholinergic interneurons, but do not rely on D2 receptors expressed by MSNs belonging to the indirect pathway. However, Kreitzer and Malenka (2007) reported that D2R-MSNs selectively display LTD, indicating that LTD might require postsynaptic D2 receptor activation. They proposed that LTD induced in D1R-MSNs may be due to a "spill-over artifact" caused by the induction.
protocol (high-intensity stimulation in the corpus callosum), which possibly would lead to broad and diffuse activation of neighboring D2R-MSNs. This in turn would evoke the D2-dependent production and release of eCBs. Since we have access to mice with selective ablation of D2R-MSNs (Adora2a-Cre+/− : iDTR+/−, A2A-DTR+/− mice) and cholinergic interneurons in striatum (ChAT-Cre+/− : iDTR+/− mice), it is a unique opportunity to clarify the mechanism underlying eCB-LTD and solve this long-lasting debate. These experiments are currently performed.

LTP in MSNs at corticostriatal synapses is much less extensively characterized than LTD. Thus the results are even more controversial compared to striatal LTD because of the variety of induction protocols (cell conditioning protocols, Mg²⁺ free or not in extracellular solution, rats versus mice, inhibition or not of the GABAergic transmission, location of the stimulation,…) (Calabresi et al., 2007; Di Filippo et al., 2009; Gardoni and Bellone, 2015). The first question is whether eCB-tLTP is expressed in both types of MSNs. To answer that, D1-eGFP mice were used in our study and we observed tLTP on both fluorescent D1R-MSNs and non-fluorescent neurons (supposed to be D2R-MSNs). In addition, in the rats, eCB-tLTP was successfully induced in the vast majority (83%) of MSNs, indicating the occurrence of eCB-tLTP in both striatopallidal and striatonigral MSNs. Because the D1R- and D2R-MSNs display different intrinsic electrical properties (Gertler et al., 2008; Planert et al., 2013), a clear segregation of I-V curves for all the MSNs further confirmed that we recorded from both subtypes of MSNs for eCB-tLTP expression in rats. We used to include biocytin in the intracellular solution for the patch-clamp pipette and did double immunofluorescence (substance P and A2A) to label the recording MSNs. Unfortunately, the results were not conclusive due to the diffusion of biocytin and the specificity of antibodies. Although it appears easier access to transgenic mice, we chose to keep in addition the rat model to make sure that similar results can be observed in two rodent species. In a future study of plasticity in rats, an alternative method that will be used is the retrograde labeling to distinguish the involvement of different subtypes of MSNs (Planert et al., 2013).
The classical view on the role of dopamine in the corticostratial plasticity is that dopamine acting on D1 receptors promotes LTP induction, whereas activation of both D1 and D2 receptors is required for LTD (Calabresi et al., 1992a, 2000a). Such conclusion is supported with experiments using tetanic stimulation (Calabresi et al., 1997; Kreitzer and Malenka, 2005, 2007b; Wang et al., 2006) and STDP protocol (Shen et al., 2008). However, Pawlak and Kerr (2008) using STDP paradigm (with 0.1Hz pairings) proposed that the D2 receptor activation does not account for the amplitude of the plasticity efficacy change but modulates the kinetic expression of corticostratial STDP. Indeed, inhibition of D2 receptors delayed the onset of tLTD while the onset of tLTP was earlier compared to control tLTP. Nevertheless, the authors did not distinguish between MSNs expressing different dopamine receptor subtypes. In our present study (Article 3), we demonstrated that D1 and D2 receptors interact synergistically to enable formation of tLTD, which is consistent with the general conclusion. But to our surprise, we failed to induce tLTP with bath-applied D2 receptor antagonist while D1 receptor antagonist had no effect on the tLTP. Such results are in apparent contradiction with earlier observations. However, LTP reported so far at corticostratial synapse are NMDA-mediated, while tLTP we are studying is a new form of presynaptic CB1R-dependent tLTP. The same downstream signaling pathway of CB1R and D2 receptor at presynaptic side indicated the involvement of the presynaptic D2 receptors in this tLTP.

In summary, tLTP induced in D2R-MSNs ablated mice (Adora2a-Cre+/−:iDTR+/− mice) suggests that the D2 receptors involved in eCB-tLTP are not postsynaptically located in MSNs. Thus, we aimed at identifying the locus of presynaptic D2 receptors underlying eCB-tLTP. Presynaptic D2 receptors are expressed at the nigrostriatal dopaminergic afferents (De Mei et al., 2009), in the cholinergic interneurons (Hersch et al., 1995) and at the corticostratial glutamatergic afferents (Bamford et al., 2004). So far, we have already excluded the first two possibilities: we observed a potent tLTP on MFB lesioned rats when supplying a bath-applied D2 receptor agonist, as well as in cholinergic interneuron ablated mice (ChAT-Cre+/−:iDTR+/− mice). Indeed, the activation of D2 receptor expressed by cholinergic interneurons would decrease acetylcholine release, which should promote LTD, but not LTP (Wang et al., 2006).
Pharmacological experiments (using bath-applied D1 and/or D2 receptors antagonists) demonstrated that dopamine is critical for the eCB-tLTP at corticostriatal synapses in MSNs. Optogenetics provided us a chance to further investigate the dopaminergic effect taking into account the dynamic change of dopamine level during STDP induction (and more specifically during each pairings). In the mice which expressed Archeo-rhodopsin-3 in DAT-expressing neurons (DAT-Cre+/− : Arch3-GFP+/+ mice), 16 seconds of opto-inhibition of the dopamine release from the dopaminergic terminals in the striatum significantly blocked the eCB-tLTP (Article 2). It is in agreement with the proposition that the selectivity of dopamine effects is achieved by the timing of dopamine release in relation to the activity of glutamatergic synapses (Arbuthnott and Wickens, 2007; Wu et al., 2015).

**Perspectives for future investigation**

1. **The use of fast-scan cyclic voltammetry to monitor the dopamine dynamics in striatal brain slices.**

We showed that the putative opto-inhibition of dopamine release inhibited the eCB-LTP, however, in fact the decisive evidence for the efficacy of this opto-treatment is missing. The fast-scan cyclic voltammetry constitutes an appropriate technique for measuring dopamine concentrations in real time, which allows a precise quantification of subtle changes in dopamine level in the nanomolar to micromolar range, on a millisecond time scale (Venton et al., 2003). Moreover, the micrometer-dimension probe assures a fine spatial resolution with minimal damage of the tissue. A growing number of studies combined fast-scan cyclic voltammetry and optogenetics to explore the link between dopamine release through optical stimulation of dopaminergic neurons in the midbrain and behavioral conditioning (Tsai et al., 2009; Bass et al., 2010; Adamantidis et al., 2011; Witten et al., 2011).

Recently, it was shown that dopamine release can be enhanced not only by opto-activation of cell bodies in the midbrain but also by opto-activation of the terminals within the striatum
(Bass et al., 2013). The amount of dopamine released in the striatum was even higher (20 folds) than in cell body regions in the VTA or SNC (Ford et al., 2010). However, little is known about the efficiency of opto-inhibition of dopamine release. A recent investigation reported that the opto-inhibition of SNC dopaminergic neurons or in dorsal striatum of the dopaminergic afferences induced aversion (Ilango et al., 2014). This finding demonstrated that opto-inhibition at striatal dopaminergic terminals was sufficient to trigger a behavioral change; but it is fair to say that the direct evidence of a dopamine decrease is still lacking. In our experiments, the low basal dopamine level (around 30 nM according to Venon et al., 2003) in ex vivo brain slices make it more difficult to measure the dopamine variation induced by an opto-inhibition compared with opto-activation of dopamine release. Recently, fast-scan cyclic voltammetry has been set up in our laboratory (Figure 37), but so far we were able to detect only high level of dopamine transient (1mM) by local puff. Many environmental factors may affect current response to dopamine transient, including local Ca$^{2+}$ and Mg$^{2+}$ concentrations, pH and O$_2$ changes. Further efforts are needed to improve the shape of the detecting probes and calibrate its sensitivity (nA/mM) to enhance the resolution of dopamine concentration.

**Figure 37.** The demonstration of stimulus evoked dopamine release from genetically defined neurons such as by combining optogenetics and carbon fiber voltammetry (A) Infrared microphotograph of the voltammetry carbon fiber and the patch-clamp pipette in the striatum (B) electrophysiological recording of one MSN (C) voltammogram trace of MSN recorded in (B), while dopamine release increase.
The use of "SNc-striatum-cortex" brain slice (a corticostriatal slice in which SNc and the nigrostriatal fibers are preserved) for optogenetics.

To mimic the in vivo condition, a novel brain slice requires preserving the intact dopaminergic pathways as much as possible. Thanks to a 3D model of rat brain set-up in the lab (Mailly et al., 2010), we developed a corticostriatal slice in which SNc and the nigrostriatal fibers are preserved (Figure 38) (unpublished data). In a forthcoming study, we will perform opto-stimulation of dopaminergic neurons in SNc to activate or specifically inhibit those neurons, mimicking various spiking activity in vivo. Combined with fast-scan cyclic voltammetry, we should be able to determine how plasticity depends on the synaptic inputs activated and dopamine dynamic levels. The goal is to better understand the dynamics of dopamine release related to various phasic activities. Such knowledge will help us improving the design of our experiments that aim at mimicking physiological situations. Indeed, a huge number of studies aimed at determining the dopaminergic control of corticostriatal information processing (mainly in pathological situations), but it is fair to say that the dopaminergic control of plasticity is far from being fully understood. Indeed, the impact of the magnitude and dynamics of dopamine concentration on STDP is currently poorly understood. Yet, physiological dopamine constitutes a highly dynamical signal with tonic and slow and fast phasic components and the molecular pathways underlying STDP are highly sensitive to the precise dynamical pattern of molecular interactions. Thus, the different temporal dopamine signals most likely affect STDP in very different ways, a central issue that currently remains unexplored. Understanding these dynamical interactions therefore represents a primary goal to assess the dopaminergic control of adaptive behavior.

