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Role of excitatory serotonergic signaling in the pathway-specific neuromodulation of striatal synaptic plasticity

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To my family

Aknowledgments

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Abstract

The dorsolateral striatum (DLS) of the basal ganglia plays a critical role in action selection and motor control. The DLS receives cortical and thalamic afferents, which are extensively modulated by monoaminergic inputs, such as dopamine and serotonin (5-HT). Dopamine and 5-HT act as circuit neuromodulators by activating both stimulatory (Gs) and inhibitory (Gi) protein-coupled receptors that regulate synaptic mechanisms of plasticity.

On a system level, 5-HT signal has been classically associated with learning of negative events, acting as an opponent of dopamine regulation of rewarding processes. Recent evidence has challenged this view, suggesting that 5HT signaling can synergize with dopamine signaling to shape reward-guided behavior. However, the molecular and synaptic correlates of this behavioral role of 5-HT at striatal circuits remain to be established.

To address this hypothesis, we investigated the role of serotonergic signaling in regulating the strength of glutamatergic synaptic connections to the Medium Spiny Neurons of the direct pathway (dMSNs), which mediate movement, reward and reinforcement. Specifically, we focused on the regulation of distinct forms of long-term synaptic plasticity that depend on both the relative timing of a neuron output and an input spike (Spike timing-dependent plasticity, STDP), and on the pattern of neuronal stimulation (high-frequency stimulation, HFS). Upon a STDP protocol, the chemo-genetic inhibition of 5-HT release resulted in a long lasting depression (STDP-LTD) of glutamatergic afferents to the dMSNs of the DLS. The synaptic effects of chemo-genetic inhibition of 5-HT release were recapitulated by the pharmacological inhibition of the Gs-coupled 5-HT4 receptor subtype (5-HT4R).

This form of LTD was independent from presynaptic CB1 receptor (CB1R) activation, it showed a postsynaptic locus of expression, and it was associated with an increased dendritic Ca^{2+} signal. We obtained similar results upon HFS; antagonism of 5-HT4R resulted in a CB1R independent form of HFS-LTD, which was associated with enhanced dendritic Ca^{2+} levels.

Collectively, these data provide molecular and synaptic insights on the neuromodulatory role of 5-HT at striatal circuits. Dysfunctional serotonergic modulation of striatal circuits has been associated with repetitive behaviors in obsessive-compulsive disorders (OCD). Thus, elucidating how 5-HT4R manipulation affects aspects of reward-guided behavior, and how this is causally relevant for defined cognitive processes implicated in action control, could facilitate the development of new pharmacological approaches to treat OCD symptoms.

Introduction

The basal ganglia consist of highly interconnected subcortical nuclei involved in the control of motor behaviors, mood and cognition. They associate the cortex to the neural systems exerting motor function, and their dysfunction leads to a wide variety of neurological and psychiatric conditions.

The striatum is their primary input nucleus, and it receives strong glutamatergic input from cortex and thalamus. Serotonergic and dopaminergic afferents modulate intrinsic glutamate neurotransmission along this circuit, to optimize circuit performance for specific aspects of executive control over behavior. While key aspects of midbrain dopaminergic modulation of glutamatergic synapses have been revealed, serotonergic effects on these circuits are not well understood.

In this thesis, I will report the results of the work I did during my PhD, aimed at investigating the function of serotonin in the neuromodulation of glutamatergic synaptic plasticity specifically in the lateral part of the dorsal striatum, which is involved in motor planning and habit formation. The purpose of this study was to provide a better understanding of the mechanisms by which serotonin influences striatal circuits, to shed light on its functional integration with the dopaminergic signaling.

The Basal Ganglia: organization and function

The basal ganglia are a group of subcortical nuclei residing in the basal forebrain, connecting the cerebral cortex with different neural systems that affect a variety of behaviors, including motor planning, procedural learning related to goal-directed and habitual behavior, action selection and motivation state. Indeed, they provide outputs to various behavior effector systems, such as the thalamic nuclei projecting to frontal cortical areas, midbrain regions including the superior colliculus, the pedunculopontine nucleus and hypothalamic systems involved in autonomic functions.

The first evidence of the basal ganglia involvement in motor function came from post-mortem studies in patients affected by brain pathologies characterized by motor impairments, such as Parkinson's Disease (PD), Huntington's Disease (HD) and hemiballismus. In all of them, pathological changes in basal ganglia structures were reported. In addition to these, several psychiatric conditions, including obsessive-complulsive disorder (OCD), schizophrenia and drug abuse, appear in part to be reflecting changes in basal ganglia function.

Although a formal definition of the basal ganglia is lacking, the term today generally indicates the striatum and its projection targets. The basal ganglia can be thus anatomically subdivided in 4 interconnected nuclei: striatum (composed in primates by the caudate, putamen, and nucleus accumbens), subthalamic nucleus, globus pallidus (further sub-divided into an internal and an external tract) and substantia nigra (divided in pars compacta, pars reticulata, and ventral tegmental area) (**Fig. 1**).

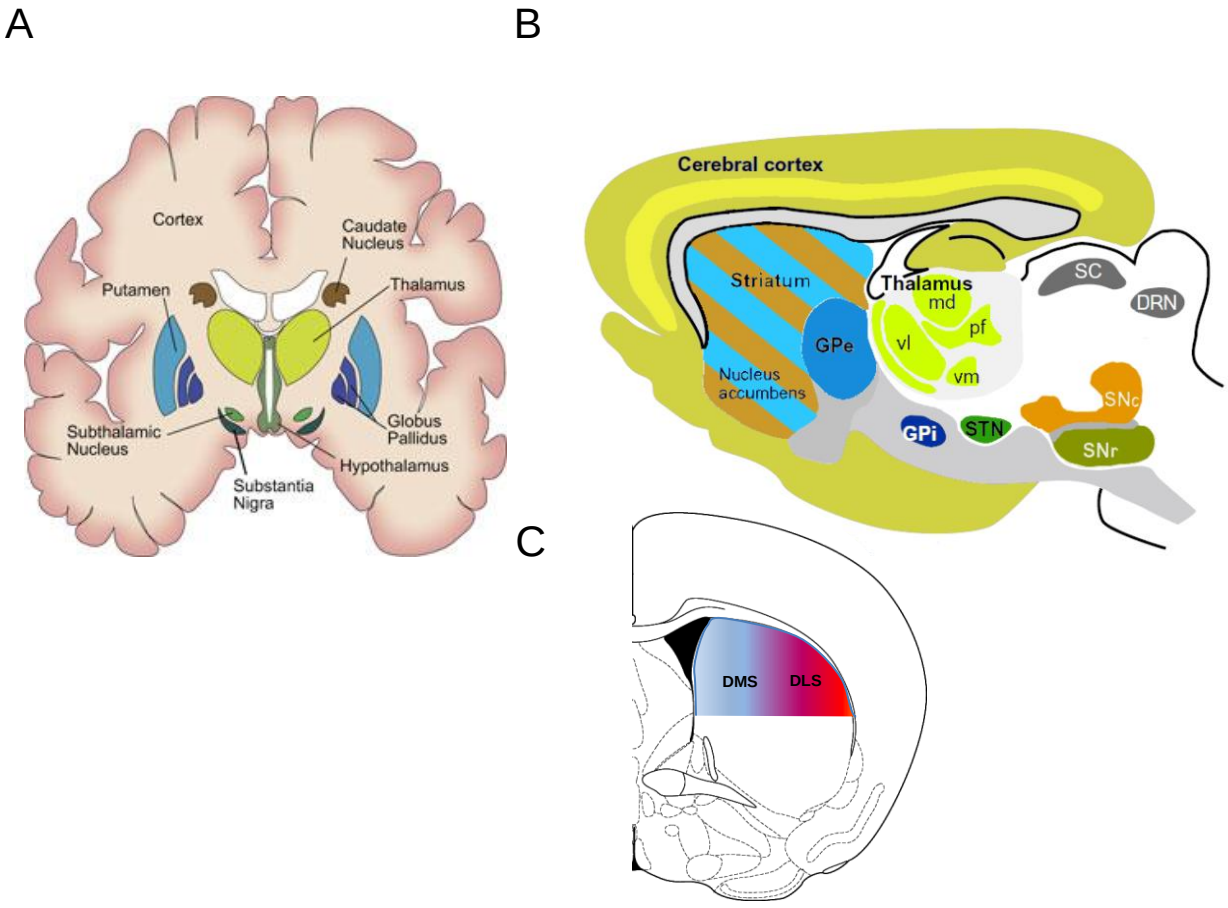


Figure 1. The basal ganglia.

*The human (**A**) and rodent (**B**) basal ganglia illustrated respectively on a coronal and a sagittal diagram. **C.** Coronal diagram of mouse brain shows the anatomical segregation in the medial and the lateral dorsal striatum, respectively corresponding to human caudate and putamen nuclei.*

The Striatum

The striatum is the main input nucleus of the basal ganglia, and its principal role is integrating cortical and thalamic inputs with signals originating from midbrain dopaminergic neurons. It has the fundamental function of processing cortical information, and sending the integrated message back to the cortex, to allow shaping of subsequent motor and cognitive processes. Its main functions are represented by the regulation of motor and procedural learning, and reward-guided behaviors. As a consequence, striatal pathological conditions (such as PD, HD, OCD, schizophrenia, and aberrant dopaminergic signaling following drug abuse and L-DOPA administration), have a profound impact on behavioral functions.

Within the striatum there are many different neuronal subtypes. The main striatal cell population is represented by the γ-aminobutyrric acid (GABA)-releasing medium spiny projection neurons (MSNs). They constitute ~95% of striatal neurons, and are so called because they constitute the projection system of the striatum (Kemp and Powell, 1971). The remaining ~5% is composed by several classes of interneurons, such as the large cholinergic interneurons, the GABAergic fast-spiking interneurons, the somatostatin-expressing neurons, and interneurons expressing the calcium-binding protein calretinin (Reiner *et al.,* 1998).

In the primate brain, the striatum is anatomically separated in caudate and putamen nuclei, and nucleus accumbens (**Fig. 1 A**). In rodents, the subdivision is only functional (**Fig. 1 B**), reflecting the topographical distribution of inputs from different cortical areas. In particular, the rodent striatum can be divided in a dorsal and a ventral part. The ventral part comprises the olfactory tubercle and the nucleus accumbens, which plays a pivotal role in translating motivational states into goal-directed behavior. The dorsal striatum roughly corresponds to the caudate and putamen nuclei, and its primary activity is the control of motor function and motivated behavior (**Fig. 1 C**). The dorsomedial striatum (DMS) roughly corresponds to the primate caudate nucleus*,* while the dorsolateral striatum (DLS) is the correspondent of the putamen (Balleine *et al.,* 2007) (**Fig. 1 C**). The DMS receives projections preferentially from associative cortices, such as the prelimbic region of the prefrontal cortex, as well as from premotor cortical areas involved in processes of action monitoring and programming implicated in executive functions (Passingham *et al.,* 1988; McGeorge and Faull, 1989; Corbit and Balleine, 2003). The posterior part of the DMS also receives inputs from the basolateral amygdala (Kelley *et al.,* 1982). Data from behavioral and electrophysiological experiments in rodent indicate that this part of the striatum is involved in goal-directed and associative learning (Hilario and Costa, 2008). The DLS is preferentially innervated by the somatosensory and motor cortices (Goldman-Rakic and Selemon, 1986) and it is involved in sensorimotor processing and the acquisition of habitual behavior (Yin *et al*., 2004; Hilario and Costa, 2008; Nazzaro *et al*., 2012).

In addition to cortical innervations, the striatum receives projections from:

- the thalamic glutamatergic neurons of the central medial complex and the parafascicular nucleus (Smith *et al.,* 2004);
- the midbrain dopaminergic neurons of the substantia nigra pars compacta (targeting mainly the dorsal striatum) and of the ventral tegmental area (reaching preferentially the ventral striatum)(Encyclopedia of Movement Disorders, Volume 1, Kompoliti and Verhagen Metman, 2010);
- the serotonergic neurons residing in the dorsal raphe nucleus (Azmitia and Segal 1978; Bobillier *et al.*, 1976);
- the GABA releasing neurons of globus pallidus externum (Gerfen *et al.,* 2010, Handbook of basal ganglia structure and function)(**Fig. 2**).

Striatal outputs target other nuclei of the basal ganglia, to ultimately reach the thalamus.

The subthalamic nucleus

The subthalamic nucleus (STN) is a small nucleus located caudally to the striatum and ventrally to the thalamus. It is an input stage of the basal ganglia, as well as the striatum, and similarly to that nucleus, it receives afferents from the cortex and the thalamus (Smith *et al.,* 1998), and it can be functionally divided in a motor (dorsolateral), an associative (ventromedial) and a limbic (medial) sector (Parent and Hazrati, 1995). In addition, the STN receives also dopaminergic afferents from substantia nigra pars compacta and GABAergic projections from the globus pallidus externum (GPe).

Most STN neurons are glutamatergic, and they provide excitatory input to the GPe and to the two output structures of the basal ganglia, the globus pallidus internum (GPi) and the substantia nigra pars reticulata (SNr)(Charpier *et al.,* 2010, Handbook of basal ganglia structure and function).

Lesion of the STN results in hemiballismus, a movement disorder which is characterized by violent movements of the arms and legs. Also in PD and epilepsy a dysfunction of STN has been reported.

The globus pallidus

The globus pallidus is positioned caudally to the striatum, and it is functionally separated in the internal (GPi) and external (GPe) tract. The GPi anatomically differs between primates and rodents in that in the primate brain it lays medially to the GPe forming with it a structurally unique nucleus, while in rodents it is separated, sited caudally from the GPe, and it has historically been termed the entopeduncular nucleus (Kita *et al.,* 2010, Handbook of basal ganglia structure and function).

The GPe receives major inputs from the striatum and the subthalamic nucleus (STN). Its GABAergic neurons send outputs to most of the basal ganglia nuclei, including the striatum, GPi, STN, and the SNr (Kita *et al.,* 2010, Handbook of basal ganglia structure and function).

The GPi receives inputs from the striatum, STN, GPe, and brainstem nuclei, including the peduculopontine nucleus, and send its outputs out of the basal ganglia. The GPi, with the SNr, represent the output nuclei of the basal ganglia, and their GABAergic neurons provide inhibitory input to thalamic relay neurons and brainstem targets (Parent and Hazrati, 1995).The GPi is involved in limb and axial movements (Gerfen et al., 2010, Handbook of basal ganglia structure and function).

The substantia nigra and the Ventral Tegmental Area

The substantia nigra is divided into a dorsal, mainly dopaminergic pars compacta (SNc), and a ventral, mainly GABAergic pars reticulata (SNr). The ventral tegmental area (VTA) lays medially to the substantia nigra, and its neurons are 50-60% dopaminergic cells, while the others are mainly GABAergic and few glutamatergic.