A major obstacle to the understanding of how dopamine controls corticostriatal STDP is that the dynamics of dopamine occurs at various time scales, each of which could have a different influence on STDP. Dopaminergic activation is seen across varying time scales (from milliseconds to hours), displaying tonic, slow phasic and fast phasic signals. For example, bursts of action potentials (sub-second modulation) have been related to dopamine outflow and to the phasic dopamine signaling of novel rewards and environmental stimuli. In normal
conditions, dopaminergic neurons exhibit a spontaneous firing activity, described as a continuum between two distinguishable rhythms: a slow regular single spike firing and a bursting mode (Grace and Bunney, 1984). Regular spiking emerges from intrinsic membrane potential oscillations (Kitai et al., 1999). The burst-firing pattern is absent in midbrain slice preparations suggesting that it critically depends on afferents to the dopaminergic neurons, emphasizing the need for in vivo studies. The afferents include glutamatergic fibers, originating mainly from the prefrontal cortex, as well as cholinergic and glutamatergic fibers originating from the tegmental pedunculopontine nucleus and the latero-dorsal tegmentum nucleus. The burst-firing mode has generated particular interest since it causes a larger dopamine release than regular spiking (Goon, 1988). The transition from regular firing to bursting activity is associated with alerting or anticipatory phases of reward (Schultz et al., 1997; 2007), as well as with stimuli associated with addictive drugs (Di Chiara and Imperato, 1988). Finally, synchronized pauses in the activity of dopamine cells could influence the dopamine receptor activation (Dreyer et al., 2010), introducing the notion that synchronization of dopamine cells is also important (Hyland et al., 2002).

Figure 38. 3D virtual SNC-striatum-cortex brain slice based on anatomical rebuilding of neurons and fibers following in vivo biocytin injections (unpublished data)
Parkinson's disease and enriched environment

As the D2 receptors are implicated in Parkinson's disease, schizophrenia and other neurological disorders, they constitute a primary target for many antipsychotics and for Parkinson's disease therapy (Seeman et al., 2005, 2007; Beaulieu and Gainetdinov, 2011; Surmeier et al., 2014). We showed that the eCB-tLTP is impaired in Parkinson's disease rat model and can be restored by the chronic L-Dopa treatment. A similar rescue of eCB-tLTP by an acute application of D2 receptor agonist in brain slice further confirmed that activation of D2 receptor is required for physiological plasticity processes, which appears to be crucial for motor control and habit learning.

In Parkinson's disease, the massive loss of dopaminergic neurons in SNC causes severe motor impairments probably due to imbalances of basal ganglia pathways. At present, the most efficient therapy for Parkinson's disease relies on L-DOPA, which is a dopamine precursor. However there is a severe side effect of L-DOPA treatment after the so-called "honeymoon" period (5 to 10 years of L-dopa treatment) for which L-DOPA leads to patients deeply attenuated motor symptoms. Thus, alternative approaches for the prevention and/or treatment of Parkinson's disease are required. Although the etiology of Parkinson's disease and factors affecting its progression remains unclear, studies on human patients suggest that the risk of Parkinson's disease might be influenced (among other factors) by lifestyle (Olanow and Tatton, 1999; Elbaz and Moisan, 2008), such as education or occupation (Frigerio et al., 2005) and physical activity (Chen et al., 2005; Thacker et al., 2008; Yang et al., 2015). An experimental proxy for "lifestyle" can be mimicked by the housing rodents in an enriched environment which would provide a sustained cognitive and sensorimotor stimulation, social interactions, and increased physical activity (Anastasia et al., 2009).

We found that continuous exposure to enriched environment for only 10 days significantly rescue the impaired eCB-tLTP in the 6-OHDA-lesioned rats. These data imply that positive life experiences, involving more cognitive and physical activities would have beneficial consequences even after the onset of Parkinson's disease. Further investigation are needed to
confirm this point and determine the mechanisms underlying the rescuing process; this should help to search for molecular targets and the development of a new therapy accompanying the classical symptomatic treatment of Parkinson's disease. It will also be interesting to find a physiological substrate for the observed improvements in synaptic plasticity. Knowing that the abnormal involuntary movement and L-DOPA induced dyskinesia (LIDS) are the main side effects of L-DOPA therapy, we will evaluate the effect of different duration of enriched environment, such as before/after 6-OHDA lesions and after the first appearance of dyskinesia. Furthermore, the in vivo neuronal activity recordings in the basal ganglia output nuclei, i.e., triphasic response in SNr (Maurice et al., 1998, 1999; Nambu et al., 2000; Kolomiets et al., 2003) are the next step to perform to investigate their modifications after enriched environment exposure.

**Huntington's disease (see ANNEXE)**

Huntington’s disease is a dominant inherited neurodegenerative disorder caused by an abnormal expansion of a polyglutamine repeat in the huntingtin (HTT) protein. Huntington’s disease is tightly related to the basal ganglia, showing the dysfunction and marked degeneration of adult striatal and cortical neurons. Our collaborator Sandrine Humbert's team used a genetic mouse model and in utero electroporation to inactivate HTT specifically in post-mitotic lineages. They found that HTT regulates the polarization of newborn cortical neurons, which is necessary for the maintenance of the bipolar morphology of cortical projection neurons during the locomotive mode of migration. Meanwhile, we analyzed the electrophysiological properties of cortical and striatal neurons and cortical and cortico-striatal excitatory synaptic transmission to assess the functional consequences of the structural changes they observed. We provided evidence that non-synaptic properties (passive and active membrane properties) of layer V pyramidal cells and MSNs showed no significant difference between control and mutant mice. Whereas, the spontaneous postsynaptic current and paired-pulse ratio of neurons in cortex and striatum were markedly changed in huntingtin (HTT) protein depleted mutant mice (see Figure 8 of Article 4). Thus, the cortical defects
resulting from the post-mitotic loss of HTT are maintained in young adult brains and affect the activities of cortical and cortico-striatal circuits. And we speculate that the long-term plasticity at these synapses would also be modified, which could be verified in the future.
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ANNEX

Short-term synaptic plasticity at cortico-striatal and cortical synapses in a new transgenic mice model of Huntington's disease (Article 4)
Huntingtin regulates the polarization, migration and functional connectivity of projection neurons during cortical development

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ABSTRACT

Mutation in huntingtin (HTT) causes the adult neurodegenerative condition, Huntington’s disease (HD). Little is known about the role of HTT during brain development. Here, we report that HTT is enriched in polarizing and migrating projection neurons during cortical development. The depletion of HTT specifically in post-mitotic projection neurons decreases cortical thickness and leads to the mislocalization of layer-specific neuronal populations in the mouse neocortex. We show that HTT is required for the polarization of projection neurons and for the maintenance of their polarity during their radial migration. HTT regulates the RAB11-dependent N-Cadherin trafficking in cortical neurons, which is known to be important for their polarization. Finally, the cortical defects resulting from the post-mitotic loss of HTT are maintained in young adult brains and affect the activity of cortical and cortico-striatal circuits. Altogether, these data provide new insight into the role of HTT during cortical development with potential implications for HD.
INTRODUCTION

Huntington’s disease (HD) is a dominant inherited neurodegenerative disorder caused by an abnormal expansion of a polyglutamine repeat in the huntingtin (HTT) protein. HD is characterized by the dysfunction and marked degeneration of adult striatal and cortical neurons. The most characteristic symptoms of HD include psychiatric disorders, cognitive decline and disturbance of muscle coordination. For obvious reasons, most studies have focused on the toxicity of mutant HTT (mHTT) in mature neurons, whereas the role of HTT and mHTT during development has received much less attention.

The mammalian cortex, the cerebral structure that controls all higher-order brain functions, is formed of six layers in which the number and the position of neurons are highly regulated during development. During cortical development, neocortical interneurons are produced in the developing ventral telencephalon and migrate tangentially to the neocortex, whereas cortical excitatory neurons (also named projection neurons) are produced in the ventricular zone (VZ) and migrate radially to the cortical plate (CP)\textsuperscript{1-4}. The proliferation of radial glial progenitors (RGPs) in the VZ generates projection neurons, either directly or indirectly through the transient amplification of intermediate progenitors. Although RGPs exhibit strong apicobasal polarity, newly born neurons lose this polarity and adopt a multipolar morphology. During this stage, cells possess multiple processes that extend and retract in a dynamic manner within the subventricular zone (SVZ) and the lower intermediate zone (IZ). This multipolar stage is transient and followed by a transition to a characteristic bipolar morphology where cells extend a leading process oriented toward the pial surface and a trailing process in the direction of the VZ. They then undergo a locomotion-based mechanism of migration along the glial fibers. Studies have suggested that the transient multipolar stage is critical for the establishment of neuronal layers identity and proper cortical lamination\textsuperscript{5}. 
The symptoms of HD only emerge during mid-adulthood, although both wild-type and mutant HTT are expressed in the developing embryo. Following the identification of the gene encoding HTT, several studies showed that it is essential for embryonic development because its inactivation in mice results in embryonic lethality at embryonic day 7.5 (E7.5)\(^6\)\(^-\)\(^8\). More specifically, HTT is important for the formation of the nervous system. Mice expressing less than 50\% of the normal levels of HTT present defects in the formation of the precursor of the epiblast, as well as malformations of the cortex and striatum, and die shortly after birth\(^9\). Furthermore, the specific inactivation of HTT in Wnt1 cell lineages leads to severe hydrocephalus, which further demonstrates the role of HTT in the formation of the nervous system\(^10\). Analysis of chimeric embryos, in which a limited number of cells are depleted of HTT, showed that this protein is essential for the differentiation of neuroblasts in the striatum, cortex and thalamus\(^11\). Notably, we previously focused on embryonic cortical neurogenesis and found that HTT is required for mitotic spindle orientation of dividing cortical progenitors\(^12\). Another study suggested that HTT is involved in the migration of cells from the VZ to the CP\(^13\). However in this study, HTT was knocked-down in all neuroepithelial cells in the neocortex by shRNA. Therefore, the migration defects observed might have been related to HTT-deficiency in RGPs. Overall, the contribution of HTT to steps other than the division of RGPs during cortical development is unknown.