The VTA is implicated in the codification of natural- and drug reward-based signals, and the dopaminergic VTA neurons are the source of the so-called mesocorticolimbic dopamine system, which connects the nucleus accumbens, the amygdala, the hippocampus and the medial prefrontal cortex; these nuclei project back to the VTA, thus creating a feedback loop.

The SNr is involved in movements of the eyes, head and neck and contains mostly GABAergic neurons, and some dopaminergic cells. It receives inputs from almost all nuclei of

the basal ganglia, and it projects to the SNc, the thalamus, the superior culliculus and the pedunculopontine nucleus with GABAergic afferents (Gerfen *et al.,* 2010, Handbook of basal ganglia structure and function).

The SNc contains mainly dopaminergic neurons and it is the principal source of monoaminergic signaling for the basal ganglia, together with the serotonergic system arising from dorsal raphe nucleus. Dopaminergic signaling codes for positively or negatively reinforcing stimuli (Schultz *et al.,* 1997) and, in the striatum, it is thought to trigger phenomena of synaptic plasticity leading to reinforcement learning of action-outcome associations (Shultz *et al.,* 1993; Hollerman and Shultz, 1998). While timing and magnitude of dopamine release are important for normal voluntary movement, the firing patterns of dopamine neurons are related to the perception of rewards. The idea that dopamine could be the brain signal for rewarding events came from evidence of *in vivo* electrophysiological recordings. Dopaminergic neurons increase their firing activity upon delivery of a reward, while they decrease their firing or cease it completely when a punishment occurs. Subsequent experiments highlighted the need of the reward to be unexpected to induce a strong response of dopaminergic neurons. The current vision considers dopamine as a code to signal a reward prediction error, acting to distinguish between subjective expected and actual reward (Glimcher, 2011; Steinberg *et al.,* 2013). The role of dopamine in the reward system will be discussed extensively further on in this thesis.

The SNc sends and receives projections from the striatum, GPe, STN and SNr. Furthermore, it receives inputs from the amygdala, cholinergic innervation from the pedunculopontine nucleus and various basal forebrain nuclei, and an inhibitory input from the lateral habenula.

Nigral dopaminergic neurons receive inputs through their 3-5 dendrites, extending also in the SNr, and they send a single axon which runs medially and rostral to SNc and coalesces into a tract called the medial forebrain bundle (Tepper *et al.,* 2010, Handbook of basal ganglia structure and function). As the axons course rostrally, they arborize sparsely in the STN (Cragg *et al.,* 2004) and in the GP (Lindvall and Bjorklund, 1979) before reaching their principal target, the striatum. In the striatum the axons branch profusely, forming large and

dense arborizations of varicose processes that occupy an average volume of ~0.5 mm^3 (Matsuda *et al.,* 2009). The dopaminergic innervation in the striatum is so dense, that even one single dopaminergic neuron can influence a very large number of striatal MSNs: it is thus not surprising that dopamine plays such a critical role in the modulation of motor behavior and learning.

For the purpose of this thesis, I will mainly focus on the striatum, analyzing aspects of the serotonergic neuromodulation responsible for the regulation of the glutamatergic synaptic transmission in this nucleus.

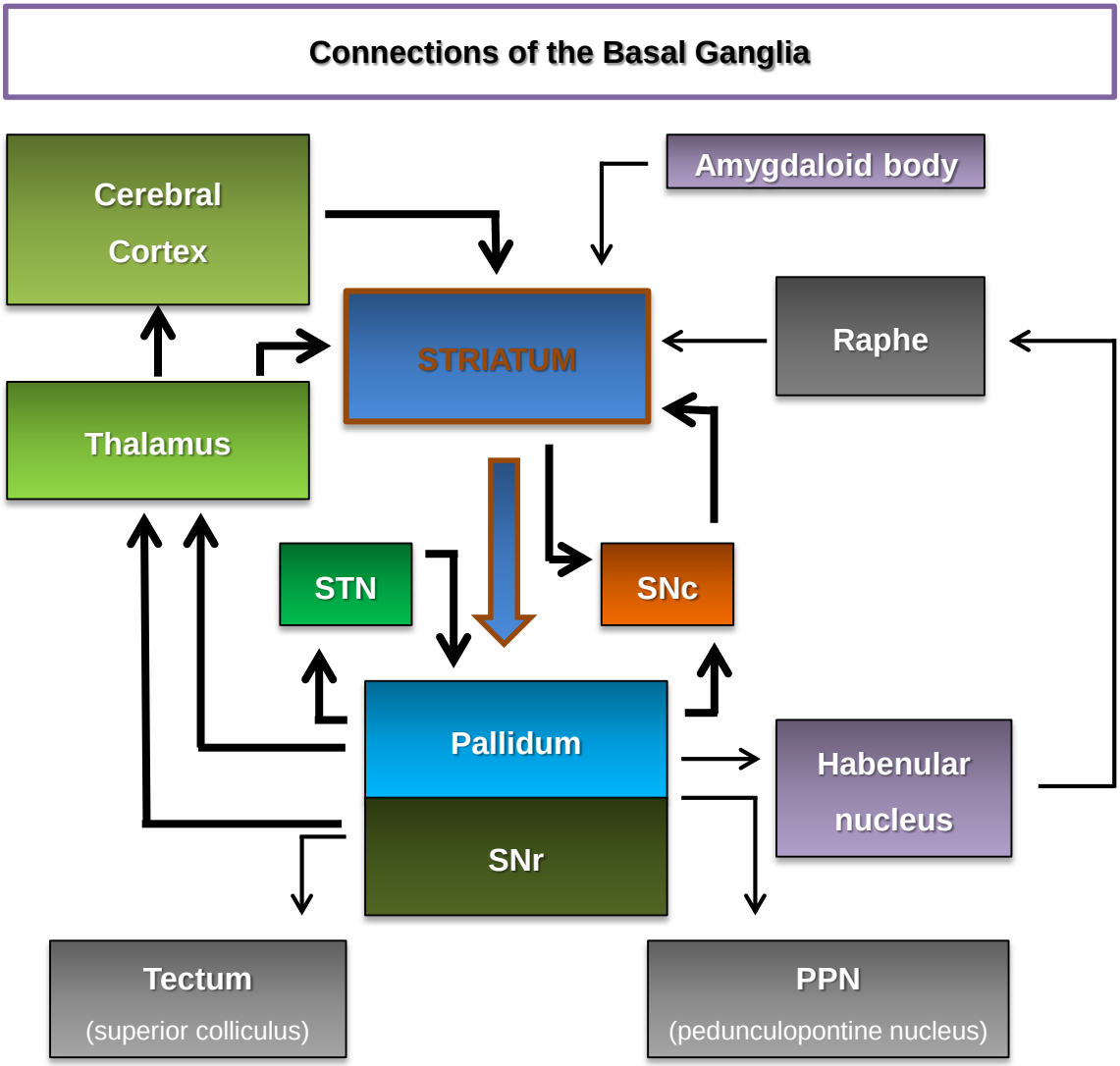


Figure 2. Simplified diagram of basal ganglia circuitry.

Flowchart illustrating the connections of the basal ganglia with their principal inputs and outputs.

Glutamatergic synaptic transmission in striatal MSNs

The principal neuronal population of the striatum, the GABAergic medium spiny projection neurons (MSNs) is reached by glutamatergic projections of pyramidal neurons residing in cortical layer 2/3 and 5 (Wall *et al.*, 2013; Kress *et al.,* 2013), and from neurons of the thalamic central medial complex and the parafascicular nucleus (Smith *et al.,* 2004).

Cortical and thalamic glutamatergic presynaptic terminals release glutamate, which activates specific receptors of the postsynaptic dendritic spine of MSNs. Those cells contain mainly 3 types of glutamate receptors: ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate (NMDA), and metabotropic glutamate (mGlu) receptors.

AMPA receptors (AMPARs), the main responsible for fast synaptic transmission in the basal ganglia, are tetramers which can be composed by different combinations of 4 subunits (GluR1-4). They have a non-selective cation channel which allows the flux of Na^+ and K^+, and also Ca^{2+} in case of absence of the Ca^{2+}-impermeable GluR2 subunit. These receptors have a desensitization mechanism that closes the channel quickly, terminating its depolarizing effect (Hammond, 2008).

NMDA receptors (NMDARs) are heteromeric ligand-gated ion channels. Each receptor can be formed by 4 subunits: 2 NR1 subunits (which are critical for the formation of functional channels), and 2 other subunits (NR2 A/B/C/D, or NR3 A/B) (Tepper *et al.,* 2010, Handbook of basal ganglia structure and function). NMDAR is a non-selective cation channel, which allows the flux of Na^+, K^+ and Ca^{2+}. At resting membrane potential, the channel is blocked by Mg^{2+}, and the putative binding of an agonist wouldn't result in the channel opening. Depolarization, which usually occurs via AMPARs, relieves the Mg^{2+} block, allowing the channel opening upon ligand binding. This condition is present in MSNs during the alternation of "up" and "down" states, as it will be better elucidated later. If glutamate is released from the presynaptic terminal when a MSN is in an "up" state, Na^+ influx through AMPARs and NMDARs causes further depolarization, keeping the NMDA channel in the unblocked state, and allowing a strong influx of Ca^{2+}. This is a trigger for several synaptic

and cellular events, such as synaptic plasticity (Hammond, 2008). NMDARs are indeed important for the induction of both long-term depression and potentiation at cortico-striatal synapses.

For the activation of AMPARs and NMDARs, generally a single presynaptic stimulus is sufficient. It produces a large and brief glutamate transient in the synaptic cleft (Clements *et al.*, 1992; Diamond and Jahr, 1997), which is limited by removal of the neurotransmitter by diffusion and glutamate transporters. When a train of stimuli reaches the synapse, instead, the release of much more glutamate is induced, also because of mechanisms of presynaptic facilitation and delayed release (Carter and Regehr, 2000). The resulting large glutamate levels may overwhelm clearance mechanisms, allowing an extended glutamate signal and even glutamate spillover to nearby sites. In this way, high affinity metabotropic glutamate receptors (mGluRs), which are often located at extrasynaptic sites, can be activated (Scanziani *et al.*, 1997).

mGluRs are G-protein coupled receptors, and they can be divided in different sub-families. Group I (mGluR1 and 5). mGluRs are coupled to the adenylyl cyclase activating Gq protein (Hammond, 2008), are facilitatory of neural transmission, and are expressed in MSNs (Tallaksen-Greene *et al.,* 1998). Group II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) mGluRs are coupled to adenylyl cyclase inhibiting Gi/o protein (Hammond, 2008) inhibit neural transmission and are expressed on the terminals of cortico-striatal afferents (Testa *et al.,* 1998).

The activity of striatal MSNs is dependent on the pattern of incoming glutamatergic signaling, which causes the activation of these different glutamatergic receptors.

MSNs are neurons with a medium cell body size (~9-17 μm), and a large and extensive dendritic tree. The average MSN has 20-60 dendritic branches, and each branch has approximately 500 input spines, so that the typical MSN integrates inputs coming from 10000-30000 synaptic boutons. This impressive number of input stations reflects the intrinsic firing properties of these neurons, whose firing activity requires the coordinated activation of many different excitatory inputs. MSNs are normally silent, kept in a “down” state (with a

hyperpolarized membrane potential of ~-80 mV) by a continuous shunting current mediated by rapidly activating inwardly rectifying potassium (Kir) channels (Wilson and Kawaguchi, 1996). In the “down” state, the synchronous input to few dendritic branches is insufficient to reach the firing threshold. Instead, when a large, synchronous release of glutamate from afferent terminals span most of the dendritic arbor, the shunting current collapses and the neuron enters a depolarized “up” state (the membrane potential reaches a voltage of ~-60--55 mV) in which firing activity may occur (Wilson and Kawaguchi, 1996; Millers, 2010). The electric potential of this “up” state is just below the neuron’s firing threshold, and a much smaller synchronous input is required to get the MSN to fire. This state may last for tenths or hundreds of milliseconds, or even a few seconds.

In the “down” state, the receptors which mediate excitatory postsynaptic potentials are mainly AMPARs, while during “up” states, the elimination of the Mg^{2+} block allows the recruitment of NMDARs. The recruitment of both receptor types leads to prolonged excitatory potentials, thus increasing the likelihood of temporal summation of incoming stimuli. Additionally, the activation of Ca^{2+}-permeable NMDARs during the “up” state allows further source of Ca^{2+} entry (in addition to Ca^{2+} channels gated by the depolarization of the neuron, called L-type voltage-gated calcium channels), which has been shown to play a pivotal role in processes of synaptic plasticity (Carter and Sabatini, 2004).

Thanks to the existence of these “up” and “down” states, the resulting effect is that only those neurons which will receive highly coordinated glutamatergic input together with permissive monoaminergic, cholinergic and GABAergic inputs (from midbrain dopaminergic and serotoninergic neurons and from striatal interneurons) will be able to signal to their downstream targets.

MSN projection pathways: the model of basal ganglia functioning

MSNs integrate glutamatergic inputs with monoaminergic signals, mainly arising from midbrain dopaminergic neurons, and from serotonergic neurons from dorsal raphe nucleus. Dopamine and serotonin bind to different classes of G-protein-coupled receptors, and activates multiple signaling cascades. By modulating the gating and trafficking of voltage-dependent and ligand-gated ion channels, those molecules can affect the MSN response to glutamatergic signals.

MSNs can be divided into two different classes, and dopamine exerts opposite effects on their activity, depending on the receptor subtype expressed by the cell. In the MSNs that predominantly express dopamine receptors of class 1 (D1, D5) (dMSNs), dopamine signal is coupled to Gαs proteins and increases neuronal excitability. In MSNs mainly expressing dopamine receptors of class 2 (D2, D3, D4) (iMSNs), dopamine stimulates Gαi proteins and results in inhibition of neuronal activity. According to the classic Albin-DeLong model (Albin et al., 1989; DeLong 1990), dMSNs constitute the first step in the propagation of cortico-thalamic inputs in the so-called striatonigral direct pathway, projecting to the internal segment of the globus pallidum (GPi) and to the substantia nigra pars reticulata (SNr). The GABAergic neurons in these two nuclei project to the thalamus, and they display a relatively high level of tonic activity, mediating a constant basal inhibition of their thalamic targets. Thus, the activation of the direct pathway leads to disinhibition of the target thalamic nuclei, promoting movement (**Fig. 3**).

The so-called striatopallidal indirect pathway is proposed to ultimately inhibit the thalamus through the activation of GPe and SNr nuclei. The activation of striatal iMSNs causes the inhibtion of the external segment of the globus pallidum (GPe). This nucleus, which contains mainly GABAergic neurons, exerts an inhibitory activity on the subthalamic nucleus (STN). The STN, instead, contains glutamatergic excitatory neurons that activate the GABAergic

GPi and SNr. The net disinhibition of the STN by iMSNs activity produces an increase in the activity of basal ganglia outputs, opposing the effect of the direct pathway and inhibiting the thalamus (Albin *et al.,* 1989; DeLong, 1990) (**Fig. 3**).

The integration of the signals originating from the two pathways occurs in the thalamic nuclei, which in turn project back to the cortex, gating movement.

Based on the Albin-DeLong model, motor dysfunctions depend on an imbalance of these two pathways, due to changes in the relative activity of dMSNs and iMSNs. Indeed, this computational model was postulated upon observation of the motor features characterizing PD. In this pathology, upon dopamine depletion, the output from iMSNs is enhanced while output from dMSNs is decreased, leading to an overall hypoactivity of the thalamus, which causes the hypokinesia (reduced movement) observed in patients. Indeed, the gold-standard treatment of PD targets these mechanisms through a dopaminergic replacement therapy with the dopamine precursor L-DOPA.

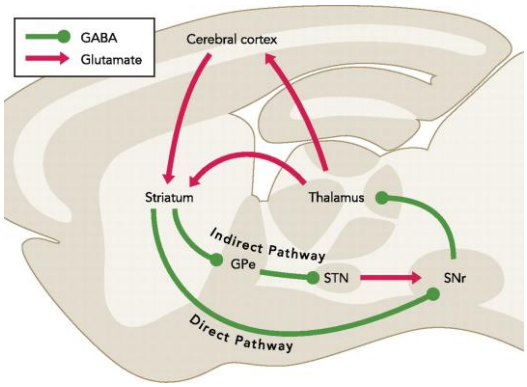
The application of the Albin-DeLong model to physiological conditions originally indicated that movement is inhibited by indirect pathway activation, while direct pathway activity favors movement. This hypothesis has been recently tested with an optogenetic approach, activating specifically the direct or indirect pathway (Kravitz *et al.*, 2010). The optogenetic strategy used, although, doesn't simulate a physiological activation of the striatal pathways, in that the MSN sub-population is activated strongly and as a whole. This implies that, while a massive activation of each pathway confirms the Albin-DeLong prediction, the interplay between the two pathways may be different under physiological conditions. The model has been recently challenged by a study measuring Ca^{2+} transients, a marker of neuronal depolarization and activation, in identified dMSNs and iMSNs of freely moving animals upon action initiation (Cui *et al.*, 2013). According to the classical model, one should predict the activation of dMSNs upon behavioral entrainment of the animal and the engagement of iMSNs in correspondence of movement cessation. What has been observed, instead, was the concurrent activation of MSNs belonging to both pathways just before the initiation of movement. Similarly, both neuronal sub-classes became relatively inactive when the

movement stopped. The explanation that the authors suggested was that both pathways are needed for fine control of motor function, with bursts of striatonigral MSNs disinhibiting specific thalamocortical targets promoting desired actions, while the activation of striatopallidal neurons would inhibit different thalamocortical targets suppressing competing motor programs. To reconcile these data with the previous results obtained by Kravitz and co-workers (Kravitz et al., 2010), they hypothesized that the simultaneous activation of the striatonigral or the striatopallidal pathway as a whole (as Kravitz and colleagues induced) can result in the activation or the inhibition, respectively, of all motor programs. In a more physiological context, instead, where actions depend on the environmental setting, the coordinated activation of clusters of neurons belonging to both pathways may be required to promote the selection and initiation of a precise movement, while inhibiting all the other ones. However, the precise cellular mechanisms shaping the coordinated activity of dMSNs and iMSNs have yet to be established. For this purpose, a recent study coming from our laboratory showed that the two pathways may be coordinated through concurrent, cell-type specific regulation of cortico-striatal synaptic plasticity at dMSNs and iMSNs subpopulations, and that the loss of this regulation disrupts motor control (Trusel *et al.*, 2015).

Although the Albin-DeLong model of the basal ganglia is still the prevailing model of computation in the basal ganglia, it is becoming clear that it is a simplification of a highly complex system (DeLong and Wichmann, 2009).

A modern theory would suggest that basal ganglia play a role not in the on-line motor control, but rather in processes controlling motor functions such as motor learning (Bar-Gad and Bergman, 2001; Doyon, 2008). It has been hypothesized that, in physiological conditions, basal ganglia could act as a store for neuronal representations of several learned behavioral patterns, and cortical inputs could induce their selection and release, producing the correct behavior. In pathological conditions, the aberrant modulation and the subsequent lack of filtering of cortical information by the basal ganglia might allow the activation of uncontrolled motor programs. This could, in turn, lead to involuntary movements, and interfere with normal cortical and thalamic activity.

A



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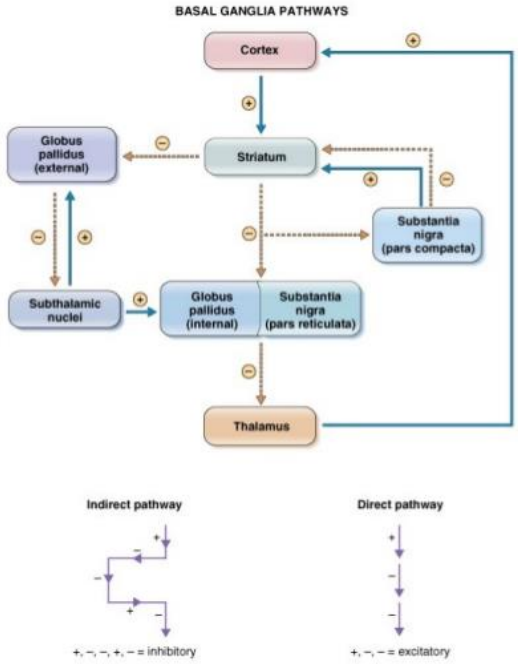


Figure 3. The Albin-DeLong model of basal ganglia.

A. *Sagittal diagram of the rodent brain showing the main projection pathways reaching the striatum and the projections from the striatum to downstream nuclei. Glutamatergic input originates from pyramidal neurons of cortical layer 5 pyramidal neurons and from the thalamic intralaminar/parafascicular complex. The MSNs projection pathways segregate in the direct and indirect pathways (from Kravitz and Kreitzer, 2012).*

B. *Flowchart illustrating the striatonigral direct and striatopallidal indirect pathways.*

Striatal interneurons

In the striatum there are various interneurons which contact and influence MSNs (Reiner *et al.,* 1998). They can provide an additional input to dopaminergic fibers from the SNc, serotonergic afferents from dorsal raphe nucleus and collateral contacts between MSNs to modify the responsiveness of MSNs to glutamatergic excitatory input. Striatal interneurons can be divided into four classes:

Cholinergic interneurons

Cholinergic interneurons represent 1-2% of total striatal neurons. They are large (>50 µm), aspiny neurons with dendritic branches covering a volume of 1 mm^3, and an axon that branches densely and profusely over a large portion of the striatum (Goldberg *et al.,* 2010, Handbook of basal ganglia structure and function). *In vivo*, they are tonically active, with a spiking frequency of 3-10 Hz (Bennett and Wilson, 1999) at rest, but during behaviorally salient stimuli they show bursting followed by a pause in firing that last for up a second (Aosaki *et al*., 1995; Ding *et al*., 2010).

The spontaneous firing activity is triggered by a persistent sodium current, which allows the neuron to reach -50 mV threshold to fire. The resulting spike is limited by the opening of the big-conductance calcium- and voltage-dependent (BK) potassium current, which is activated by the opening of voltage-dependent calcium channels and which repolarizes the resting membrane potential (Bennett *et al.,* 2000; Goldberg and Wilson, 2005). Calcium channels also activate small-conductance calcium-dependent potassium channels (SK), which sustain an after hyperpolarization (AHP) of the neurons after the action potential firing and which set the rhythm of discharge of these neurons (Bennett *et al.,* 2000; Goldberg and Wilson, 2005).

Cholinergic interneurons release acetylcholine (ACh), which plays an important role in modulating glutamatergic transmission, neuronal excitability, synaptic plasticity and ultimately action selection and decision-making. The effects of ACh signaling are due mainly to its action on muscarinic receptors expressed on striatal neurons (Koós and Tepper, 2002; Zhou

et al., 2002; Wilson, 2004). ACh can also activate nicotinic receptors expressed on the axons of dopaminergic neurons and on fast-spiking GABAergic interneurons (Goldberg *et al*., 2010, Handbook of basal ganglia structure and function).

Muscarinic acetylcholine receptors (mAChRs) are G protein-coupled receptors (GPCRs) widely expressed on axon terminals to the striatum and by striatal neurons. They can be divided in two distinct classes: M1-like (M1, M3, and M5) receptors are functionally linked with Gαq/11 proteins, activate phospholipase C and mediate a phosphoinositide-dependent signaling pathway. M2-like (M2 and M4) receptors are coupled to Gαi/o proteins, thus they lead to inhibition of adenylyl cyclase, thereby decreasing cAMP production and protein kinase A (PKA) activity (Peralta *et al.,* 1988). In MSNs the main muscarinic receptors expressed are M1, which are found in both dMSNs and iMSNs (Bernard *et al.,* 1992; Hersch *et al.,* 1994; Yan *et al.,* 2001), while M4 are more highly expressed in dMSNs (Hersch *et al.*, 1994; Ince *et al.,* 1997; Santiago and Potter 2001).

Fast-spiking interneurons

Parvalbumin-immunoreactive (PV^+) striatal interneurons are medium to large sized (16-18 µm diameter) cells, and have 5–8 aspiny, often varicose, dendrites which branch relatively sparsely, originating a restricted dendritic arborization (200–300 µm diameter). The axon branches overlap and extend well beyond the limits of the dendritic field of the cell of origin, thus creating the densest arborization of all striatal neurons: a single interneuron can connect to 135-541 MSNs, allowing for bursts from single cells to significantly affect the excitability of numerous MSNs (Koos and Tepper, 1999; Tepper *et al*., 2010, Handbook of basal ganglia structure and function). PV^+ neurons make up a very small percentage of the total striatal neuronal pool, accounting for the 0.7% of striatal neurons (Rymar *et al.,* 2004), with a dorsal to ventral and lateral to medial gradient of expression, being more present in the dorsoltareal striatum (Luk and Sadikot, 2001), suggesting an important role in sensorimotor integration.

They receive strong, multiple excitatory inputs from the cortex and differently from MSNs, they are contacted multiple times by each cortical fiber (Ramanathan *et al.,* 2002). They also

receive dopaminergic (Kubota *et al.,* 1987), cholinergic (Chang and Kita, 1992), pallidal (Bevan *et al.,* 1998) and thalamic afferents (Kita, 1993). Their GABAergic projections synapse on or near the soma of dMSNs and iMSNs (Koos and Tepper, 1999; Sidibe and Smith, 1999; Planert *et al.*, 2010), although a preferential connectivity with dMSNs is reported (Gittis *et al*., 2010).

These neurons display a hyperpolarized resting membrane potential (~-80 mV), very brief action potentials with a rapid and large amplitude, and brief duration spike afterhyperpolarization. These properties allow for the fastest spiking mode among striatal interneurons (Koós and Tepper, 1999; Taverna *et al.,* 2007). They co-contain GABA, parvalbumin (PARV), and the hexapeptide LANT6.

Spiking activity of PV^+ interneurons induces inhibitory post-synaptic potentials (IPSPs) in contacted MSNs: their activation is thus the primary mechanism of feed-forward inhibition, contributing to action selection by inhibiting MSNs in circuits with competing inappropriate actions (Parthasarathy and Graybiel, 1997; Gage *et al*., 2010). Gap-junctions connect PV^+ interneurons to each other (Koós and Tepper, 1999), allowing the synchronization or desynchronization (depending on the firing mode) (Russo *et al.,* 2013) of many interneurons, thus creating an inhibitory syncytium exerting a strong and synchronous inhibitory control over a large number of MSNs (Koos and Tepper, 1999).

SS/NPY/NOS interneurons

SS/NPY/NOS interneurons are, after the giant cholinergic interneurons, the largest cell population in the striatum, with a soma diameter of 15–25 µm. Their dendritic tree is simple and unbranched, extending up to 600 µm in diameter, while their axonal arborization, with 1 or 2 main axons (Kawaguchi, 1993), is the sparsest and longest of any striatal neuron (Tepper *et al*., 2010, Handbook of basal ganglia structure and function). Although not all the three markers, somatostatin (SS), neuropeptide Y (NPY) and nitric oxide synthase (NOS), are always simultaneously expressed by these neurons, they are considered a unitary class (Tepper *et al.*, 2010, Handbook of basal ganglia structure and function).

SS/NPY/NOS interneurons receive monosynaptic inputs from the cortex (Kawaguchi, 1993), as well as dopaminergic (Kubota *et al.,* 1987; Li *et al.,* 2002), cholinergic (Tepper *et al*., 2010, Handbook of basal ganglia structure and function), and pallidal GABAergic afferents (Bevan *et al.,* 1998). Their resting membrane potential is quite depolarized (~-50--60 mV), and their action potential is characterized by a long duration (Kawaguchi, 1993; Kubota and Kawaguchi, 2000; Centonze *et al.,* 2002). They are also referred to as low-threshold spiking interneurons due to their firing properties, and they constitute another feed-forward circuit in the striatum (Tepper *et al*., 2010); indeed, their firing induces inhibitory post-synaptic currents (IPSCs) in MSNs. A part from this, little is known of their functional importance, but their innervation of distal dendrites in MSNs hampers their understanding (Gittis *et al*., 2010). It has been proposed that they could be responsible for the regulation of Ach interneurons activity (Sullivan *et al.,* 2008).

Calretinin (CR)-expressing interneurons

Calretinin (CR)-expressing interneurons are medium sized cells (12-20 μm in diameter) with a small number of aspiny dendrites that branch sparingly (Bennett and Bolam, 1993). They represent a small population of striatal neurons, accounting, in rodents, for ~0.8% of total neurons (Rymar *et al.,* 2004). In primates, however, their number seems to be 3-4 fold higher (Wu and Parent, 2000). Not much is known about these neurons, as it is for other striatal interneuronal classes (e.g. tyrosine hydroxilase expressing neurons).

Neuromodulation of striatal activity: focus on monoaminergic inputs to the striatum

In the striatum, several signaling molecules, defined synaptic neuromodulators, regulate both glutamatergic and GABAergic inputs, and MSNs intrinsic excitability. On a cellular level, these molecules are able to shape synaptic strength, acting through G-protein coupled mechanisms, which regulate the release of neurotransmitters, modify ion channel activity and

affect neuronal excitability. On a system level, synaptic neuromodulators transduce salient environmental stimuli, and integrate animal's internal states, into behavioral patterns.
In pathological conditions, alteration of neuromodulatory pathways may lead to aberrant assignment of salience to environmental events, or to the lack of representation of internal states, ultimately causing symptomatic cognitive and behavioral dysfunctions.

In the following paragraphs, I will focus on the characteristics of monoaminergic signaling mechanisms, which are relevant for the synaptic processes investigated in my project.

At MSNs synapses, the main glutamatergic input arising from cortical and thalamic afferents is integrated with monoaminergic signals, mainly from midbrain dopaminergic neurons encompassed in the substantia nigra pars compacta and the ventral tegmental area, and from serotonergic neurons from dorsal raphe nucleus. I will first describe these two modulatory afferents separately; next, I will analyze their integrated function in shaping glutamatergic transmission.