Here, we investigated the role of HTT in post-mitotic newborn neurons, which we found to be enriched in this protein. We used a genetic mouse model and in utero electroporation to inactivate HTT specifically in post-mitotic lineages. We found that HTT regulates the polarization of newly generated cortical neurons, which enables their correct migration, positioning and activity.
RESULTS

**Huntingtin is enriched in polarizing neurons in the developing cerebral cortex**

We first examined the precise expression pattern of HTT in the developing mouse cortex. We analyzed the abundance of HTT protein by immunoblotting extracts from microdissected cortices. HTT protein levels were high between embryonic day 12.5 (E12.5) and E18.5, and were lower at post-natal day 0 (P0), when most projection neurons terminate their migration to the CP and undergo terminal differentiation (Fig. 1a,b). We next performed immunostaining on cortical sections at different developmental stages (Fig. 1c). HTT protein was detected throughout the cortex at E12.5, where it colocalized with the RGC marker, Nestin, and with the neuronal differentiation marker, MAP2 (Fig. 1c and Supplementary Fig. 1a). At E14.5, HTT was more strongly expressed in the IZ than in the VZ and CP layers (Fig. 1c). Strikingly, most HTT was restricted to the upper IZ and the lower CP layers at E16.5. However, HTT was also expressed at low levels in the CP, where it was coexpressed with MAP2 (Fig. 1c and Supplementary Fig. 1a). Thus, the expression pattern of HTT at E16.5 correlates with the polarization of cortical neurons and with the beginning of their radial migration.

**The presence of huntingtin in postmitotic neurons ensures proper cortical development**

To deplete HTT in differentiated neurons, we inactivated the mouse *HTT* gene in NEX-expressing cell lineages by crossing *HTT*<sup>lox/lox</sup> mice harboring floxed *HTT* alleles<sup>14</sup> with transgenic mice expressing the CRE recombinase under the control of the NEX promoter, which is active at around E11.5 in post-mitotic neurons<sup>15</sup>. In NEX<sup>CRE+/−</sup> embryos, CRE is expressed throughout cortical development in projection neurons derived from the cortical VZ, which migrate radially in the neocortex<sup>15</sup>. HTT immunostaining of E14.5 cortices revealed that in the mutant cortex (*HTT*<sup>lox/lox</sup>;NEX<sup>CRE+/−</sup>), HTT was expressed in
neuroprogenitors in the VZ but was absent from neurons in the upper IZ and CP (Fig. 1d). By contrast, in control embryos (HTT<sup>lox/lox</sup>;NEX<sup>+/+</sup>), HTT was expressed in the VZ, the upper IZ and the CP. Furthermore, immunoblotting and immunocytochemical analysis of primary cultures of E14.5 cortical neurons showed that the abundance of HTT protein was substantially lower in mutant embryos expressing CRE recombinase than in control neurons (Fig. 1e,f and Supplementary Fig. 1b).

We next analyzed cortical development by immunolabeling control and mutant brains for Nestin and β-III-tubulin, markers of progenitor cells and neurons, respectively (Fig. 1g). We measured the thickness of the VZ, IZ and CP at E14.5 and E16.5. The CP was significantly thinner in mutant than in control embryos at both stages of development (E14.5: 78.1% ± 1.1% in mutant versus 100% ± 1.4% in control mice; E16.5: 81% ± 1.9% in mutant versus 100% ± 3.1% in control mice; Fig. 1h,i). We also analyzed cell density in control and mutant IZ and CP. Cell density was similar in control and mutant CP, but was higher in mutant IZ than in control IZ (123.1% ± 1.9% in mutant versus 100% ± 2.3% in control mice; Fig. 1j). Consistent with these observations, the cortex was thinner in HTT<sup>lox/lox</sup>;NEX<sup>CRE/+</sup> embryos than in control embryos (E14.5: 92.5% ± 0.6% in mutant versus 100% ± 0.7% in control mice; E16.5: 91.2% ± 0.7% in mutant versus 100% ± 1.5% in control mice; Fig. 1h,i). These differences in cortical thickness were not associated with a high rate of cell death (data not shown) or defects in radial glia integrity (see Nestin immunolabeling in Fig. 1g). Thus, HTT depletion in differentiated neurons impairs corticogenesis.

**Huntingtin is required for the establishment of cortical layers**

We then investigated whether the absence of HTT affects corticogenesis at later stages. We first analyzed the overall cortical cytoarchitecture in mice at post-natal day 0. At this stage, most projection neurons are terminating their migration. Hematoxylin and eosin staining of
P0 sections revealed that the cortex was thinner in mutant than in control mice (88.1% ± 1.9% in mutant versus 100% ± 3.3% in control mice; Fig. 2a,b). To examine more precisely the organization of the cortical layers, we then immunostained P0 cortical sections for Tbr1, Ctip2, Foxp1 and Cux1, which are markers of layers VI, V, IV/V and II/III/IV, respectively (Fig. 2c,d). Tbr1, Foxp1 and Cux1 labeling revealed that the thickness of layers VI and II/III/IV was similar between mutant and control mice (Fig. 2e). By contrast, Ctip2 immunolabeling showed that layer V was thinner in mutant than in control animals (75.1% ± 6% in mutant versus 100% ± 8.4% in control mice). Furthermore, although the number of Tbr1-positive neurons in layer V was similar in both conditions (Fig. 2f), the number of Ctip2- and Cux1-positive cells in deeper layers was higher in mutant than in control mice (Ctip2 in layer VI: 142.7% ± 3.2% in mutant versus 100% ± 10.4% in control mice; Cux1 in layer V/VI: 231.6% ± 17.5% in mutant versus 100% ± 14.8% in control mice; Fig. 2g,h). This localization defect was maintained in P2 animals (Supplementary Fig. 2) and suggests that migration is impaired in the absence of HTT.

We thus used a BrdU birthdating assay to compare the laminar position of BrdU-positive neurons in mutant mice and their control P0 littermates (Fig. 2i-k). We quantified the proportion of neurons in six arbitrarily defined layers of the same size along the cortex. As expected, in P0 animals born to mice administered BrdU at E12.5 (the time at which layer VI neurons are born), most BrdU positive neurons were located in deep layers of the cortex, with no differences between control and mutant brains (Fig. 2i,j). However, in P0 mutant animals born to mice administered BrdU at E15.5 (the time at which neurons of the upper layers are born), a significant proportion of neurons failed to migrate to the upper layers and stayed in the deeper layers of the cortex (44.8% ± 3% in mutant versus 19.4% ± 1.3% in control mice) (Fig. 2i,k). Collectively, these results show that HTT-depleted neurons fail to reach their position during corticogenesis leading to altered cortical layering. Notably, the enrichment of
cells in the SVZ/VZ in HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} embryos (Fig. 1j) further suggests that HTT is involved neuronal migration.

**Conditional removal of HTT impairs the polarization of newborn projection neurons**

To decipher the mechanisms underlying the migration defect observed in HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} animals, we depleted HTT in projection neurons using *in utero* electroporation. We electroporated HTT\textsuperscript{lox/lox} E14.5 embryos with plasmids expressing CRE and GFP or GFP only under the control regulatory sequences of the *NeuroD* promoter (ND:CRE-GFP and ND:GFP respectively). Under this regulatory sequence, NeuroD is expressed in newborn neurons, but not in RGPs and Tbr2-positive intermediate progenitors (*Supplementary Fig.* 3a,b). Immunohistochemistry of coronal sections of electroporated brains confirmed that NeuroD:CRE-GFP electroporated neurons expressed CRE recombinase (*Supplementary Fig.* 3c) and lower levels of HTT than neighboring unelectroporated cells (*Supplementary Fig.* 3d).

Consistent with previous studies\textsuperscript{16}, two days after the electroporation of E14.5 embryos, control neurons were migrating through the upper IZ and CP (also named the radial migration zone, RMZ) (*Fig.* 3a-c and *supplementary Fig.* 3e). By contrast, HTT-depleted neurons were mostly confined to the lower IZ and upper SVZ (also named the multipolar migration zone, MMZ). Neurons migrating in the RMZ are known to adopt a bipolar morphology. A smaller proportion of HTT\textsuperscript{lox/lox} ND:CRE-GFP neurons than control neurons adopted this morphology (63.3% ± 1.9% in HTT\textsuperscript{lox/lox} ND:CRE-GFP neurons versus 77.9% ± 3.2% in HTT\textsuperscript{lox/lox} ND:GFP neurons) (*Fig.* 3d,e). By contrast, a significantly higher proportion of HTT\textsuperscript{lox/lox} ND:CRE-GFP neurons than HTT\textsuperscript{lox/lox} ND:GFP neurons showed highly branched leading processes (25.7% ± 1.9% in HTT-depleted neurons versus 3% ± 0.9% in control neurons).

Extensive branching of leading processes can result from defects in the transition from the multipolar to bipolar stage\textsuperscript{17,18}. We used the position of the Golgi apparatus as a marker of
cell polarity because when cells polarize, they orient the Golgi apparatus toward the CP. We stained cortical sections from HTT$^{lox/lox}$ electroporated embryos for the Golgi matrix protein GM130 (Fig. 3f,g). In embryos electroporated with ND:GFP, the Golgi apparatus of most upper MMZ neurons was oriented toward the CP (84.3% ± 3.02%), whereas the Golgi apparatus was misoriented in cells electroporated with ND:CRE-GFP (53.3% ± 1.63%). These observations suggest that absence of HTT alters neuronal polarization.

To examine further the role of HTT during the acquisition of bipolar morphology, we used time-lapse video-microscopy to analyze the movement of multipolar neurons in the upper MMZ and lower RMZ (Fig. 4a,b and movies 1,2). In contrast to the control neurons, many HTT-depleted neurons extended and retracted their processes without adopting the bipolar morphology required to start radial migration. Indeed, a smaller proportion of HTT-depleted neurons than control neurons underwent a multipolar to bipolar transition (45.9%±2.2% in HTT-depleted neurons versus 75.2% ± 1.2% in control neurons) (Fig. 4c). Furthermore, the analysis of the migratory paths showed that control multipolar neurons in this region moved straight up toward the CP (Fig. 4d and movies 3,4). By contrast, most HTT-depleted neurons moved in random directions, which further suggests that HTT is required for multipolar cells to adopt a bipolar morphology and to migrate toward the CP.

Newborn bipolar neurons attach to the radial glia to initiate their locomotive mode of migration. We investigated whether loss of HTT affected this process. To examine the location of HTT in bipolar neurons, we electroporated E14.5 embryos with ND:GFP and mcherry-tagged full-length HTT (FLHTT) vectors (Fig. 4e), which enabled us to study individual neurons and to analyze the distribution of HTT. HTT was present in the cell body and the leading process of newly generated cortical neurons. To quantify the attachment of leading processes to radial glia fibers, we measured the angle formed between the leading process of ND:CRE-GFP and ND:GFP electroporated neurons, and the adjacent radial glia in
the lower part of the RMZ (Fig. 4f,g). Not only were there fewer ND:CRE-GFP than ND:GFP cells becoming bipolar and attaching to the radial glial fiber in HTT-depleted neurons (Fig. 4c), the angle of attachment was higher in HTT-depleted than in control neurons (25.8° ± 1.3° in mutant versus 8.5° ± 1.1° in control) (Fig. 4f,g). We conclude that HTT regulates the polarization of cortical neurons and their attachment to the radial glia fibers.

**Huntingtin is necessary the maintenance of the bipolar morphology of cortical projection neurons during the locomotive mode of migration**

We then investigated how the depletion of HTT affects later stages when cells migrate through the upper IZ and CP. We electroporated E14.5 HTTlox/lox embryos with ND:CRE-GFP and ND:GFP constructs and examined the position of control and HTT-depleted cells in the cortical wall 4 days later (Fig. 5a and Supplementary Fig. 4a). As before (Fig. 2j,k), we quantified the proportion of neurons in six arbitrarily defined layers of the same size along the cortex. At E18.5, most control neurons had reached the upper layers, whereas many CRE-expressing neurons failed to do so (94% ± 1.4% in control mice versus 74.3% ± 1.7% in mutant mice) (Fig. 5a,b). The ectopic expression of a construct encoding a full-length HTT tagged with mcherry (HTTFL) restored radial migration in electroporated neurons to levels similar to those observed in control conditions (89.6% ± 6.2% of neurons reached the upper cortex; Fig. 5a,b and Supplementary Fig. 4b). Interestingly, HTT-depleted projection neurons were still detected in the deep layers after birth at post-natal day 2 (P2) and most neurons trapped in deep layers expressed the upper-layer marker Cux1 (Supplementary Fig. 4c). This suggests that HTT depletion impairs migration but not laminar specification.

To assess the dynamic of these events, we electroporated E14.5 HTTlox/lox embryos in utero with NeuroD:CRE-GFP or NeuroD:GFP constructs and analyzed organotypic brain cultures using time-lapse videomicroscopy three days later (E17.5) (Fig. 5c-5h). We
specifically focused on neurons that had adopted a bipolar shape and were migrating in the CP (Fig. 5d). GFP-positive neurons were recorded for 8 hours. The velocity of migrating HTT\textsuperscript{lox/lox} ND:CRE-GFP neurons was lower than that of control neurons (10.2 ± 0.8 μm/h ± in HTT-depleted neurons versus 13.9 ± 0.6 μm/h in control neurons) (Fig. 5e). However, HTT-depleted neurons paused more often than control neurons (Fig. 5f). When pauses were subtracted from the analysis, the motility index was similar in both conditions (Fig. 5g). Furthermore, bipolar neurons electroporated with NeuroD:CRE-GFP lost their shape more readily than NeuroD:GFP neurons during the recording (Fig. 5c,h and movies 5,6). Collectively, these results show that HTT ensures progression through the successive phases of radial migration by regulating both the initial multipolar to bipolar transition and the maintenance of polarization during migration.

**HTT monitors neural cadherin trafficking in cortical neurons**

We sought to determine how HTT mediates its effects during the first steps of neuronal migration. Neural cadherin (NCAD) is a transmembrane protein that mediates cell-cell adhesion by calcium-dependent homophilic binding. NCAD regulates neuronal polarization and migration\textsuperscript{16,19,20}. We hypothesized that HTT affects neuronal polarization and maintenance through NCAD. Indeed, these proteins colocalize in the developing neocortex from E12.5 to E16.5 (Fig. 6a). To describe HTT and NCAD distribution in individualized migrating neurons, we electroporated E14.5 embryos with ND:GFP. We found that HTT and NCAD colocalized in leading process of migrating neurons (Fig. 6b).

We next isolated cortical neurons from HTT\textsuperscript{lox/lox},NEX\textsuperscript{+/+} and HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} mice at E14.5. Immunoblotting showed that the loss of HTT specifically in cortical neurons did not perturb the overall expression of NCAD (Fig. 6c). We also analyzed the subcellular distribution of NCAD by immunostaining. In control neurons, NACD was present both in the
cell body and growing neurites, whereas in mutant cells, it accumulated in the perinuclear region, suggesting that HTT is required for the trafficking of NACD to neuronal processes (Fig. 6d,f).

RAB11 regulates the trafficking of NACD during neuronal migration\textsuperscript{20} and HTT binds RAB11 and regulates RAB11-dependent recycling activity in neurons and epithelial cells\textsuperscript{21,22}. HTT may thus affect the RAB11-mediated trafficking of NCAD in cortical neurons. We expressed different variants of GFP-tagged RAB11 in HTT\textsuperscript{lox/lox};NEX\textsuperscript{+/+} and HTT\textsuperscript{lox/lox};NEX\textsuperscript{Cre/+} neurons (Fig. 6c,f,g). In control neurons, the expression of wild-type RAB11 (RAB11\textsuperscript{WT}) or the constitutively active RAB11\textsuperscript{Q70L} (a permanently GTP-bound state) did not influence the distribution of NCAD. The dominant-negative RAB11\textsuperscript{S22N} (a permanently GDP-bound state) accumulated in the cytoplasm and affected the localization of NCAD. We then examined whether the expression of the RAB11 variants rescued the defects in NCAD localization induced by the absence of HTT. Remarkably, NCAD was located in neurites in HTT\textsuperscript{lox/lox};NEX\textsuperscript{Cre/+} neurons expressing RAB11\textsuperscript{Q70L} but not in those expressing RAB11\textsuperscript{WT} or RAB11\textsuperscript{S22N}.

Overall, these findings suggest that HTT regulates NACD trafficking through a RAB11-dependent process, which may affect neuronal polarization and migration.

**Cortical defects induced by the post-mitotic loss of HTT are maintained in young adult brains**

We then used the HTT\textsuperscript{lox/lox};NEX\textsuperscript{Cre/+} genetic mouse model to test whether HTT depletion in newly generated post-mitotic neurons affects the brain in young adults. We performed BrdU birthdating assays to compare the laminar fates of BrdU positive neurons in P21 HTT\textsuperscript{lox/lox};NEX\textsuperscript{+/+} and HTT\textsuperscript{lox/lox};NEX\textsuperscript{Cre/+} animals. There were no differences between control and mutant animals born to mice administered BrdU at E12.5 (data not shown). In
HTT\textsuperscript{lox/lox},NEX\textsuperscript{+/+} and HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} animals born to mice administered BrdU at E15.5, most BrdU positive cells were located in the upper cortical layers (Fig. 7a,b). However, in HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} animals, a significant proportion of neurons failed to migrate and stayed in the deep cortical layers (14.1% ± 2% in mutant versus 5% ± 0.6% in control mice). Consistent with these findings, the deep layers and the lower part of the superficial layers contained substantially more Cux1-expressing neurons in mutant mice than in their control littermates (Fig. 7a).

We next investigated whether the depletion of HTT affects neuronal morphology; therefore, we stained control and mutant cortical coronal sections using the Golgi-Cox method (Fig. 7c). We traced the dendrites of pyramidal neurons in layer II/III and V (Fig. 7d,g). Sholl analysis revealed that in mutant mice, the dendrites of layer II/III neurons were shorter than in control mice (655.2µm ± 45.4µm in mutant versus 1006.5µm ± 136µm in control; Fig. 7e) and dendritic arborization was less complex (two-way ANOVA, p<0.001; Fig. 7f). By contrast, the dendrites of layer V neurons were longer in mutant than in control mice (1808µm ± 158.9µm in mutant versus 1136.4µm ± 67.7µm in control, Fig. 7h) and the dendritic arborization was more complex (two-way ANOVA, p<0.001; Fig. 7i).

Thus, the defects of cortical lamination induced by the specific loss of HTT after neuronal differentiation are maintained in young adults and are accompanied by changes in the arborization of projection neurons.