Dopaminergic signaling

In the dorsal striatum the main dopaminergic signaling comes from cells residing in the SNc, producing tipically from 500 to 5000 dopamine inputs per MSN (Millers, 2010). Dopaminergic terminals often contact the spine neck of MSNs, thus modulating the glutamatergic signal transmitted by cortical or thalamic afferents contacting the spine head. The specificity of the dopaminergic signaling is not given by a spatial organization of the synapses. Dopamine released from a single synapse acts as a volume transmitter, affecting the synapses in a supposed surrounding volume of ~7 µm. The effects of dopamine signaling are limited to specific synapses by its coincidence with incoming glutamatergic drive (Arbuthnott and Wickens, 2007). As previously discussed, dopamine signaling has the behavioral significance of marking the salient environmental stimuli and to code unexpectancy of rewards (Hollerman and Schultz, 1998; Matsumoto and Hikosaka, 2009; Steinberg *et al.*, 2013).

In the resting state, dopaminergic cells display a tonic firing rate which produces a uniform basal concentration of ~6.5 nM dopamine in the striatum (Sam and Justice, 1996). When an unexpected reward occurs in response to an action, a tonic increase in firing activity of dopaminergic neurons may bring this concentration to ~250 nM (Garris and Wightman, 1995). This causes a reinforcement of the behavioral sequence that led to the reward (Mirenowicz and Schultz, 1994; Hollerman and Schultz, 1998; Matsumoto and Hikosaka, 2009; Steinberg *et al.*, 2013). When instead an expected reward is omitted, dopaminergic neurons stop their firing activity, causing a drop in dopamine concentration. By this mechanism, dopamine strengthens the appropriate cortico-striatal synapses, thus contributing to adaptive behaviors (Mirenowicz, and Schultz, 1994; Hollerman and Schultz, 1998; Matsumoto and Hikosaka, 2009; Steinberg *et al.*, 2013).

Serotonergic signaling

After dopamine, serotonin (5-hydroxytryptamine, 5-HT), is the most abundant amine neurotransmitter in the basal ganglia. 5-HT projections reach the striatum from the dorsal raphe nucleus (DRN) (Azmitia and Segal, 1978; Bobillier *et al.*, 1976), whose fibers project to all areas of the basal ganglia, including the striatum, GP, SN and STN. The 5-HT ascending pathway sends collaterals to each region as it travels to the frontal cortex (van der Kooy and Hattori, 1980). The medial raphe nuclei also send fibers to the basal ganglia, but those projections appear not to be 5-HT (De Deurwaerdere *et al.*, 2013). In the striatum, serotonergic afferents are predominantly characterized by varicosities, and they form en passant synapses, whereas classical terminals are less often observed, particularly in the lateral striatum (Soghomonian *et al.*, 1989); conversely, a higher proportion of proper synaptic terminals have been described in the SNr, SNc and VTA (Corvaja *et al.*, 1993; Moukhles *et al.*, 1997; De Deurwaerdere *et al.*, 2013).

5-HT receptors can be divided in different families (Hannon and Hoyer, 2008)(**Fig. 4 B**):

- **5-HT1 family**, which comprises the 5-HT1a, 5-HT1b, 5-HT1d, 5-HT1e and 5HT1f receptors, which are Gi/o protein coupled receptors, negatively coupled to adenylyl

cyclase. In the basal ganglia, 5-HT1a receptor levels are extremely low, while all the other receptors of the family are expressed in this region. In particular, there is high density of 5-HT1b receptor sites, especially in the substantia nigra, globus pallidus, ventral pallidum and entopeduncular nucleus. Strong evidences support the idea that 5-HT1b receptor has a role as both a 5-HT autoreceptor and 5-HT heteroreceptor. In the striatum, not only the protein, but also 5-HT1b receptor mRNA is present at high levels of expression in MSNs. 5-HT1d receptor sites are also present in basal ganglia, and 5-HT1b and d receptors can form homo or heterodimers. A functional role for 5-HT1b receptor in the striatum has been proposed by Mathur and colleagues, who demonstrated that pharmacological activation of 5-HT1b receptor was able to induce a form of long-term depression of cortico-striatal synaptic activity. This form of synaptic plasticity reciprocally occluded the classical form of striatal long-term depression mediated by endocannabinoids (Mathur *et al.*, 2011).

- **5-HT2 family**, comprising 5-HT2a, b, and c receptors, which couple preferentially to Gq/11 to increase inositol phosphates and cytosolic Ca^{2+} concentrations via phospholipase C. 5-HT2b receptor expression in the brain has been controversial; recent data show that it seems to be restricted to a few brain regions, which do not include the basal ganglia. 5-HT2a receptor expression in basal ganglia is intermediate, but there is no evidence of striatal mRNA in man and monkey; both protein binding sites and mRNA expression, instead, were reported for 5-HT2c receptor in basal ganglia. In the striatum, 5-HT2 receptors seem to exhibit a powerful excitatory control on cholinergic interneurons, by suppressing the AHPs associated with calcium-activated potassium currents (Blomeley and Bracci, 2005).
- **5-HT3 receptors**, belonging to the ligand-gated ion channel receptor superfamily. They trigger a rapid depolarization due to the opening of non-selective cation channels (Na^+, Ca^{2+} influx, K^+ efflux), and the response desensitizes and resensitises rapidly. 5-HT3 receptors are expressed in the striatum on MSNs, but not on dopaminergic nigrostriatal fibers.

- **5-HT5 receptors**. The function of these receptors is still unclear; they could be coupled to Gi/o or possibly Gs. Their distribution seems to be widespread in both rat and mouse brain.
- **5-HT receptors that preferentially couple to Gs**. 5-HT4, 5-HT6 and 5-HT7 receptors all couple preferentially to Gs and promote cAMP formation, by activation of different adenylate cyclases. In turn, cAMP as an intracellular messenger interacts with various targets, the protein kinase A (PKA), but also cyclic nucleotide-gated ion channels, leading to the modulation of calcium ion flux and membrane excitability and other cellular processes. PKA can phosphorylate cAMP responsive transcriptional factors, such as cAMP response element binding protein (CREB), leading to gene expression modifications, which can promote long term changes in cellular responses. cAMP can also interact with Epac (exchange proteins directly activated by cAMP) sensors, which mediate PKA-independent signal transduction, e.g. activation of Rap and Ras GTPases.

  5-HT7 receptor binding is present in different regions of the basal ganglia, but no mRNA transcripts were seen in those regions. Its expression seems to be higher in the limbic system, suggesting a role for 5-HT7 receptor in sleep, circadian rhythmic activity and mood. It is also important for synapse development (Ciranna and Catania, 2014).

  5-HT6 receptor seems to be involved in phenomena concerning learning and memory, and it is highly expressed in the striatum. Recent work by Eskenazi and colleagues showed that 5-HT6 receptors play a role in the dorsolateral and dorsomedial striatum in these behaviors that serve to temper goal-directed and habitual actions. 5HT6 receptor activated both dMSN and iMSN pathways to differentially impact instrumental learning and habitual responding (Eskenazi *et al.*, 2015).

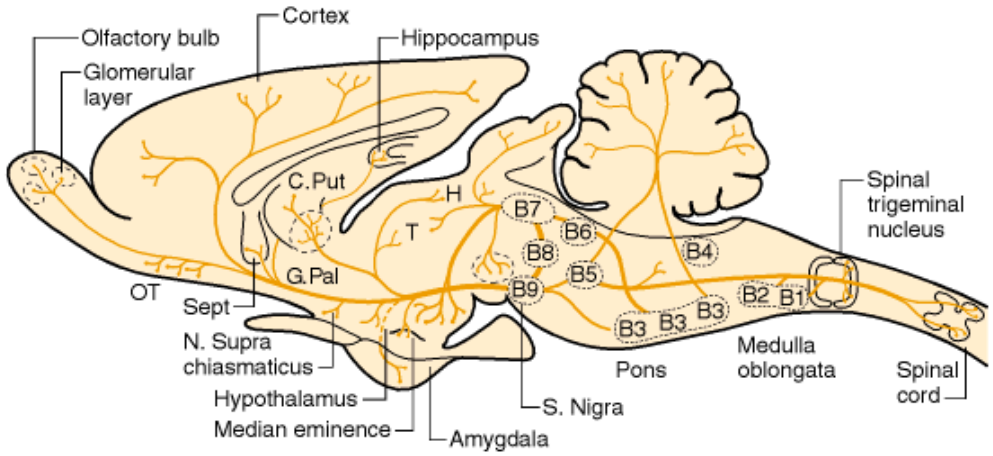
  5HT4 receptor is also coupled with a Gs, but, in addition, a direct coupling to K^+ channels and voltage-sensitive calcium channels has been proposed (Hannon and

Hoyer, 2008). The 5-HT4 receptor KO mouse is behaviorally normal in a standard environment, but displays very low locomotor activity accompanied with hypophagia in response to novelty and stress (Compan *et al.*, 2004). Expression of the 5-HT4 receptor in the striatum has been documented, and this receptor seems to be present in the somato-dendritic compartment of MSNs (Waeber *et al.*, 1993; Waeber *et al.*, 1996; Patel *et al.*, 1995; Vilaro *et al.*, 2005). Its functional implication on striatal function, however, it is still to be elucidated.

The DRN is located in the midbrain, and it is rich in 5-HT neurons that provide the principal source of 5-HT innervation in the forebrain (**Fig. 4 A**). It is generally agreed that *in vivo* 5-HT neurons spontaneously fire broad spikes in a slow and regular firing pattern (Aghajanian *et al.*, 1978; Sawyer *et al.*, 1985; Jacobs and Fornal, 1991). In many species, significant numbers of DRN neurons contain 5-HT, but neurons containing GABA, dopamine, glutamate, Ach, or one of a variety of neuropeptides are also present, accounting for almost one third of all the DRN neurons (Charara and Parent, 1998; Allers and Sharp, 2003; Hioki *et al.*, 2010). These non 5-HT cells are thought to contribute to the regulation of the activity of 5-HT neurons through local circuits and/or their mediation of the effects of afferent inputs (Monti, 2010). Recent evidence also suggests a role for these non 5-HT DRN neurons in reward circuitry participation (McDevitt *et al.*, 2014).

The role of 5-HT signaling in the striatum is still unclear. 5-HT neurons of DRN have been reported to fire coincidently with the initiation and termination of selected voluntary movements (Fornal *et al*., 1996), thus suggesting a role for 5-HT in modulating motion and, putatively, MSNs activity. A much debated issue is whether 5-HT can control dopamine release from nigrostriatal projections: indeed, both an enhancement and an inhibition of dopamine release have been observed in different experimental conditions (Navailles and De Deurwaerdere, 2011).

A



B

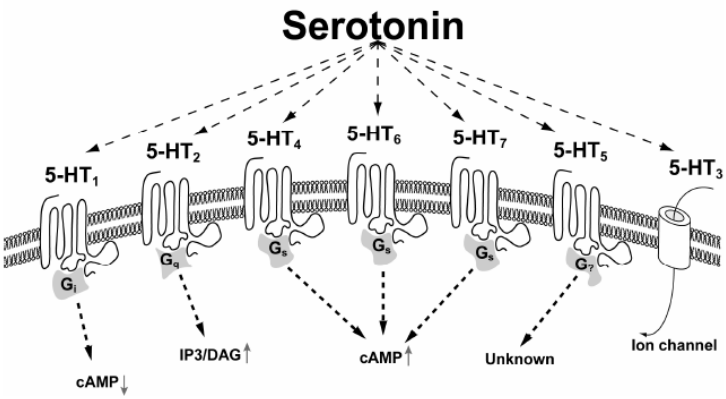


Figure 4. Simplified diagram of basal ganglia circuitry.

A. *Sagittal diagram of a rodent brain showing the main projection pathways of serotonergic neurons from the raphe.* ***B.*** *Schematic representation of the different serotonin receptors subtypes.*

Dopamine and Serotonin integration in reward related systems

Reward and punishment play critical roles in shaping animal behavior over short and long timescales (Rolls, 2005; Somerville *et al.*, 2013). On short timescales, moment-to-moment expectations of reward or punishment increase or decrease motivation to perform a specific action associated with the predicted outcome. On long timescales, repeated exposure to reward or punishment can induce long-lasting positive or negative emotional states, or

"moods", that influence the frequency of performing reward-seeking actions (Niv *et al.*, 2006; Cools *et al.*, 2011; Somerville *et al.*, 2013; Wang *et al*., 2013). A wealth of past research in reward primarily focused on dopamine (Berridge, 2007; Kranz *et al.*, 2010), while the role of 5-HT in reward processing has been largely neglected. In the last few years, increasing amount of work provides argument for 5-HT as a fundamental mediator of emotional, motivational and cognitive aspects of reward representation, which makes it possibly as important as dopamine for reward processing (Kranz *et al.*, 2010).

There are different theories concerning the role of 5-HT: the classical one proposes that 5-HT regulates aversive learning and negative motivation in response to punishments (Daw *et al.*, 2002). Accordingly, 5-HT opposes the positive reinforcement and behavioral activation regulated by dopamine. Whereas dopaminergic neurons signal appetitive prediction errors (Schultz *et al.*, 1997), 5-HT neurons could signal punishments, adjusting future behavior to avoid actions previously associated with a negative outcome. Although several studies provide data to support this theory (Crockett *et al.*, 2009; Shin and Ikemoto, 2010), in awake animals there is little evidence for 5-HT neurons to code for punishments (Montagne-Clavel *et al.*, 1995; Schweimer and Ungless, 2010).

A second theory proposes 5-HT as a signal for global reward states, such as tracking average reward (Daw *et al.*, 2002) and modulating mood (Savitz *et al.*, 2009). 5-HT in this view would be able to provide its targets with long-term signals regarding the value of the environment. This hypothesis has support from clinical (Fava and Kendler, 2000) and genetic (Donaldson *et al.*, 2013) studies, but does not find any neurophysiological evidence.

A third theory implies 5-HT in waiting for reward (Miyazaki *et al.*, 2011; Fonseca *et al.*, 2015): according to this, activation of 5-HT neurons promotes patience (Miyazaki *et al.*, 2014), or slows movements that allow an animal to wait for a delayed reward.

In light of these conflicting views on the role of 5-HT in reward behavior, a recent study (Cohen *et al.*, 2015) tested whether the firing of optogenetically-identified DRN 5-HT neurons correlated with rewards and punishments on different timescales. The research showed that a large fraction of 5-HT neurons shows tonic firing modulation depending on state value on

long timescales (tens of seconds to minutes); punishments induces a phasic response of 5-HT neurons (mostly by excitation), but a subset of 5-HT neurons is phasically excited by reward-predicting cues. Collectively, the authors propose that many single 5-HT neurons are activated by both reward-predictive cues and punishment, and they can use tonic as well as phasic firing to convey reward information. These firing patterns appear to be inconsistent with the idea that 5-HT neurons are just opponent to dopaminergic neurons: a possibility could be that 5-HT could combine with dopamine signal to form logical combinations postsynaptically (e.g. 5-HT AND dopamine codes for reward, whereas 5-HT AND NOT dopamine codes for punishment) (Cohen *et al.*, 2015; Cohen 2015).