The expression of HTT in post-mitotic neurons is essential for the activity of cortico-cortical and cortico-striatal circuits

To assess the functional consequences of the structural changes we observed, we analyzed the electrophysiological properties of cortical and striatal neurons and cortical and cortico-striatal excitatory synaptic transmission in P21 HTT\textsuperscript{lox/lox},NEX\textsuperscript{+/+} and HTT\textsuperscript{lox/lox},NEX\textsuperscript{CRE/+} brains.
We first examined non-synaptic properties (17 passive and active membrane properties; Supplementary Table 1) of layer V pyramidal cells and found no significant difference between control and mutant mice. Both the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) of layer V neurons were lower in mutant than in control mice \((p<0.0001 \text{ for both frequency and amplitude})\) (Fig. 8a-c). This suggests that the number of release sites \((n)\) at the presynapse or the probability of release \((Pr)\) is lower in mutant than in control neurons. A common method to assess changes in \(Pr\) is to analyze paired-pulse ratios (PPR), which are independent of \(n\)\(^{23,24}\). We thus recorded EPSCs evoked by paired-pulse stimulations in layers II/III at various frequencies (between 50 and 500 ms) with patch-clamp recordings of layer V pyramidal cells to assess the probability of release (Fig. 8d). PPR analysis revealed that cortical short-term plasticity displayed depression at 250 and 500 ms inter-stimulus intervals in control mice \((81 \pm 5\%, p = 0.003 \text{ and } 79 \pm 4\% \text{ with baseline, } p<0.0001)\) whereas no significant plasticity was observed in mutant mice \((105\% \pm 8\%, p = 0.555 \text{ and } 102 \pm 7\%, p = 0.771 \text{ with baseline; } p = 0.0063 \text{ and } p = 0.0161 \text{ with control mice; Fig. 8e,f})\). Furthermore, no plasticity was observed for paired-stimulation intervals of 25, 50 and 100 ms \((96 \pm 8\%, p = 0.610; 101 \pm 7\%, p = 0.868 \text{ and } 93 \pm 9\%, p = 0.445 \text{ with baseline, respectively})\) in control mice. By contrast, mutant mice showed facilitation at the same stimulus intervals \((25 \text{ ms: } 137 \pm 9\%, p = 0.004; 50 \text{ ms: } 146 \pm 5\%, p<0.0001; 100 \text{ ms: } 134 \pm 9\%, p = 0.005 \text{ with baseline, respectively; } p = 0.0031, p = 0.0002 \text{ and } p = 0.0043 \text{ with control mice, respectively})\). In addition to alterations in sEPSC frequency, the fact that PRR was higher in mutant than in control mice indicates that the probability of release in layer II/III neurons is lower in mutant mice.

We then determined the effect of HTT depletion on cortico-striatal synaptic transmission by analyzing sEPSCs in striatal medium-sized spiny neurons (MSNs). We first examined the non-synaptic properties (Supplementary Table 2) of MSNs and found no
significant difference between control and mutant mice (although the resting membrane potential was slightly more hyperpolarized in mutant than in control mice). The frequency of sEPSCs was higher in mutant than in control MSNs (p = 0.008; Fig. 8g,h), but their amplitude was similar (p = 0.083; Fig. 8g,i). These altered frequencies of sEPSCs suggest presynaptic modifications. PPR analysis revealed that cortico-striatal short-term plasticity was biphasic in control mice, consistent with previous reports\(^{25}\). Indeed, control MSN EPSCs were facilitated for paired-stimulations at an interval of 50 ms (126% ± 6%, p = 0.003 with baseline) and were depressed at 500 ms (78% ± 5%, p = 0.002 with baseline) (Fig. 8j-l). No significant paired-pulse plasticity was detected at an interval of 100 ms (102% ± 6%, p = 0.711 with baseline). By contrast, in mutant mice, no facilitation was observed at a 50 ms inter-stimulus interval (86% ± 10%, p = 0.184 with baseline; p = 0.0068 with control mice) and depression was observed at intervals of 100 and 500 ms (83% ± 6%, p = 0.009 with baseline, and 75% ± 3%, p<0.0001 with baseline, respectively; p = 0.0281 and p = 0.6510 with control mice, respectively). PPR at 50 and 100 ms intervals was significantly lower in mutant than in control mice (50 ms, p = 0.0033; 100 ms, p = 0.0178); therefore, the probability of glutamate release by layer V neurons was higher in mutant than in control mice.

Thus, the deletion of HTT during corticogenesis alters the activity of cortical circuits and has different effects on short-term plasticity depending on the recorded synapses.
DISCUSSION

HTT is known to be expressed in the developing brain\textsuperscript{26,27}. Here, we describe the expression of HTT specifically during cortical development. The expression pattern of HTT in the neocortex is consistent with its role during the various steps of corticogenesis. Indeed, HTT is expressed in nestin-positive progenitors at E12.5. We previously showed that HTT controls the orientation of the spindle pole during the mitosis of cortical progenitors\textsuperscript{12}. We report here that HTT is highly enriched in the IZ at E14.5 and the upper IZ and lower CP layers at E16.5. We show that HTT plays an important role in these regions because it regulates the transition of neocortical projection neurons from a multipolar to bipolar state. Depletion of HTT results in the mispositioning of neurons along the CP. Consistent with our results, Tong and collaborators showed that the knockdown of HTT in all cells of the E12.5 and E14.5 neuroepithelium affects neuronal migration and, depending on the developmental stage analyzed, cell survival\textsuperscript{13}. However, they could not determine whether HTT directly controls neuronal migration because the loss of glial scaffold integrity, poor cell survival and defects in proliferation and the specification of RGCs that they observed could alter radial migration. In our experiments and in previous studies\textsuperscript{12,28}, the depletion of HTT at different stages during cortical development did not promote apoptosis. Overall, our data clearly show that the post-mitotic expression of HTT is required for the proper positioning of neurons along the CP.

HTT is a large stable protein that is thought to act as a scaffold in several cellular pathways including the regulation of intracellular dynamics of various cargoes\textsuperscript{29}. Notably, HTT mediates vesicular recycling by binding to and regulating RAB11\textsuperscript{21,30}. We recently showed that HTT also regulates RAB11-dependent recycling during epithelial morphogenesis\textsuperscript{22}, which ensures the microtubule- and kinesin-1-dependent apical vesicular translocation of PAR3-aPKC. Kawachi and colleagues reported that RAB11 is involved in the transition from multipolar to bipolar migration\textsuperscript{20}. They suggested that RAB11 regulates
the trafficking of NCAD to cortical neuronal processes via recycling endosomes. We found that in HTT-deficient neurons, NCAD accumulates in the cell body, not in neuronal processes. This phenotype is rescued by an active form of RAB11. We thus propose a model in which HTT is required for the transition from multipolar to bipolar morphology, which may occur through the RAB11-dependent trafficking of NCAD.

Kawauchi and colleagues also identified RAB5 as a key player required for neuronal migration\textsuperscript{20}. They found that in the absence of RAB5, cells were stalled near the border between the IZ and the CP, similar to what we observed following the depletion of HTT. However, in contrast with the loss of HTT, RAB5 deletion promoted the accumulation of round cells with no processes and neurons with bipolar morphology and an abnormally thick trailing process. These phenotypes suggest that RAB5 mediates the initial endocytosis of NCAD, which enables cells to detach from radial glia cells at the VZ. RAB5 may also act synergistically or concomitantly with RAB11 to transport NCAD from the cell body to neuronal processes during neuronal polarization\textsuperscript{19}. RAB11 may then mediate the membrane recycling of NCAD, which leads to the attachment of neurons to the radial glia cells\textsuperscript{31}. HTT may be involved in these processes because both the initial attachment of bipolar neurons to the radial glia fiber and the maintenance of this attachment during migration are altered in HTT knockdown conditions.

Both the loss of HTT in RGPs and neurons (this study and \textsuperscript{12}), and the depletion of NCAD result in defects in neuronal differentiation and migration\textsuperscript{16,20,32-34}. NCAD is crucial for the cell-cell adhesion of RGPs, the polarization of post-mitotic neurons and controls neuronal migration by regulating the interaction between migrating neurons and RGC fibers\textsuperscript{19,31,33}. In the only study linking HTT and NCAD, Lo Sardo and colleagues reported that these proteins cooperate to ensure neuroepithelial cell adhesion both in mouse and zebrafish embryos\textsuperscript{35}. They proposed that the lack of HTT promotes the activity of the metalloprotease
ADAM10 and the cleavage of NCAD. Interestingly, the knock-down of ADAM10 leads to the premature differentiation of neural progenitor cells into neurons, which depletes the progenitor population\textsuperscript{36}. Furthermore, ADAM10-depletion and the resultant premature differentiation of neurons is associated with aberrant neuronal migration and a disorganized laminar architecture in the neocortex. Thus, NCAD, ADAM10 and HTT are important in RGCs but also in post-mitotic neurons. We previously found that HTT regulates the orientation of the mitotic spindle in progenitors. It is not known whether HTT affects NCAD-mediated adhesion in these progenitors. In addition, further studies are required to determine the relative contribution of NCAD cleavage by ADAM10 and its RAB(s)-mediated recycling during corticogenesis.

The NEX-driven depletion of HTT altered the dendritic morphology and electrophysiological properties of neurons. A previous study also reported that the loss of HTT in Emx1 progenitor lineages leads to abnormal post-natal cortical and striatal excitatory synapses\textsuperscript{28}. Although some of our electrophysiological data differ with those described in this study, we both report that the absence of HTT affects the dendritic morphology of neurons in layer II/III and V. This strongly suggests that these morphological defects are related to the loss of HTT in post-mitotic neurons and not to an earlier role of HTT in neuroprogenitors. These alterations in morphology are consistent with our electrophysiological data: the deletion of HTT has differential effects on the morphology and subsequent activity of post-natal neurons depending on the nature of the neuronal subpopulation generated in the absence of HTT. Notably, HTT specific deletion did not affect non-synaptic properties of Layer V pyramidal cells and striatal MSNs from 5-8-weeks-old mice. However, the loss of HTT led to a decrease of the synaptic activity in layer V pyramidal cells while an increase of sEPSCs was observed in striatal MSNs, in line with results obtained by \textsuperscript{28}. Analysis of paired-pulse experiments show that the early loss of HTT induced opposite effects in cortical circuits since
layer II/III and layer V pyramidal cells displayed, respectively, a decrease and an increase in the probability of glutamate release.