According to an increasing amount of experimental evidence, we can say that dopamine and 5-HT probably play complementary rather than simply opponent roles in the reinforcement and control processes that underpin choice behaviors (Cools *et al.*, 2011; Rogers, 2011).

In the context of basal ganglia, which are supposed to mediate reinforcement learning processes, computational models posit that the striatum learns reward prediction and action selection based on the “reward prediction error” represented by the dopaminergic input. Correlation of striatal activity with reward prediction error could be due to dopamine-dependent plasticity of cortico-striatal synapses (Tanaka *et al.*, 2004). It has been suggested that a possible role for 5-HT from DRN is to control the effective time scale of reward prediction (Tanaka *et al.*, 2004) by differentially regulating activities within the striatum (Tanaka *et al.*, 2007).

There are increasing evidences supporting the integration between dopamine and 5-HT signal in mediating aspects of decision making from the viewpoint of reinforcement learning theory (Balasubramani *et al.*, 2015; Carli and Invernizzi, 2014), and different tests performed on humans are now pointing to the complementary role of these modulators in all the aspects of decision making (evaluation of the action candidates depending on how much reward or punishment they would bring; selection of the appropriate action; learning: reevaluation of the action based on the reached outcome) (Tanaka *et al.*, 2004; Tanaka *et al*., 2007; Kranz *et al*., 2010; Schweighofer *et al*., 2007; Doya, 2008; Palmintieri *et al*., 2012).

Interestingly, now key aspects of the molecular and cellular basis of dopaminergic modulation of striatal activity in relation to behavioral reinforcement have been revelaed. Hence, a recent study by Yagishita and colleagues clarified the molecular and cellular basis of reinforcement plasticity at the level of single dendritic spines, showing that, in striatal dMSNs, dopamine promoted spine enlargement only during a narrow time window (0.3 to 2 s) after the glutamatergic inputs. The temporal contingency was detected by a rapid regulation of cAMP in thin distal dendrites, where PKA was activated only within the time window because of a high phosphodiesterase activity. Thus, dopaminergic role in reinforcement plasticity has now clearer molecular basis (Yagishita *et al.*, 2014).

This is not the case for 5-HT, because the cellular correlates of these behaviors are still unclear. Given the increasing evidence of the behavioral relevance of 5-HT in reward-guided behaviors, and the complexity of its interplay with dopamine, it is clear that it will be fundamental to unravel the cellular and molecular mechanisms of action of 5-HT in the context of the striatal neuromodulation. To shed more light on mechanisms of monoaminergic synaptic regulation of the striatum will possibly contribute to a better understanding of basal ganglia associated behaviors, as well as to open new avenues for intervention in pathological states.

Synaptic plasticity in the striatum

At the level of the dorsolateral striatum (DLS), convergent glutamatergic inputs from sensorimotor cortex and thalamus integrate with dopaminergic and serotonergic signals coding for the motivational state of the animal (Kreitzer and Malenka, 2008). This information processing is thought to occur by mechanisms of synaptic plasticity. Synaptic plasticity is the ability of functional connections between neurons to adapt over time in response to very transient signals. It can be observed either a persistent increase or decrease in synaptic strength, respectively referred to as long-term potentiation (LTP), and long-term depression

(LTD). Once established LTP can be reversed to control levels by an active phenomenon defined “synaptic depotentiation” (Picconi *et al.*, 2005).

Mechanisms of striatal synaptic plasticity have been widely investigated in the past two decades, showing that, depending on experimental protocols and conditions, cortico-striatal synapses can display both long- and short-term potentiation and depression and that these phenomena are not just present in *ex vivo* brain slices, but have been proven to occur also in awake animals *in vivo* (Kawagoe *et al*., 1998; Lauwereyns *et al*., 2002, Yin *et al.*, 2009). To study synaptic plasticity, several different protocols have been used: high frequency stimulation (HFS, 100Hz) or low frequency stimulation (LFS, 1-20 Hz) of afferent fibers (with micro or macroelectrodes placed in the striatum, in the corpus callosum, or in deep cortical layers), and spike-timing dependent plasticity (STDP) (reviewed in Cerovic *et al*., 2013).

In brain slice preparations, depending on the experimental conditions, application of high frequency stimulation (HFS) to afferent fibers has been reported to result in both LTP and LTD (Calabresi *et al*., 1992a; Calabresi *et al.*, 1992b; Calabresi *et al.*, 1992c). In particular, HFS *per se* results in LTD, whereas by adopting experimental conditions able to increase NMDAR recruitment, it is possible to unmask LTP (Calabresi *et al.*, 1992c; Wickens *et al.*, 1996). The need to somehow “forcing” the experimental conditions to obtain LTP on *ex vivo* brain slices raised the question of whether striatal LTP represents the expression of a pathological process in which over-active NMDARs would lead to excitotoxicity and successively result in neurodegeneration. In 1997, Charpier and Deniau showed for the first time that HFS of cortical afferents *in vivo* causes LTP of cortico-striatal excitatory transmission (Charpier and Deniau, 1997). This evidence, together with data from an *ex vivo* study in which both LTP and LTD could be induced at the same synapses by applying different electrical stimulation protocols (Fino *et al.*, 2005) confirmed that striatal LTP is a physiological form of plasticity expressed at cortico-striatal synapses. Typically, plasticity induction in HFS combines strong somatic depolarization with high frequency stimulation of the afferent fibers. *In vivo*, this might be accomplished by convergent synaptic input that triggers state transitions in dendrites, which are the site of signal integration and plasticity

triggering, or by the temporal convergence of synaptic input and back-propagating action potentials (Plotkin *et al*., 2011; Plotkin *et al*., 2013).

Also low frequency stimulation (LFS) protocols have been reported to induce LTD (Ronesi and Lovinger, 2005; Lerner and Kreitzer, 2012) on *ex vivo* brain slices, or LTP (Charpier *et al.,* 1999) if applied *in vivo*.

Another established form of synaptic plasticity reported in many brain areas, including the striatum, is the so-called spike timing dependent plasticity (STDP). This form of plasticity is based on the relative timing of afferent stimulation and postsynaptic spikes. Depending on the exact order and time interval between the synaptic input and the postsynaptic action potential, this stimulation can result in either LTP or LTD. In brain structures other than the striatum, delivering a presynaptic stimulus just after postsynaptic spike (post-pre) within a critical time window resulted in LTD. LTP, instead, was induced by the inverse protocol (pre-post) (Markram *et al.*, 1997; Dan and Poo, 2004; Dan and Poo, 2006). In the striatum, contrasting evidences have been reported. Experiments conducted by Fino and colleagues showed that, without any drug application, post-pre pairings induced STDP-LTP, while pre-post pairings induced STDP-LTD (Fino *et al.*, 2005). Conversely, when $GABA_A$ transmission was pharmacologically blocked, LTD was induced after post-pre protocol, and LTP could be observed with pre-post pairings (Pawlak and Kerr, 2008; Shen *et al.*, 2008; Fino *et al*., 2010; Nazzaro *et al.*, 2012).

To give a global overview of striatal synaptic plasticity, I will briefly describe the principal molecular players that influence the different forms of MSNs synaptic plasticity. Then, the putative signaling mechanisms responsible for LTP and LTD in dMSNs and iMSNs will be presented.

DARPP-32

The phosphoprotein DARPP-32 (dopamine and cyclic AMP-regulated phospho-protein, relative molecular mass 32,000) (Walaas *et al.,* 1983; Svenningsson, *et al.,* 2004) can be

considered a striatal regulator of neurotransmission, given its ability to influence the direction of plasticity of MSNs.

In mice lacking DARPP-32, both LTD and LTP are abolished (Calabresi *et al.,* 2000). This striatal-enriched protein is expressed in really high concentration (~50 μM) both in dMSNs and iMSNs where it has the potential to fine-tune the activity of various phosphatases and kinases, determining the activity of the whole cell and its response to incoming stimuli (Svenningsson *et al.,* 2004).

PKA (protein kinase A) and the multifunctional Serine/Threonine phosphatase PP-1 are two main targets (but also modulators) of DARPP-32 signaling. Their activity oppositely modulates the function of NMDA, AMPA and GABA receptors, and affects N/P-type Ca^{2+} channels and Na^{+}/K^{+}-ATPase. Moreover, DARPP-32 modulates regulators of gene expression including MAP kinase, CREB, c-Fos and ΔFosB (reviewed in Svenningsson *et al*., 2004). Each of these molecular players can have a role in the induction of synaptic plasticity.

DARPP-32 can be phosphorylated at different sites, and, depending on the site of phosphorylation, it can promote the signaling leading to LTP (through PKA-dependent enhanced Thr34 phosphorylation and Thr75 dephosphorylation), or, vice versa, promote the LTD by the opposite balance of Thr34/Thr75 levels of phosphorylation. PKA and PP-1 proteins, being not only targets, but also DARPP-32 modulators, take part to a feedback loop, potentiating the effects of DARPP-32 dependent signaling (Svenningsson *et al*., 2004).

DARPP-32 influences the processes of striatal synaptic plasticity mainly through the phosphorylation levels of AMPARs, either triggering their insertion on the plasma membrane (resulting in LTP) or their recycling (producing an LTD). The activation of this pathway can be activated by the signaling cascades downstream from many neurotransmitters, such as dopamine, glutamate, GABA, adenosine and 5-HT.

Dopamine acts on DARPP-32 pathway in a bidirectional way, depending on the receptor subtype. The dopaminergic D1 receptor is positively coupled to adenylyl cyclase (AC) through Golf protein (Herve *et al.,* 1995). AC, through the synthesis of cAMP, causes the

activation of PKA. PKA phosphorylates DARPP-32 at Thr34, while activating the specific PP-2A isoform that catalyzes the dephosphorylation of Thr75 (Nishi *et al.,* 2000). Thr75-DARPP-32 normally catalyzes the inhibition of PKA. Enhanced dopaminergic transmission via D1 receptors leads then to a decreased phosphorylation of Thr75-DARPP-32, and thereby facilitates signaling via the PKA/Thr34-DARPP-32/PP-1 cascade.

D2 receptor, instead, is coupled to Gαi/o protein, which inhibits AC. The decrease in cytoplasmatic concentration of cAMP facilitates the phosphorylation of DARPP-32 at Thr75, ultimately activating PP-1. In iMSNs, D2 signaling on DARPP-32 is counteracted by adenosine, acting on the adenosine receptor subtype A2A. As mentioned before, A2A is expressed specifically in iMSNs, and its activation stimulates the phosphorylation of DARPP-32 at the Thr34 residue. In iMSNs, A2A receptor mimics the activity of D1 receptors on DARPP-32 in dMSNs, and opposes the effects of D2 receptor activation (Svenningsson *et al.,* 2004).

Metabotropic glutamate receptors (mGlu) of group I can favor the phosphorylation of DARPP32. Activation of group I mGlu5 receptors stimulates DARPP-32 Thr34 phosphorylation through a mechanism dependent on activation of adenosine A2A receptors, but not of D1 receptors. Group I mGluRs regulate the phosphorylation of DARPP-32 also at Thr75 in a PLC- and Ca^{2+} dependent manner (Liu *et al.,* 2001; Liu *et al.,* 2002).

Glutamate can influence DARPP-32 phosphorylation state also through NMDA/AMPA receptors. Their activation induces the dephosphorylation of DARPP-32 at Thr34 (Svenningsson *et al.,* 2004).

GABA is reported to increase Thr34 phosphorylation and, finally, 5-HT-mediated signaling causes an increase in phosphorylation of DARPP-32 at Thr34 and a decreased phosphorylation at Thr75 (Svenningsson *et al.,* 2004). These serotonergic dependent DARPP-32 modifications have been proven to occur via 5-HT4 and 5-HT6 receptor activation, and have been validated both *in vitro* and *in vivo* (Svenningsson *et al*., 2002). This evidence further strengthen the interplay between dopaminergic and serotonergic

neurotransmission, and identify DARPP-32 as a possible intracellular candidate for the integration of the two signals.

Of course, DARPP-32 can influence the direction of MSNs plastic changes also through many other signals, which are not reported in this paragraph (see Svenningsson *et al.,* 2004 for extensive review).

Endocannabinoid-mediated synaptic plasticity

In the striatum, eCB signaling can influence glutamatergic synapses to MSNs. The first evidence of eCB involvement in striatal plasticity was reported in 2002 by Gerdeman and colleagues (Gerdeman *et al*., 2002); since that, a lot of work has been done to dissect the eCB role in regulating synaptic plasticity at MSNs connections. eCBs are small lipidic signaling molecules that, in the striatum, can act both presynaptically, via CB1 receptors, and postsynaptically, through TRPV-1.

eCB-LTD via CB1 receptor

The CB1 receptor (CB1R) is the receptor with the highest expression level throughout the brain, and it is coupled to Gi/o proteins; its activation, thus, leads to a decrease of synaptic transmission. In order to generate a long-lasting depression of the synapse, CB1R activation has to give rise to an intracellular signaling cascade, activating protein synthesis (Yin *et al*., 2006) within the presynaptic terminal of glutamatergic afferents. This leads to a persistent decrease in the release probability of the neurotransmitter, with no need of a change in the postsynaptic responsiveness (Chevaleyre *et al*., 2006; Lovinger, 2008; Gerdeman and Lovinger, 2001; Hoffman and Lupica, 2001; Huang *et al*., 2001; Robbe *et al*., 2001; Nazzaro *et al*., 2012).

To this, a strong activation of the eCB synthetic machinery is needed: indeed, to induce eCB-LTD at glutamatergic synapses to MSNs, moderate or high frequency stimulations are necessary (Gerdeman *et al*., 2002; Robbe *et al*., 2002; Choi and Lovinger, 1997; Ronesi *et al.*, 2004; Kreitzer and Malenka, 2005; Ronesi and Lovinger, 2005; Wang *et al*., 2006, Nazzaro *et al*., 2012).

In the striatum, eCBs production through the activation of specific enzymes occurs after a strong electrical activation of excitatory (cortical and thalamic) fibers, which release glutamate, depolarizing the MSN through AMPAR and NMDAR activation, and postsynaptic mGluR I activation. MSN depolarization leads to intracellular Ca^{2+} concentration increase through the activation of L-type voltage-gated calcium channels (L-VGCCs), and this increase, coupled to the release of Ca^{2+} from internal stores (Wang *et al*., 2006; Plotkin *et al*., 2013), stimulates the eCB production. Ca^{2+} increase via VGCCs alone is not sufficient to induce eCB production and release in the striatum, but requires the additional activation of mGluR I (Kreitzer and Malenka, 2005). Once produced, eCBs are released from the postsynaptic MSN, and retrogradely travel to the presynaptic CB1R. eCB-LTD, thus, is a form of LTD which is induced postsynaptically, but has a presynaptic site of expression.