The early lethality of HTT knock-out mice initially suggested that the loss of the normal HTT does not contribute to the pathogenesis of HD. However, this view has been challenged and the scientific community currently agrees that the loss of the normal functions of wild-type HTT is involved in the pathological process. The most compelling genetic evidence for this idea comes from a study showing that the inactivation of wild-type HTT in the adult brain leads to signs of neurodegeneration in the striatum and cortex\textsuperscript{14}. HTT appears to be crucial during several steps of cortical development, and it is unclear whether these steps are perturbed in HD. We previously found that mHTT leads to changes in the developing cortex, which affect the brain of young adults\textsuperscript{37}. This suggests that development is abnormal in HD. Consistent with the idea, the intracranial adult brain volume of carriers of the HD mutation is smaller than that of controls, even before the onset of the disease, which probably reflects abnormal development\textsuperscript{38}. The evaluation of basic anthropometric measurements shows that children at risk of HD have disproportionately small heads, suggestive of abnormal brain growth\textsuperscript{39}. We now provide evidence that cortical layering and the activity of projection neurons depends on HTT. It is tempting to speculate that this is affected in HD. Consistent with this hypothesis, several mouse models of HD show intra-cortical dysfunction and progressive disconnection between the cortex and striatum, both of which are associated with disease progression\textsuperscript{40,41}. More importantly, projection neurons from cortical layers III, V and VI are known to degenerate in HD patients\textsuperscript{42-45} and their susceptibility may be related to the earlier developmental abnormalities described in our study. Finally, developmental defects may underlie the prodromal neurological signs of HD\textsuperscript{46}, which are becoming increasingly studied because preventive treatment strategies targeting the presymptomatic stages are of major importance given the monogenetic nature of HD.
ONLINE METHODS

Mice and genotyping. To conditionally inactivate the Huntingtin gene in mice (HTT), HTT^{lox/lox} mice\(^{14}\) were crossed with NEX^{CRE/+} mice\(^{15}\) to obtain HTT^{lox/+} NEX^{CRE/+} mice, and genetic invalidation of HTT was performed after crossing the latter with HTT^{lox/lox}. The resulting HTT^{lox/lox}, NEX^{+/+} or HTT^{lox/+}, NEX^{CRE/+} (controls) and HTT^{lox/lox}, NEX^{CRE/+} (HTT conditional knockout) littermates were analyzed. Genotyping of individual mouse was performed by isolating DNA from the tail, and PCR amplification was performed using the following primers to detect HTT lox alleles: HTT forward (CATTGATTCTTACAGGTAGCTG) and HTT reverse (CTAAAGCGCATGCTCCAGACTG). The following primers were used to detect CRE recombinase: CRE forward (GAGTCGGATCATGCTTTTTC) and CRE reverse (CCGCATAACCAGTGAAACAG). All experiments were performed in accordance with local animal welfare committees (Center for Interdisciplinary Research in Biology Ethical Committee, Institut Curie) and EU guidelines (directive 2010/63/EU). Every precaution was taken to minimize stress and the number of animals used in each series of experiments.

DNA constructs. Plasmids DNA were prepared using a Plasmid Endofree Maxi Kit (Qiagen, Hilden, Germany). pNeuroD-IRES-GFP (ND:GFP) and pNeuroD-IRES-CRE-GFP (ND:CRE-GFP) were as in \(^{47}\). To obtain pCAGGS-pARIS-FL-mcherry construct (referred to herein as HTTFL-mcherry), the AgeI-BstBI fragment of the corresponding pARIS-FL-mcherry construct\(^{48}\) was inserted in the pCAGGS plasmid. GFP-RAB11A wild-type (WT), dominant-negative (S22N) and constitutively active (Q70L) (referred to herein as RAB11\(^{WT}\), RAB11\(^{S22N}\) and RAB11\(^{Q70L}\), respectively) were obtained from B. Goud (Institut Curie, France).
**In utero electroporation.** Timed-pregnant mice were deeply anesthetized with isoflurane in oxygen carrier at E14.5, and the uterine horns were exposed through a midline abdominal incision under sterile conditions. Plasmid solutions containing 3 µg/µl of DNA, mixed with 0.05% Fast Green, were injected into the lateral ventricles of the embryos using a heat-pulled capillary and a Femtojet microinjector (VWR International). Electroporation (five pulses of 35V for 50 ms with an interval of 950 ms) was performed using 5 mm platinum tweezers electrodes (CUY650P5, Sonidel, Ireland) connected to a NEPA21 electroporator (NepaGene). The uterine horns were then returned to the abdominal cavity and the abdominal wall was sutured. The pregnant mice were injected with buprenorphine (Buprecare®, Animalecare, UK). The embryos were removed at the indicated day after electroporation and the brains were processed.

**Tissue processing.** Embryonic brains were dissected in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) and fixed with 4% paraformaldehyde (PFA) solution in PBS at 4°C overnight. For postnatal brains, animals were deeply anesthetized on ice (for P0 and P2 animals) or by intraperitoneal injection of a solution containing 7.4 mg/kg xylazine (Rompun, 2%; Bayer) and 146 mg/kg ketamine (Imalgène 500; Mérieux) for P21 animals. Animals were perfused transcardially with PBS followed by 4% PFA. Brains were dissected and post-fixed overnight in 4% PFA. Fixed samples were cryoprotected overnight in 20% sucrose in PBS at 4°C, then embedded in OCT Compound (VWR International, Leuven, Belgium), cryosectioned (10-20 µm), and placed onto slides for analyses (SuperFrost®-plus, Thermoscientific).

**Preparation and transfection of mouse primary cortical neurons.** Primary cortical cultures were prepared from E15.5 mouse embryos. The cerebral cortices were treated with trypsin (0.025% at 37°C for 20 min) and the reaction was stopped with trypsin inhibitor
solution containing DNase. Neurons were dissociated by several passages through a Pasteur pipette. Transfection of dissociated neurons was performed by the Amaxa Nucleofector system (Lonza). The cells were then plated on glass coverslips or on culture dishes coated with polylysine and laminin (Sigma). The neurons were maintained in Neurobasal medium supplemented with glutamax and B27 (all reagents from Invitrogen).

**Preparation of cell Extracts, immunoblotting and immunoprecipitation experiments.**

Dissociated cortical neurons plated for 48 h were lysed in NP40 buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40 (NP40), 0.02% NaN3), containing protease inhibitor cocktail (Sigma), and centrifuged at 11,000 x g for 10 min at 4°C. Samples were heated at 95°C for 5 min before loading on a SDS-polyacrylamide gel. After transfer, nitrocellulose membranes were blocked with 5% nonfat dry milk and incubated with primary antibodies. After thorough washing, membranes were incubated with secondary IgG-HRP antibodies raised against each corresponding primary antibody

**BrdU injection.** E12.5 or E15.5 pregnant female received a single injection of BrdU (Sigma) at 50 mg/kg. Brains were perfused, fixed and sectioned at P0 or P21. Sections were treated with 1 M HCl at 45 °C for 30 min, then incubated at 4 °C overnight with mouse antibody to BrdU (1:200, rat, AbD Serotec).

**Immunohistochemistry and immunocytochemistry.** Samples were washed with PBS-Triton (0.3%; PBST) and blocked at room temperature for 2 hours in PBS containing 10% normal goat serum (NGS, Life Technologies) before incubation with primary antibodies diluted in 5% NGS in PBST. All primary antibodies were incubated overnight at 4°C. The following primary antibodies were used: mouse anti-HTT 4C8 (1:400; rabbit, Euromedex).
anti-βIII tubulin (TUJ1, 1:250, mouse, Merck Millipore), anti-Nestin (1:250, rabbit, Abcam), anti-Cre recombinase (1:500, rabbit, Covance), anti-MAP2 (1:200, rabbit, Merck Millipore), anti-CDP (Cux1, 1:500, rabbit, Santa Cruz), anti-Tbr2 (1:500, rabbit, Merck Millipore), anti-Tbr1 (1:500, rabbit, Merck Millipore), anti-Ctip2 (1:300, rat, Abcam), anti-Brdu (1:200, rat, AbD Serotec), anti-GFP (1:500, chicken, Merck Millipore), anti-GM130 (1:500, mouse, BD Transduction Laboratories), mouse anti-α-tubulin (1:500 for Western blotting and 1:1000 for immunocytochemistry; kindly provided by C. Janke; Institut Curie, France), anti-mcherry (from platform of Institut Curie, France), anti-NCAD (1:500, rabbit, Abcam), anti-RAB11A (1:250; rabbit, Abcam). After several rinses in 5% NGS-PBST, samples were incubated with appropriate AlexaFluor-conjugated secondary antibodies (Molecular Probes, Invitrogen) for 1 hour at room temperature. After several rinses in 5% NGS-PBS, nuclei were counterstained with Dapi (Roche) and samples were mounted in fluorescent mounting medium (Dako).

**Time-lapse videomicroscopy.** HTT$^{lox/lox}$ embryos were electroporated with appropriate plasmids at E14.5. Two or three days later, embryos were dissected and brains processed for organotypic slice culture as described$^{40}$. Briefly, brains were embedded in 3% agarose and sliced (300 µm) with a vibratome (Leica, Germany). Brain slices were cultured up to 4 hours before acquisition in semi-dry conditions (Millicell inserts, Merck Millipore) in a humidified incubator at 37 °C in a 5% CO$_2$ atmosphere in wells containing Basal Medium Eagle supplemented with 25% Hank’s balanced salt solution, 5% normal horse serum, 2% glucose, 1% glutamine and 1% penicillin/streptomycin (Gibco, Life Technologies). Individual slices were imaged with an inverted confocal microscope (SP5, Leica) in a humidified and thermo-regulated chamber. Movies were composed of sequential 500 ms stills, taken at 30 min intervals over periods of at least 10 hours. ImageJ software was used for quantitative
evaluation of migration velocities and time pausing by manually recording the distance traveled by the cell body of GFP positive neurons over the imaging period.

**Golgi-Cox staining and dendritic arborization analysis.** Golgi-Cox stainings were performed on mutant mice and their gender-matched littermate controls. Briefly, mice were deeply anesthetized and perfused with PBS. After dissection, brains were incubated two days in Golgi-Cox solution in the dark then in 30% sucrose in PBS at 4°C for one week. 100 µm thickness brain sections were rinsed in distilled water, mounted on slides (SuperFrost®-plus, Thermoscientific) and treated with ammonium hydroxide for 30 minutes. Sections were then rinsed in distilled water, fixed with X and dehydrated in 70%, 80%, 95% and absolute ethanol, cleared twice in xylene and mounted in Eukitt mounting medium (Sigma). To analyze dendritic length and arborization, cell bodies, apical and basal dendrites were traced using the Neurolucida software (MBF Bioscience) at 40X magnification. Total basal dendrite outgrowth and Sholl analysis were calculated using the Neurolucida software.

**Image analysis.** Confocal images were acquired using confocal microscopy (SP5; Leica) and analyzed using NIH ImageJ (PC version: [http://rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)). VZ, IZ, and CP were distinguished based on cell organization and density and staining (Nestin- and Tuj1-positive cells). Nearly identical areas in the cortex of individual brains were chosen for analysis. For analysis of cell density, same size areas were selected in regions of interest and cells numbers were directly counted in ImageJ. For the binned analysis, the cortex was divided into six equal bins and all of the labeled neurons (GFP+ or BrdU+) in each bin were counted. Subdivisions (lower and upper parts) of the IZ and CP were delineated by an equal partitioning of each zone into subregions. For analysis of neuronal morphology in the lower part of RMZ at E16.5, the percentage of neurons exhibiting the following morphologies was
calculated: multipolar (that is, harbouring at least three processes), unipolar/bipolar, unipolar/bipolar stage with highly branched leading processes or no processes. Scan function of ImageJ was used to reveal fluorescence intensity (pixels/area) of NCAD along neurites. Only neurites longer than one soma diameter were taken into account. All images were collected under identical non-saturating conditions to allow proper fluorescence intensity quantification. For the quantitative estimation of perinuclear NCAD accumulation, primary cortical neurons were immunostained with anti-NCAD and anti-GFP antibodies. The criterion for perinuclear NCAD accumulation was the high fluorescence intensity of NCAD at the perinuclear region, which was defined by ImageJ software. As indicated by the color bar in Supplementary Fig. 5, white or blue represents strong or weak fluorescent intensity. We considered a white color signal in perinuclear region as a perinuclear NCAD accumulation.

**Brain slices preparation and electrophysiology recordings.** Horizontal brain slices containing the somatosensory cortex and the corresponding corticostriatal projection field were prepared according to the methods previously published\(^{50,51}\). Corticostriatal connections (between somatosensory cortex layer V and dorsal striatum) are preserved in a horizontal plane. Briefly, horizontal brain slices with a thickness of 300µm were prepared from mice (P20-P30 males and females) using a vibrating blade microtome (VT1200S, Leica Microsystems, Nussloch, Germany). Brains were sliced in a 5% CO2/95% O2-bubbled, ice-cold cutting solution containing in mM: 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1 MgCl\(_2\), 1 pyruvic acid, and then transferred into the same solution at 34°C for one hour and then moved to room temperature.

Patch-clamp recordings were performed as previously described\(^{50,51}\). Briefly, borosilicate glass pipettes of 4-6MΩ resistance contained for whole-cell recordings (in mM): 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Na, 0.3 EGTA.
(adjusted to pH 7.35 with KOH). The composition of the extracellular solution was (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 10μM pyruvic acid bubbled with 95% O₂ and 5% CO₂. Signals were amplified using an EPC10-2 amplifier (HEKA Elektronik, Lambrecht, Germany). All recordings were performed at 34°C using a temperature control system (Bath-controller V, Luigs&Neumann, Ratingen, Germany) and slices were continuously superfused at 2-3 ml/min with the extracellular solution. Slices were visualized on an Olympus BX51WI microscope (Olympus, Rungis, France) using a 4x/0.13 objective for the placement of the stimulating electrode and a 40x/0.80 water-immersion objective for localizing cells for whole-cell recordings. The series resistance was not compensated. Current-clamp recordings were filtered at 2.5 kHz and sampled at 5 kHz and voltage-clamp recordings were filtered at 5 kHz and sampled at 10 kHz using the Patchmaster v2x32 program (HEKA Elektronik).

**Paired-pulses protocols.** Electrical stimulations were performed with a bipolar electrode (Phymep, Paris, France) placed either in the layer V or the layer II/III of the somatosensory cortex for the corticostriatal or cortical paired-pulse experiments, respectively. Electrical stimulations were monophasic at constant current (ISO-Flex stimulator, AMPI, Jerusalem, Israel). Currents were adjusted to evoke 50-200pA EPSCs. Repetitive control stimuli were applied at 0.1Hz. 20 successive EPSCs were individually measured and then averaged. Variation of series resistance above 20% led to the rejection of the experiment.

**Data analysis.** GraphPad Prism 6.0 software (San Diego, CA) was used for statistical analysis. Complete statistical analyses with number of measures are detailed in Extended Experimental Procedures.
For electrophysiological data, off-line analysis was performed using Fitmaster (Heka Elektronik) and Igor-Pro 6.0.3 (Wavemetrics, Lake Oswego, OR, USA). Paired-pulse ratio was calculated by the mean (EPSC2 amplitude/EPSC1 amplitude)\(^{25}\). sEPSCs were evaluated by amplitude or inter-event interval using the Kolmogorov–Smirnov (K–S) test on their cumulative distributions. Data were analyzed with Mini Analysis (version 6.0.3; Synaptosoft, Decatur, GA). In all cases “n” refers to the number of repetitions of an experiment (each experiment being performed on different brain slices) from single slice. Experimenters were blind to the genotype of control and mutant mice during electrophysiological recordings and analysis. All results were expressed as mean ± SEM and statistical significance was assessed using two-sided Student’s t test or the one sample t test when appropriate at the significance level (p) indicated.
SUPPLEMENTARY INFORMATION

Supplemental Information includes 5 figures, 2 tables, 6 movies, Supplementary Figures legends, movies legends and complete statistical analyses with number of measures.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.B. and S.H. designed research, analyzed data and wrote the manuscript; L.V. designed the electrophysiological experiments, analyzed the related data and wrote the manuscript. E.A., H.X., H.Y. and C.B. performed research.
FIGURES LEGENDES

**Figure 1** HTT depletion in post-mitotic neurons impairs corticogenesis. (a) HTT and α-tubulin (α-tub) immunoblotting analyses of lysates from E12.5, E14.5, E16.5, E18.5 and P0 cortices. (b) Quantitative assessment of HTT levels detected by immunoblotting in (a) (n = 3 independent experiments). (c) Immunostaining for HTT and Nestin of E12.5, 14.5 and E16.5 wild-type cortical coronal sections. Nuclei are counterstained with Dapi. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. (d) HTT immunostaining of E14.5 HTT^{lox/lox};NEX^{+/+} (control) and HTT^{lox/lox}; NEX^{CRE/+} (mutant) cortical coronal sections. Nuclei are counterstained with Dapi. (e) HTT, CRE recombinase and α-tubulin immunoblotting analyses of lysates from E14.5 control and mutant cortices (extracts from 3 cortices for each genotype are shown). (f) Quantitative assessment of HTT levels detected by immunoblotting in (e) (n = 3 for each genotype). (g) βIII-tubulin and Nestin immunostainings of E14.5 and E16.5 control and mutant cortical coronal sections. Nuclei are counterstained with Dapi. (h,i) Quantitative analysis of VZ, IZ, CP and overall cortical thicknesses of E14.5 (h) and E16.5 (i) control and mutant embryos (E14.5, control: n = 4, mutant: n = 3; E16.5, control n = 3, mutant n = 4). (j) Quantitative analysis of cell density in the CP and IZ of E16.5 control (n = 3) and mutant (n = 4) brains. All scale bars, 50 µm. Student’s t test; error bars, SEM; ns, not significant, **p < 0.01, ***p < 0.001.

**Figure 2** HTT depletion in post-mitotic neurons leads to impaired cortical lamination. (a) Hematoxylin and Eosin stained sections from P0 HTT^{lox/lox};NEX^{+/+} (control) and HTT^{lox/lox}; NEX^{CRE/+} (mutant) coronal sections. (b) Quantitative analysis of the cortical thickness of control (n = 13 brains) and mutant (n = 10 brains) P0 animals. (c,d) Tbr1 and Ctip2 or Cux1 and Foxp1 immunostainings of control and mutant P0 cortical sections. Nuclei are
counterstained with Dapi. (e) Quantitative analysis of the thicknesses of layers VI, V and II-IV of control (n = 13 brains) and mutant (n = 10 brains) P0 animals. (f-h) Tbr1- (Tbr1+) (f), Ctip2- (Ctip2+) (g) and Cux1- (Cux1+) (h) positive neurons in layers V and VI of P0 control and cortices (n = at least 5 brains per condition). Data are expressed as percentage of control.

(i) Pregnant mice were injected with BrdU at E12.5 (BrdU E12.5) or E15.5 (BrdU E15.5). Coronal sections of brain collected at P0 were analyzed by immunostaining for BrdU. Nuclei are counterstained with Dapi. Scale bars, 200 µm. (j,k) Cortical sections were divided into six equal bins. Quantitative assessment of the percentage of BrdU-positive neurons in each bin (n = at least 3 brains per condition). All scale bars, 100 µm. Student's t test, error bars, SEM; ns, not significant; **p < 0.01, ***p < 0.001.