Also dopamine plays an important role in striatal eCB-LTD, as Calabresi and colleagues demonstrated in different works (Calabresi et al., 1992a, 1997). Specifically, D2 receptor activation was shown to be involved in modulation of plasticity induction (Giuffrida *et al.*, 1999; Kreitzer and Malenka, 2005; Adermark and Lovinger, 2007).

It is a matter of debate, however, if eCB-LTD exists both on dMSNs and iMSNs, or it is expressed only on D2 receptor containing iMSNs. Lovinger's and Surmeier's groups provided a model in which both dMSNs and iMSNs support eCB-LTD via a cholinergic interneuron D2 receptor-dependent reduction in M1 receptor tone (Wang et al., 2006). Furthermore, the delivery of high-frequency stimulation with a macroelectrode placed in the white matter overlaying the striatum has been shown to induce eCB-LTD in MSNs belonging to both pathways (Wang *et al*., 2006; Adermark and Lovinger, 2007), even if other investigators didn't replicate this result (Kreitzer and Malenka, 2007).

A complementary hypothesis proposes that D2 receptors responsible for eCB-LTD contribution are segregated on iMSNs. In light of these, eCB-LTD would be preferentially induced in iMSNs upon cortical stimulation of glutamatergic inputs with STDP (Nazzaro *et al*., 2012), intrastriatal stimulation of cortical and thalamic inputs by either a HFS (Kreitzer and Malenka, 2007) or a STDP (Shen *et al*., 2008) protocol. Interestingly, D2 receptor activation

has been proved to facilitate eCB production via disinhibition of mGluR I signaling pathway via a RGS4 dependent mechanism (Lerner and Kreitzer, 2012). At dMSNs, instead, the eCB-LTD was gated only by pharmacological inhibition of D1 receptor dependent signaling (Shen *et al*., 2008), showing an opposite modulation by dopamine of synaptic plasticity mechanisms on dMSNs and iMSNs depending on their dopamine receptor expression.

Recent work from our laboratory demonstrated that stimulation of cortical afferents to the striatum by a HFS triggered eCB-LTD at iMSNs, but induced a previously unknown form of adenosine mediated LTD at dMSNs. In this cell population, eCB-LTD was observed only by forcing the eCB production by pharmacological activation of mGluR I receptor coupled to cell depolarization to recruit L-VGCCs. These data support the hypothesis that, in dMSNs, HFS-LTD is not primarly gated by eCBs, which can, however, be released to depress cortical inputs in response to the direct pharmacological activation of mGluR I (Trusel *et al*., 2015).

To summarize, eCB-LTD induced by high frequency stimulation of glutamatergic afferents requires CB1R, mGluR and D2 receptor activation, Ca^{2+} influx, and postsynaptic depolarization, suggesting that this form of plasticity occurs during MSN “up” states, when the MSN integrates a convergent and highly coordinated glutamatergic and dopaminergic input.

eCB-LTD via TRPV1 receptor

In the striatum, eCBs can also bind to another receptor, which is found postsynaptically on MSNs: the transient receptor potential vanilloid 1 (TRPV1), a Ca^{2+} permeable channel which was originally found to be sensitive to heat, opening at temperatures higher than 42ºC. TRPV1 was later found to be sensitive to lipid ligands, including eCBs, which are bound to the intracellular face of the channel (Kauer and Gibson, 2009). In the nucleus accumbens, LFS of afferents induced a LTD that was selectively expressed in iMSNs, and was only partially dependent on CB1R. The authors, indeed, found a component of this depression to be mediated by postsynaptic TRPV1 activation, which triggered a rise in postsynaptic Ca^{2+} that caused AMPAR endocytosis (Grueter *et al*., 2010).

eCB-SynDep

Synaptic depotentiation (SynDep) is a form of synaptic plasticity that occurs selectively at previously potentiated synapses following low-frequency stimulation (2 Hz) of excitatory afferents (Picconi *et al.,* 2003).

Data obtained in our laboratory indicate that eCB signaling contributes to SynDep at excitatory afferents to the lateral part of the dorsal striatum (Nazzaro *et al.,* 2012). In contrast to LTD, eCB modulation of SynDep does not involve VGCCs; rather, it requires the activation of mGluR5 (Nazzaro *et al.,* 2012).

In the striatum, the induction of SynDep appears to be dependent on the activation state of D1/PKA/DARPP-32 signaling pathway (Picconi *et al.,* 2003), but also muscarinic ACh receptors seem to play a role (Picconi *et al.,* 2006).

In conclusion, eCB signaling, by integrating glutamatergic, cholinergic and dopaminergic signaling with voltage-gated Ca^{2+} signals, is an important player in the neuromodulation of plasticity, and consequently, is an attractive putative candidate for the regulation of striatal-based behavior (**Fig. 5**).

The MEK/ERK signaling pathway

An additional cellular signaling cascade which acts as coincidence detector for dopaminergic, glutamatergic and Ca^{2+} signals is the Ras/Raf/MEK/ERK pathway. ERK is a kinase which gates the activation of several signaling proteins, and it is involved in several physiological processes.

The main signal for ERK activation is a rise in intracellular Ca^{2+} concentration. Ca^{2+} influx can activate ERK through two different pathways. The most common one is dependent on the Ras guanine nucleotide releasing factors (RasGRFs) (Iida *et al.,* 2001), which triggers ERK phosphorylation, that, in turn, can mediate the phosphorylation of cytoplasmatic targets (such as the ribosomal protein S6) and lead ERK translocation to the nucleus, where it activates downstream kinases, transcription factors (such as CREB, SRF, c-Jun, c-Fos), and proteins

regulating chromatin remodelling (histone H3) (Sgambato *et al.,* 1998, Vanhoutte *et al.,* 1999, Soloaga *et al.,* 2003), mediating long-term changes in cellular response to incoming stimuli.

A parallel, Ca^{2+}- and cAMP-dependent pathway, is the Rap1 and B-Raf pathway (Grewal *et al.,* 2000), which triggers the phosphorylation of ERK in an independent and possibly alternative way to Ras signaling.

It is possible that the interplay between Ras and Rap signaling cascades could shape ERK activation: indeed, they are recruited with different kinetics, and this could explain the dual pattern of ERK activation. Experiments conducted by Baldassa and colleagues, thus, showed that Ras activation was strong, transient, and rapid (2-5 min), while the Rap1 response was reported to be weaker, slower, and more sustained (Baldassa *et al.,* 2003).

In the striatum, the ERK signaling pathway seems to be primarly involved in the induction - but not in the mainteinance- of LTP and of its reversal SynDep (Picconi *et al.,* 2003; Cerovic *et al.,* 2013). Pharmacological blockade of ERK completely prevents the induction of HFS-LTP in cortico-striatal slices, both in dMSNs and iMSNs, leaving HFS-LTD intact. In dMSNs, Ras-GRF1 was identified as specific trigger for ERK signaling-mediated LTP, while in iMSNs, LTP induction was independent from this pathway, suggesting that the ERK modulation of LTP might occur with different upstream mechanisms in the direct and indirect pathways (Cerovic *et al.,* 2013). The importance of ERK in mediating phenomena of synaptic plasticity has been further investigated in our laboratory: recent experiments demonstrated that ERK activation can interfere with a novel form of adenosine dependent LTD specifically in the dMSNs (Trusel *et al*., 2015).

Molecular mechanisms driving plasticity in iMSNs

MSNs of the striatopallidal pathway, differently from striatonigral MSNs, express dopaminergic D2 and adenosine A2A receptor (Fink *et al.,* 1992). D2 receptor is coupled to the adenylyl cyclase-inactivating G-protein Gαi/o. Conversely, A2A receptor, is positively

coupled to the cAMP/PKA pathway through Gs protein. The interplay between these two receptors strongly influences synaptic plasticity in iMSNs.

To induce the indirect-pathway LTD, the activation of dopamine D2 receptors, but also lack of activation of postsynaptic adenosine A2A receptors is required (Shen *et al.,* 2008; Lerner *et al.,* 2010). Indeed, D2 receptors activation removes the RGS4-mediated inhibition of group I mGluR signaling pathway, favoring eCB mobilization (Lerner and Kreitzer, 2012), while A2A receptors activation counteracts this mechanism, activating adenylyl cyclase and then RGS4. On the other hand, the induction of LTP requires A2A receptor activation as well as a lack of activation of D2 receptors (Shen *et al.,* 2008; Flajolet *et al.,* 2008). The application of an antagonist of A2A prevented the induction of STDP-LTP in D2 MSNs, while the co-application of an A2A agonist and a D2 antagonist it is permissive (Shen *et al.,* 2008). Experimental evidences suggest that D2 and A2A receptors modulation of PKA activity could play an important role in their regulation of LTP (Lerner *et al*., 2010). Upon impairment of PKA signaling by application of inhibitors or by the genetic deletion of adenylyl cyclase, indeed, both HFS-induced LTD and LTP are prevented (Centonze *et al.,* 2003; Kheirbek *et al.,* 2009). This effect may be dependent on the role played by PKA on DARPP-32 and ERK signaling pathways, which are known to be involved in the induction of plasticity. Mice carrying a genetic deletion of DARPP-32 gene show disrupted HFS-LTD and LTP (Calabresi *et al.,* 2000). DARPP-32 is crucial also for the induction of STDP-LTP, as proven by a recent study where it was shown that the lack of DARPP-32 prevents this form of plasticity (Bateup *et al.,* 2010). Finally, DARPP-32 causes the disinhibition of the striatum-enriched phosphatase (STEP) (Fitzpatrick and Lombroso, 2011). When STEP is blocked, HFS induced LTP is disrupted, while SynDep is favored (Tashev *et al.,* 2009).

The supposed role of DARPP-32 in LTP induction involves the regulation of NMDA and AMPA receptors via PP1 activation (Greengard *et al.,* 1999), but consistent evidence to support this idea is still missing.

Molecular mechanisms driving plasticity in dMSNs

In dMSNs, the A2A/D2 receptors interplay typical of iMSNs is mirrored by the specific expression of dopaminergic D1, coupled to a Gαs, and cholinergic M4, coupled to a Gαolf, receptors (Kreitzer and Malenka, 2008; Herve *et al.,* 1995). D1 and M4 receptors, thus, oppositely regulate cAMP-mediated signaling. Specifically, LTP induction requires D1 receptors activation: LTP, indeed, is abolished using specific D1 antagonists, or in genetic models lacking D1 receptor expression (Calabresi *et al.,* 2000; Kerr and Wickens, 2001; Lovinger *et al.,* 2003). Also pharmacological dopamine depletion causes loss of LTP (Centonze *et al.,* 1999). Conversely, antagonism of D1 receptors allowed the induction of STDP-LTD in dMSNs (Shen *et al.,* 2008). The role of M4 receptors in LTD has not been explored so far, but the coupling of this receptor to Gαolf signaling makes it an attractive candidate for a D2 analog in dMSNs (Lerner and Kreitzer, 2011). Moreover, our laboratory provided evidence that, in dMSNs, HFS-LTD in cortico-striatal slices is not dependent on eCB production and mobilization, but is mediated by adenosine, through the activation of presynaptic A1 receptor (Trusel *et al*., 2015).

Similarly to iMSNs, LTP in dMSNs is NMDA-depentent (Calabresi *et al.,* 1992c; Dang *et al.,* 2006; Shen *et al.,* 2008), and the activation of the DARPP32-PP1 cascade is necessary for its induction (Calabresi *et al.,* 2000). Moreover, also the Ras-ERK, through Ras-GRF1, pathway is reported to be necessary to induce LTP (Cerovic *et al.,* 2013).

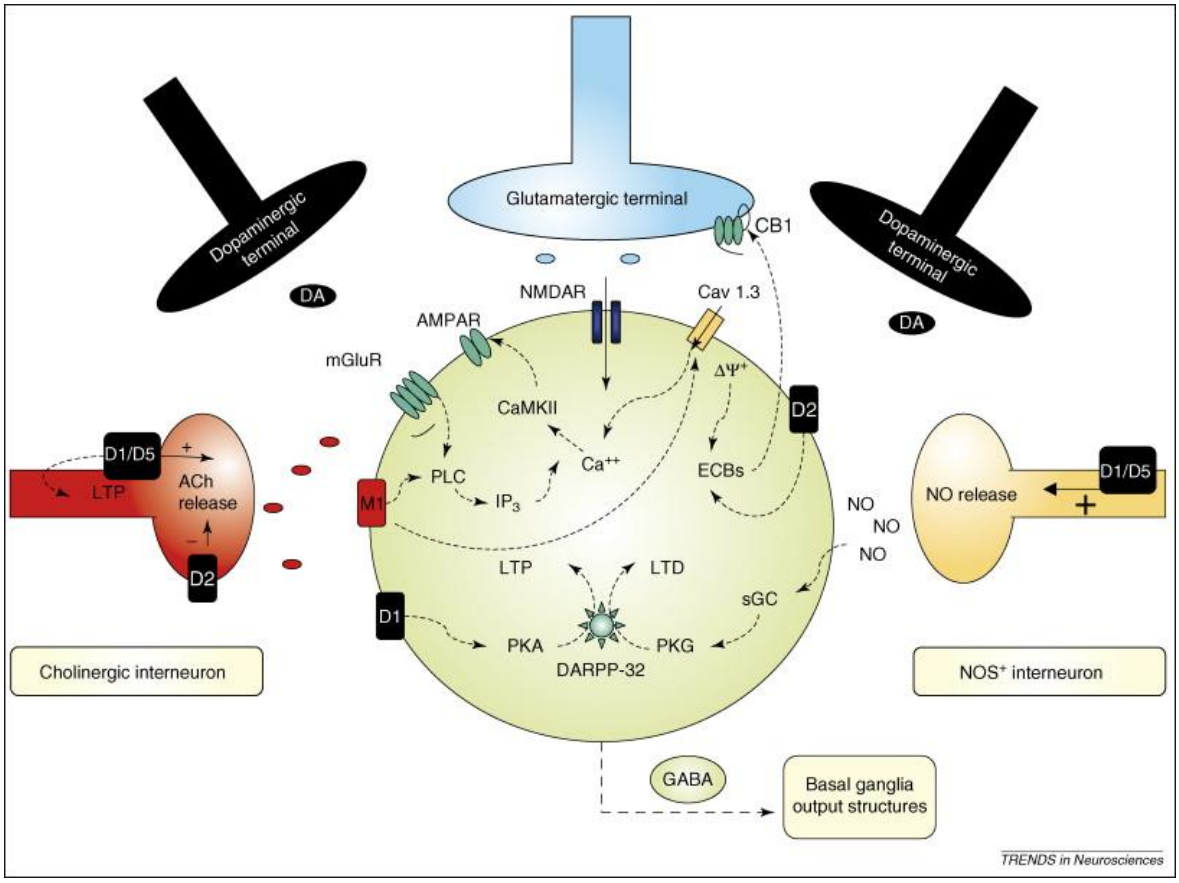


Figure 5. Schematic representation of the principal components driving synaptic plasticity at MSNs connections. (From Calabresi et al., 2007).