Figure 3 Acute HTT depletion in post-mitotic neurons alters neuronal polarization. HTT is depleted in postmitotic projection neurons by in utero electroporation of ND:GFP (control) or ND:CRE-GFP plasmids in E14.5 HTT^{lox/lox} embryos. Cortices were analyzed at E16.5. (a) Coronal sections of E16.5 embryos are immunostained for GFP. Nuclei are counterstained with Dapi. Lower panel: computer-based reconstruction of the shapes of representative GFP-positive neurons. Scale bars, 50 µm. (b) Cartoon illustrating the region shown in (a). (c) Quantitative analysis showing the distribution of GFP positive (GFP+) neurons across the different cortical regions (n = at least 4 brains per condition). uCP, upper cortical plate; lCP, lower cortical plate; uIZ, upper intermediate zone; lIZ, lower intermediate zone; SVZ, subventricular zone. (d) Inset illustrating the various morphologies of neurons. (e) Percentage of electroporated neurons in lower radial migration zone (RMZ) with bipolar morphology (unbranched/branched), multipolar morphology or without process after in utero electroporation of ND:GFP (n = 7) or ND:CRE-GFP (n = 4) plasmids. (f) Immunostainings for GFP and GM130 (arrowheads) after in utero electroporation of ND:GFP (n = 6) or ND:CRE-
GFP (n = 4). MMZ, multipolar migration zone. RMZ, radial migration zone. (g) Percentage of electroporated neurons with Golgi apparatus facing the CP in upper MMZ. Cartoon illustrating a multipolar neuron with the Golgi apparatus facing the CP. Scale bars, 25 µm. Student’s t test; error bars, SEM; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4 Acute HTT depletion in post-mitotic neurons delays the exit from the multipolar stage. (a) Organotypic slices are obtained two days after in utero electroporation of ND:GFP (control) or ND:CRE-GFP plasmids in E14.5 HTTlox/lox embryos. Sequences from a 10 hours time-lapse experiment are shown. Scale bars, 25 µm. (b) Cartoon illustrating the region shown in (a). (c) Percentage of neurons exiting the MMZ in a bipolar shape during the 10 hours recording (ND:GFP: n = 4 brains; ND:CRE-GFP: n = 5 brains). (d) Drawing paths illustrating individual neurons exiting the MMZ (colored lines, see also supplemental movie 3). (e) To allow the visualisation of individual cells, E16.5 control cortices were electroporated with plasmids expressing mcherry-tagged full-length HTT (FLHTT-mcherry). Immunostainings for GFP, mcherry and Nestin are shown. Scale bars, 10µm. (f) Electroporation of ND:GFP or ND:CRE-GFP plasmids were performed in E14.5 HTTlox/lox embryos and brains analyzed two days later. Immunostainings for GFP and Nestin are shown. Scale bars, 25 µm. MMZ, multipolar migration zone; RMZ, radial migration zone. (g) Distribution of the angles between the glial fiber and HTTlox/lox neurons expressing ND:GFP or ND:CRE-GFP plasmids. Measures were performed in the RMZ (n = 9 brains per conditions). Cartoon showing the angle formed by the leading process of bipolar neurons (green) with respect to adjacent radial glia (red). Student’s t test; error bars, SEM; **p < 0.01, ***p < 0.001.
**Figure 5** HTT regulates neuronal polarization and radial migration. (a) Cortical coronal sections of E18.5 embryos electroporated at E14.5 with ND:GFP or ND:CRE-GFP plasmids and HTT-mcherry. Sections are immunostained for GFP and mcherry. Nuclei are counterstained with Dapi. Scale bars, 100 µm. (b) Quantitative analysis showing the distribution of GFP+ neurons across the cortex divided into 6 equal bins (n= 4 brains per condition). One-way ANOVA; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001. (c) Organotypic slices are obtained three days after *in utero* electroporation of ND:GFP or ND:CRE-GFP plasmids in E14.5 HTTlox/lox embryos. Sequences from a 8 hours time-lapse experiment are shown. Drawings in the right panels illustrate the typical neuronal morphologies observed during radial migration. Scale bars, 25 µm. (d) Cartoon illustrating the regions analyzed in (e-h). (e-h) Quantitative analysis of time-lapse experiments as in (c). Migration velocity (e), average pause number (f), motility index (g) and percentage of neurons undergoing bipolar-multipolar-bipolar transition (h) are shown (n = 4 independent time-lapse experiments per condition). Student’s t test; error bars, SEM; **p < 0.01, ***p < 0.001.

**Figure 6** HTT mediates NCAD trafficking in a RAB11-dependent manner in embryonic cortical neurons. (a) Immunostaining for NCAD and HTT of E12.5, 14.5 and E16.5 wild-type cortical coronal sections. Nuclei are counterstained with Dapi. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bar, 50 µm. (b) Immunostaining for GFP, HTT and NCAD after *in utero* electroporation of ND:GFP in E14.5 wild-type embryos. Scale bar, 20 µm. (c) NCAD and α-tubulin (α-tub) immunoblotting analyses of lysates from primary cultures of HTTlox/lox, NEX+/- (control) and HTTlox/lox, NEXcre+/+ (mutant) cortical neurons (E14.5). (d) Tubulin and NCAD immunostaining of primary cultures of control and mutant cortical neurons (E14.5). Scale bar, 25 µm. (e) Neurons were electroporated with the
indicated plasmids and immunostained for GFP and NCAD. (f) Representative line scan analysis showing the distribution of NCAD in the indicated conditions. (g) Percentage of control and mutant cortical neurons transfected with the indicated plasmids showing a perinuclear accumulation of NCAD. One-way ANOVA; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 7** NEX-CRE driven HTT depletion impairs post-natal cortex layering and neuronal morphology. (a) Pregnant HTT<sup>lox/lox</sup>;NEX<sup>+/+</sup> (control) and HTT<sup>lox/lox</sup>; NEX<sup>CRE/+</sup> (mutant) mice were injected with BrdU at E15.5 and coronal sections of brain collected at P21 were analyzed by immunostaining for Cux1 and BrdU. Scale bars, 200 μm. (b) Cortical sections were divided into six equal bins. Quantitative assessment of the percentage of BrdU-positive neurons in each bin (n = at least 5 brains per condition). (c) Golgi-Cox staining of brain sections from P21 control and mutant mice. Scale bar, 200 μm. (d-i) Representative traces (d,g), quantification of dendritic outgrowth (e,g), and Sholl analysis (f,i) of cortical pyramidal neurons in layer II/III and V from P21 mutant and control mice (n = 4 brains per condition). Student’s t test (e,h) and Two-way ANOVA (f,i); error bars, SEM; ns, not significant; **p < 0.01, ***p < 0.001.

**Figure 8** HTT is required for transmission and short-term plasticity at cortical and striatal synapses. Comparison of transmission and short-term plasticity in HTT<sup>lox/lox</sup>;NEX<sup>+/+</sup> (control) and HTT<sup>lox/lox</sup>; NEX<sup>CRE/+</sup> (mutant) mice at cortical (a-f) and cortico-striatal synapses (g-l). (a) Representative sEPSCs in control and mutant mice at cortico-cortical synapses. (b,c) Cumulative distributions of the inter-event intervals (b) and sEPSCs peak amplitude (e) show a significant decrease (p<0.0001, K-S test) of the sEPSC frequency in mutant mice with a significant decrease of the peak amplitude (p<0.0001, K-S test) (control, n = 6; mutant, n =
10. (d) Scheme of the recording (layer V) and stimulating (layer II/III) sites (horizontal cortico-striatal slices). (e,f) Representative synaptic currents (e) and average graph (f) of the paired-pulse experiments in control and mutant mice (control, n = 10; mutant, n = 8). (g) Representative sEPSCs in control and mutant mice in control and mutant mice at cortico-striatal synapses. (h,i) Cumulative distributions of the inter-event intervals (h) and sEPSCs peak amplitude (i) show a significant increase (p = 0.008, K-S test) of the sEPSC frequency in mutant mice without significant change of the synaptic weight (p = 0.083, K-S test) (control, n = 7; mutant, n = 6). (j) Scheme of the recording (dorsal lateral striatum) and stimulating (cortical layer V) sites in cortico-striatal slices. (k,l) Representative synaptic currents (k) and average graph (l) of the paired-pulse experiments in control and mutant mice (control, n = 10; mutant, n = 12). Student’s t test; error bars, SEM; *p < 0.05, **p < 0.01, ***p < 0.001.
REFERENCES


FIGURE 4

a) ND:CRE-GFP E14.5 → E16.5

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b) Transition MMZ-RMZ

IZ/SVZ/VZ
CP

Transition multipolar-bipolar

Proportion of GFP returns (%)

ND/GFP
ND:CRE-GFP

100 80 60 40 20 0

MMZ RMZ MMZ RMZ

GFP Nestin

RMZ

RMZ

Angle of leading process, with respect to radial glide (°)

ND:GFP
ND:CRE-GFP

***
FIGURE 7

**a**

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**b**

BrdU E15.5

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Proportion of BrdU labeled cells (%)

**c**

HTT^{lox/lox}, NEX^{+/+} vs HTT^{lox/lox}, NEX^{CRE/+}

**d**

Layer II/III

HTT^{lox/lox}, NEX^{+/+} vs HTT^{lox/lox}, NEX^{CRE/+}

**e**

Length (μm)

**f**

Interactions

**g**

Layer V

HTT^{lox/lox}, NEX^{+/+} vs HTT^{lox/lox}, NEX^{CRE/+}

**h**

Length (μm)

**i**

Interactions