Behavioral relevance of synaptic plasticity in the striatum

Synaptic plasticity is an activity-dependent process with the functional significance of adapting neuronal connections to shape brain responses to stimuli originating from the environment and from internal states. Impairments of striatal synaptic plasticity associate with behavioral deficits in motor and procedural tasks (Trusel *et al*., 2015; Nazzaro *et al*., 2012).

The most common behavioral test used to measure motor functions in rodents is the rotarod test. By measuring the animal ability to balance and walk on a rotating rod, it is possible to assess locomotion, motor coordination, balance and motor skill learning (Shiotsuki *et al.*,

2010; Ardayfio *et al.*, 2008; Colebrooke *et al.*, 2006; Kelly *et al.*, 1998; Beeler *et al*., 2010; Yin *et al*., 2009). Processes of synaptic plasticity in the DLS are involved both in the early and late phases of training in motor skill-learning in rotarod tasks (Yin *et al*., 2009).

Rotarod performance is impaired in animals showing striatal synaptic plasticity dysfunctions. In particular, loss of dopaminergic signaling (Blundell *et al*., 2008), deletion of dopaminergic D2 receptors (Calabresi *et al*., 1997) and dopamine depletion by neurotoxic lesion (Iancu *et al*., 2005; Monville *et al*., 2006) all lead to behavioral impairments as measured by the rotarod test.

Interestingly, recent work from our laboratory has strengthened the link between synaptic plasticity and motor function. Experimental evidence indicated that proper regulation of mechanisms of synaptic plasticity in dMSNs and iMSNs is able to shape motor behavior; disruption of synaptic plasticity regulation in dopamine-depleted mice resulted in motor abnormalities (Trusel *et al.*, 2015).

In addition to motor behavior, the striatum is also involved in reward-guided instrumental learning. Specifically, the associative DMS is preferentially involved in the acquisition of action-outcome contingencies and the expression of goal-directed actions. The sensorimotor DLS, instead, is involved in the expression of stimulus-response behavior (habits) (Yin *et al.*, 2004, 2005).

It is well known that dopamine and dopamine-dependent synaptic plasticity play a fundamental role in these mechanisms. Lesion of nigrostriatal projections causes the loss of habitual behavior, and favors the acquisition of goal-directed contingencies (Faure *et al*., 2005). Chronic administration of dopamine-related drugs, such as amphetamine and cocaine, or of cannabinoid drugs (e.g. THC) also affects these processes. It is documented that these drugs cause the loss of different forms of striatal synaptic plasticity, dysregulating goal-directed behavior and leading to habitual responses (Nelson and Killcross, 2006; Nordquist *et al*., 2007; Nazzaro *et al.*, 2012). In particular, our group showed that the chronic administration of the CB1R agonist THC causes the disruption of eCB-LTD in the DLS and a shift towards habitual behavior (Nazzaro *et al*., 2012).

The behavioral impact of serotonergic modulation of striatal synaptic plasticity and circuit function is less known, but it is possible that dynamic interaction of striatal serotonergic and dopaminergic signaling cascades could provide an additional mean to fine tune cortico-striatal input enabling proper action selection in instrumental learning paradigms. Indeed, it is known that serotonergic neuronal activity correlates with repetitive or sustained behavior (Kane *et al.*, 2012), and the notion of striatal 5-HT influencing action control is consistent with the clinical use of selective serotonin reuptake inhibitors (SSRIs) for the treatment of obsessive compulsive disorder (OCD) (Pizarro *et al.*, 2014). Moreover, 5-HT, via 5-HT6 receptor, seems to positively interact with dopamine in cocaine reinforcement (Valentini *et al*., 2013). The role of 5-HT in instrumental learning has been evaluated further by a recent work by Eskenazi and colleagues. They demonstrated that 5-HT6 receptors activate both dMSN and iMSN pathways to differentially impact instrumental learning and habitual responding. In the DLS, 5-HT6 signaling could facilitate behavioral flexibility by opposing the differential activation of the MSN output pathways by dopamine (Eskenazi *et al*., 2015).

In addition to this, evidence for a role of 5-HT in modulating instrumental and habitual behaviors has been provided by demonstration that 5-HT DRN neurons activation positively reinforces behaviors and signals reward (Liu *et al*., 2014).

This possible serotonergic influence on reward-based mechanisms is particularly relevant if we consider that common basal ganglia circuits are likely to mediate not only movement, but also reinforcement and reward. Specifically, it has been suggested a link between motor activity and hedonic feelings, with positive feelings sharing common neural circuitry with cells that drive movement, and negative feelings sharing common circuitry with cells which inhibit movement (Kravitz and Kreitzer, 2012). In this framework, movement could be mediated by anatomical connections between basal ganglia and motor circuitry. Reward, which refers to hedonic value that is assigned to a stimulus, may be mediated by relationship between brain states and hedonic states. Finally, reinforcement, which is the process that maintains or increases behavior, and can be caused by the addition of a positive stimulus or the removal of a negative stimulus, can be mediated by plasticity in dMSNs and iMSNs or in their afferent

inputs (Kravitz and Kreitzer, 2012). In particular, it has been shown that direct pathway activation is reinforcing, whereas indirect pathway activation is punishing, and activation of direct and indirect pathways may evoke primary hedonic responses, such that direct pathway activation is rewarding and indirect pathway activation is aversive (Kravitz *et al*., 2012). Starting from this evidence, it is possible to suggest that plasticity of the inputs onto these cell types would alter future motor or hedonic responses, as in reinforcement or punishment. Specifically, a positive reinforcement may be associated with plasticity that enhances synaptic efficacy onto dMSNs, whereas a positive punishment may be associated with plasticity that enhances synaptic efficacy onto iMSNs. Conversely, a negative reinforcement may be associated with plasticity that depresses synaptic efficacy onto iMSNs, whereas a negative punishment, such as the removal of a positive stimulus may be associated with plasticity that depress synaptic efficacy onto dMSNs.

It is thus possible that differences in plasticity onto dMSNs and iMSNs can differentially contribute to reinforcement and punishment (Kravitz and Kreitzer, 2012). Based on the recent evidence of the rewarding effects of 5-HT (Cohen *et al.*, 2015; Cohen 2015;Cools *et al.*, 2011; Rogers, 2011; Tanaka *at el*., 2004; Tanaka *et al*., 2007), the possibility that 5-HT could exert a positive regulation of synaptic mechanisms of plasticity in the reward-related direct pathway is intriguing.

The emerging scenario is that striatal synaptic plasticity, by shaping the processing of cortical inputs through the basal ganglia, allows a proper control of motor and operant behavior. This can occur through the fine neuromodulation of glutamatergic input by integrated dopaminergic and serotonergic signals. Dysregulation of these processes may affect striatal function, and possibly leading to the behavioral impairments characterizing pathological states such as drug addiction and OCD.

Aims and Relevance

The experimental evidence described in the previous paragraphs indicates that, for a proper control of striatal circuit function, the coordinated and cell-type specific neuromodulation of striatal synaptic plasticity is crucial.

Focusing on monoaminergic modulation of glutamatergic inputs to the striatum, it is increasingly clear that dopaminergic and serotonergic interplay plays a central role in the acquisition and expression of reward-guided behaviors. Whilst key aspects of midbrain dopaminergic modulation of cortico-striatal synapses have been revealed, serotonergic effects on these circuits are not well understood. Serotonergic innervation originates from 5-HT neurons located in the dorsal raphe nucleus, and the striatum expresses a multitude of 5-HT receptors coupled with both Gs and Gi protein signaling cascades. This raises the possibility that expression of specific receptor subtypes selectively on dMSNs or iMSNs can provide a pathway-specific gating mechanism for activity-dependent plasticity.

On a system level, 5-HT signal has been classically associated with learning of negative events, acting as an opponent to dopamine regulation of rewarding processes. Recent evidence has challenged this view, suggesting that 5HT signaling can synergize with dopamine signaling to code reward. However, the molecular and synaptic correlates of the rewarding properties of 5-HT at striatal circuits remain to be established.

The global aim of this project is to investigate the role of 5HT in the neuromodulation of glutamatergic synaptic plasticity specifically in the lateral part of the dorsal striatum (DLS), which is involved in motor planning and habit formation. This will provide a better understanding of the integrated function of DA and 5HT at striatal circuits. At these circuits, direct and indirect pathway neurons are known to exert opposing control over motor output. Furthermore, it is increasingly clear that dMSNs also mediate reinforcement and reward, while iMSNs mediate punishment and aversion, and the future motor or hedonic responses may be possibly altered by changes in synaptic efficacy of the inputs onto dMSNs and iMSNs (Kravitz and Kreitzer, 2012).

In dMSNs, dopamine D1 and 5-HT4 receptor (5HT4R) subtypes are both functionally coupled to Gs proteins. In light of this, we will test the ***central hypothesis*** that 5HT4R-mediated signaling can act as an alternative/cooperating player to D1 receptor-mediated signaling to regulate activity-dependent synaptic plasticity specifically at dMSN synapses, which are known to be related to reward coding (Kravitz and Kreitzer, 2012).

Providing insights on the neuromodulatory role of 5-HT at striatal circuits, and how this is causally relevant for defined cognitive process implicated in action control, could help to better clarify how dysfunctional 5-HT-mediated regulation of striatal synaptic plasticity might contribute to some of the behavioral alterations that characterize neuropsychiatric disorders such as Obsessive Compulsive Disorder (OCD). This could facilitate the development of new pharmacological approaches to treat symptoms of OCD.

Materials and Methods

Animals

Male C57Bl6/J mice (postnatal day 28-35 for electrophysiology) were used in this study. Moreover, some DREADD pharmacology experiments were conducted on transgenic animals gently provided by M. Pasqualetti laboratory of University of Pisa and A. Gozzi of IIT of Rovereto. Double transgenic hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ mice expressing the inhibitory receptor hM4Di selectively in the serotonergic neurons originated crossing a Pet1(210)-Cre transgenic mouse line with a loxPlox2722-hM4Di-mCherry mouse line.

Moreover, some experiments were conducted on drd1a-tdTomato^{tg}/drd2-EGFP^{tg} double transgenic mice (Gong *et al.,* 2003; Shuen *et al.,* 2008). drd1a-tdTomato^{tg}/drd2-EGFP^{tg} mice were originally obtained by bacterial artificial chromosome (BAC) transgenesis. This technique involves the insertion of a transgene (the fluorescent protein EGFP or Tomato) upstream of the ATG start codon of the gene of interest (the D2 or D1 receptor respectively) on a selected BAC clone. The transfer of this BAC clone in an organism allows the insertion of the gene coding for the fluorescent protein downstream the promoter and regulatory elements for the expression of the specific dopaminergic receptor, thus differently and specifically labeling MSNs belonging to the striatopallidal or striatonigral pathway (Gong *et al.,* 2003; Shuen *et al.,* 2008). The two strains were then crossed to obtain a double-fluorescent mouse line.

Animals were housed in a temperature- and humidity-controlled room under a 12:12 h light/dark cycle with lights on at 07:00.

Substances

Ascorbic acid, Triton X, NaN_3, NaCl, KCl, NaH_2PO_4, $MgCl_2$, $CaCl_2$, $NaHCO_3$, D-glucose, Choline chloride, $KMeSO_4$, HEPES, $MgCl_2$, Na_2-ATP, Na_3-GTP, Clozapine-N-Oxide were purchased by Sigma Aldrich.

Gabazine (SR 95531 hydrobromide), RS39604, GR113808, AM251 were purchased by Tocris Bioscience.

A2A antibody was purchased by VinciBiochem. SP antibody was purchased by Merck-Millipore.

Fluo-4 and Fluo-5F were purchased by Life Technologies.

Alexa 568 was purchased by Invitrogen.

Cell sorting of striatal medium spiny neuronal populations

drd1a-tdTomato^{tg}/drd2-EGFP^{tg} double transgenic male mice (post-natal day 60-80) express the tdTomato and EGFP proteins under control of the drd1 (D1 receptor) and drd2 (D2 receptor) promoters respectively, which enable fluorescent-activated cell sorting (FACS) of the distinct striatal drd1- and drd2-positive MSN populations. Mice were sacrificed by decapitation after which brains were carefully removed from the skull and placed in an ice-cold brain matrix (Zivic Instruments, Pittsburgh, PA, USA). Coronal slices containing the DLS were obtained and subsequent dissection of the DLS was performed using a scalpel and an ice-cold petri dish. The DLS was subsequently put in 4 ml of dissociation solution (870 mM NaCl, 25 mM KCl, 10 mM NaH_2PO_4, 70 mM $MgCl_2$, 750 mM sucrose, 24 mM $NaHCO_3$, 10 mM glucose and 0,5 mM ascorbic acid). To enable dissociation of the cells 2 mg/ml Pronase (Sigma-Aldrich, Germany) was added and the solution containing the tissue was incubated at 37°C for 10 min, after which the cells were dissociated by serial trituration and sorted using the FACS Aria^{TM} II cell sorter (BD Biosciences, San Jose, CA, USA). Sorted cells (yield for tdTomato-positive cells: ~15.000, EGFP-positive cells: ~10.000) were collected in RNA*later*

RNA stabilization reagent (Qiagen, Germany), after which total mRNA was isolated using the RNAeasy® Plus Micro Kit (Qiagen), according to manufacturer's instructions.

cDNA synthesis and PCR analyses

Complementary DNA was synthesized on a Peltier Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the GoTaq® 2-step RT-qPCR kit (Promega, Fitchburg, WI, USA), according to manufacturer's instructions (cycle conditions: 25°C for 5 min, 42°C for 1 h, 70°C for 10 min). Subsequent qPCR analysis was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) with 40 cycles of 45 s at 60°C and 15 s at 95°C, followed by a melting curve from 60-95°C. The same cDNA was used for end-point PCR analysis (40 cycles of 15 s at 95°C, 15 s at 60°C and 72°C for 30 s) using GoTaq® Master Mix (Promega). The samples were loaded on a 1.5% agarose gel for electrophoresis and subsequent visualization. The following primers were used for drd1 (NM_010076), drd2 (NM_010077.2) and 5-HT4R (NM_008313.4): DRD1_FW: AGATGACTCCGAAGGCAGCCTT, DRD1_RV: GCCATGTAGGTTTTGCCTTGTGC; DRD2_FW: CCTGTCCTTCACCATCTCTTGC, DRD2_RV: TAGACCAGCAGGGTGACGATGA; 5-HT4R_FW: AGCTGGTCCAAGACATCTGG, 5-HT4R_RV: CTCCCAACATTAATGCGATGC. The primers were designed to span an exon-exon boundary in order to prevent unwanted amplification of genomic DNA.

Electrophysiology

Slice preparation

To prepare *ex-vivo* brain slices, mice were anesthetized with isofluorane, perfused with artificial cerebrospinal fluid (aCSF) containing 115 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 25 mM D-glucose and aerated with 95% O_2 and 5% CO_2. The animals were then decapitated, and their brains were transferred to ice-cold dissecting artificial cerebrospinal fluid (aCSF) containing 110 mM choline chloride, 2.5

mM KCl, 1.25 mM NaH_2PO_4, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 25 mM D-glucose, 11.6 mM ascorbic acid, saturated with 95% O_2 and 5% CO_2. Coronal sections (250 µm thick) or horizontal slices (270 µm thick) were cut using a Vibratome 1000S slicer (Leica), then transferred to aCSF containing 115 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 25 mM D-glucose and aerated with 95% O_2 and 5% CO_2. Following 20 min of incubation at 32°C, slices were kept at 22–24 °C. During experiments, slices were continuously superfused with aCSF at a rate of 2 ml/min at 28°C.

Whole cell patch clamp recordings

When a dissection of the neuronal network and of the electrical properties of single neurons is required, the most widely used technique is whole cell patch clamp. By establishing a physical and electrical connection between the recording electrode and the cell membrane, it is possible to measure the voltage and current variations in the cell upon different stimuli.

Patch clamp recordings were made on neurons of the DLS in horizontal corticostriatal slices or in neurons of the DRN in coronal slices. This slice configuration was chosen on the basis of the work of Fino and colleagues, who showed that somatosensory cortical afferents to the dorsal striatum are best preserved with horizontal slices (Fino *et al.,* 2005). Intrastriatal electrical stimulation was performed, observing striatal neuronal response to this local input. Whole-cell recordings were made under direct IR-DIC (infrared differential interference contrast) visualization of neurons in the DLS, which were identified as medium spiny neurons (MSNs) on the basis of morphological and electrical properties (Nazzaro *et al.,* 2012).

In patch–clamp experiments, the recording electrode (a borosilicate patch pipette, 4-6 MΩ) is in physical continuity with the cytoplasm of the cell. This requires the solution filling the electrode to mimic the cytoplasmatic ionic composition. The intracellular solution contained (in mM): 130 $KMeSO_4$, 5 KCl, 5 NaCl 10 HEPES, 0.1 EGTA, 2 $MgCl_2$, 0.05 $CaCl_2$, 2 Na_2-ATP, 0.4 Na_3-GTP (pH 7.2-7.3, 280-290 mOsm/kg).

Excitatory postsynaptic potentials (EPSPs) were evoked in the presence of the $GABA_A$ receptor antagonist gabazine (10 μM) by paired intrastriatal stimuli (130 ms interpulse interval) delivered with a bipolar electrode (FHC). STDP-LTD was induced by 20 bouts of EPSPs paired with action potentials, delivered 10 s apart. Each bout consisted of five bursts (120 ms apart) each composed of three action potentials at 50 Hz followed by one EPSP (negative timing). HFS-LTD was induced by 4 × 1-s-long 100-Hz trains repeated every 10 s. During train stimulation, postsynaptic cell was depolarized from -80 to -50 mv, to mimic cortically-induced up-states. Data were excluded when the input resistance (*R*inp) changed >20%.

Action potentials (APs) were generated by a series of 400-ms current pulses (25 or 50 pA between each current pulse).

Data are reported without corrections for liquid junction potentials.

Data were acquired using a Multiclamp 700B amplifier controlled by pClamp 10 software (Molecular Device), filtered at 10 kHz and sampled at 20 kHz (current-clamp) with a Digidata 1322 (Molecular Device).

To distinguish between striatopallidal and striatonigral neurons, MSNs were filled with Neurobiotin (0.3 mg/ml) (DBA Italia) during recordings, and subsequently processed for immunostaining against A2A receptor (for iMSNs) and substance P (for dMSNs).

Data Analysis

The occurrence and magnitude of synaptic plasticity was evaluated by comparing EPSP normalized amplitudes from the last 5 min of baseline recordings with the values between 20–30 min after conditioning.

The plasticity loci (pre- vs postsynaptic) were deduced from the change in the paired-pulse ratio (PPR) after the delivery of the stimulation protocol.

2-photon Calcium Imaging

Ca^{2+} transients were imaged with 100 µM Fluo-4 (for STDP experiments) or 100 µM Fluo-5F (for HFS experiments) dissolved in the intracellular recording solution. Alexa 568 (10 µM) was used for visualization of cell bodies and dendrites. Neurons were filled with Alexa 568 and Fluo-4 or Fluo-5F via the patch electrode for 20 min before imaging to allow dye equilibration in the proximal dendrites (Plotkin *et al*., 2013). Whole cell maximum-projection images of the soma and dendrites were acquired with 0.4 μm^2 pixels with 15 µs pixel dwell time; ~120 images were taken with 0.4 µm focal steps. Drugs were bath applied for 10 min.

Two-photon Ca^{2+} imaging was performed with a Leica SP5 AOBS upright DM6000 CFS microscope coupled with a 2P laser Chameleon Ultra Coherent and equipped with a Leica 25x NA 0.95 water-immersion objective. For all experiments, the laser wavelength was 800 nm. The fluorescence emission was collected with an external non-descanned photomultiplier detector equipped with a 525/50 emission filter.

Green fluorescence line-scan signals were acquired at 6 ms and 256 pixels per line, with 0.1 µm pixels and 20 µs pixel dwell time. The laser-scanned images were acquired with 800 nm light pulsed at 90 MHz (pulse duration: ~250 fs). The line scan was started 750 ms before the stimulation protocol (1-s-long 100 Hz train coupled with cell depolarization from -80 to -50 mV) and continued for 4 s after the stimulation, in order to obtain the background fluorescence and to record the decay of the optical signal after stimulation.

Images were collected with LAS AF Leica software and analyzed using ImageJ software (version 1.45, http://rsb.info.nih.gov/ij/) and Origin 9.1 (MicroCal software).

For each recording, background fluorescence was determined from a cell-free area of comparable size to that of the line-scan image. After subtracting the averaged background signal, fluorescence values were recorded for 200 ms before the triggering of APs and averaged to give the basal fluorescence (F_{basal}). The amplitude of the fluorescence transients at the recording sites was expressed as the fractional change in basal fluorescence, $(F-F_{basal})/F_{basal}(F/F)$, which is approximately proportional to the changes in intracellular Ca^{2+}.

Over the course of the experiment, F_{basal} remained within the standard error of F_{basal} measured under control conditions. For data analysis, transients were digitally filtered off-line (adjacent-averaging routine, smoothing factor n = 5; Origin 9.1).

A2A and SP immunofluorescence

To check the MSN subtype (dMSN or iMSN) of the cells recorded in the DLS of C57Bl6/J mice, MSNs were filled with Neurobiotin (0.3 mg/ml) (DBA Italia) during recording, and processed post-hoc by immunostaining of markers of the striatonigral and striatoplallidal pathways, respectively the neuropeptide substance P (SP) and the A2A adenosine receptor (A2A).

After the end of the experiment, the slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer 7.4 pH (PB) overnight at 4°C, and then stored in PB with 0.02% of the preservant sodium azide (PB-N3). The slices were subsequently rinsed 3x15 min in PB, and incubated 30 min in 50 mM sodium citrate pH 8.5 at 80°C for antigen retrieval. After rinsing 3x15 min, the slices were incubated in a primary antibody cocktail (rabbit polyclonal antibody to A2A (1:250, Alexis Biochemicals) and rat monoclonal antibody to substance P (1:200, Millipore)) in PB containing 0.3% Triton X-100 and 0.02% NaN_3 (PB-TX-N_3) 24 h at RT, and 48 h at 4°C. The brain sections were subsequently rinsed 3x15 min in PB, incubated 4 h at RT in alexa 568-streptavidin (1:5000 in PB-TX-N_3, Invitrogen) to label neurobiotin-filled cells, rinsed again, and then incubated with Alexa 647– and Alexa 488–conjugated secondary antibodies (1:200 in PB-TX-N_3, Invitrogen) for 48 h at 4°C. After rinsing the slices 3x15 min, the nuclei were stained with Hoechst solution (1:400 in PB), rinsed again, mounted on glass slides, and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Images (1024x1024 pixels, pixel size: 240.5 nm voxel width, dwell time 2.4 µs) were acquired at 100X magnification (HCX PL APO lambda blue 63.0x1.40 OIL UV) with a Leica TCS SP5 AOBS TANDEM laser scanning confocal microscope, and the expression of the markers was

evaluated to identify the cells as a dMSN or a iMSN. Only cells confirmed as dMSNs were considered for analysis.

Statistics

Electrophysiological data were analyzed by One-way ANOVA for repeated measure for planned comparison within a group (RM1W), and by two-way repeated measure or two-way ANOVA for planned comparison among groups (RM2W)(GraphPad Prism 5 software). *Post-hoc* analyses (Tukey and Bonferroni's multiple comparison tests) were performed only when ANOVA yielded a significant main effect. Two groups were tested for statistical significance using an independent two-population *t* test (GraphPad Prism 5 software). Calcium imaging data were analyzed by *t* test (GraphPad Prism 5 software).

Results

MSNs of the DLS integrate information arising from glutamatergic inputs with the neuromodulatory signals provided by dopamine and 5-HT. These neuromodulators regulate synaptic mechanisms of plasticity by activating both stimulatory (Gs) and inhibitory (Gi) protein coupled receptors.

In MSNs of the direct pathway, dMSNs, dopamine D1 and 5-HT4 receptor subtypes (D1R and 5-HT4R) are both functionally coupled to Gs proteins. In light of this, we tested the central hypothesis that 5HT4R-mediated signaling can act as an alternative/cooperating player to D1R-mediated signaling to regulate synaptic plasticity specifically at dMSN synapses. To this, we assessed the impact of both chemo-genetic manipulation of 5-HT release in the DLS and pharmacological manipulation of 5-HT4R on specific forms of synaptic plasticity. We then evaluated the effect of these manipulations on intracellular Ca^{2+} dynamics.

Results provided here aim at clarifying the role of 5HT in the neuromodulation of glutamatergic synaptic plasticity in the DLS.

Learning, action planning and adaptation are thought to occur by mechanisms of synaptic plasticity. Thus, understanding the synaptic role of 5-HT in the DLS could be an important step towards the understanding of the cellular and molecular determinants of DLS dependent behaviors.

Chemo-genetic modulation of 5-HT release impacts synaptic plasticity at dMSNs connections

Validation of an animal model for the selective expression of DREADD receptor hM4Di in serotonergic neurons

Recently, the Roth's laboratory developed a tool for the selective inhibition or activation of specific neuronal populations. It is referred to as chemo-genetics or "DREADD pharmacology", where DREADD stands for designer receptors exclusively activated by designer drugs (Armbruster *et al*., 2007; Alexander *et al*., 2009). It is based on the directed molecular evolution of the muscarinic receptors, to generate a family of G-protein coupled receptors that are activated exclusively by a pharmacologically inert drug-like and bioavailable compound cloxapine-N-oxide (CNO), but not by their native ligand ACh. Those modified receptors are powerful tools for selectively modulating signal-transduction pathways *in vitro* and *in vivo* in a noninvasive way (Armbruster *et al*., 2007). The evolution of receptors coupled to Gi/o (Armbruster *et al*., 2007) or to Gαq (Alexander *et al*., 2009) and Gs (Farrell *et al*., 2013) proteins allows the precise control of the three major G-protein coupled receptor signaling pathways in the cells expressing the DREADD receptors (Zhu and Roth, 2014)(**Fig. 6**). DREADD technology can be thus used to gain remote control over neuronal signaling, via DREADD expression in the cell-type of interest via either transgenic or viral technology, and subsequent CNO administration (Zhu and Roth, 2014).

Expression and activation of Gq or Gs DREADDs in neurons leads to their depolarization and increase in firing rate (Alexander *et al*., 2009; Farrell *et al*., 2013), while Gi DREADD receptors induce hyperpolarization and neuronal silencing (Armbruster *et al*., 2007).

The possibility to selectively express DREADD receptors in serotonergic neurons is thus an attractive strategy to dissect the role of 5-HT in neurotransmission and synaptic plasticity.

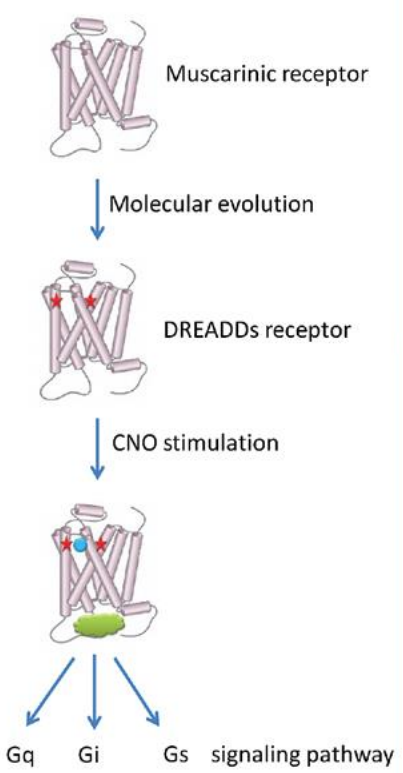


***Figure 6. DREADD chemo-genetic approach to modulate G-protein coupled receptor signaling* in vivo *and* in vitro.**

The molecular directed evolution of the muscarinic receptors creates DREADD receptors that can precisely control the Gq, Gi or Gs signaling pathways (From Zhu and Roth, 2014).

To this purpose, the laboratories of A. Gozzi (IIT of Rovereto) and M. Pasqualetti (University of Pisa), generated a transgenic mouse line in which the inhibitory DREADD receptor hM4Di was selectively expressed in Pet expressing neurons. The line was obtained by crossing a Pet1(210)-Cre transgenic mouse line (Pelosi *et al.,* 2014) with a loxPlox2722-hM4Di-mCherry line.

Pet1 mRNA expression promotes the expression of genes defining the mature serotonergic phenotype such as tryptophan hydroxylase 2 (Tph2) and serotonin transporter (SERT). Hence, genetic tools based on Pet1 specific expression represent a valuable approach to study the function of the serotonergic system. In the Pet1(210)-Cre transgenic mouse line the Cre recombinase is expressed under the control of a 210 kb fragment from the Pet1 genetic locus to ensure a reliable and faithful control of somatic recombination in Pet1 cell lineage.

Thus, Pet1(210)-Cre transgenic mouse line faithfully drives Cre-mediated recombination in all Pet1 expression domains representing a valuable tool to genetically manipulate serotonergic Pet1 cell lineages (Pelosi *et al*., 2014).

The loxPlox2722-hM4Di-mCherry line has been generated through h4MDi knock-in. The hM4Di receptor is fused in frame with the vital reporter mCherry in the ROSA-26 gene locus. To obtain high level of expression, the CMV enhancer fused to β-actin chicken promoter has been added. The cDNA of the hM4Di receptor is antiparallel to the promoter, and it is flanked by two loxP-lox2722 sites, to obtain the DREADD receptor transcription only after Cre mediated somatic recombination (Giorgi *et al*., unpublished data)(**Fig. S3**).

Hence, in the DREADD/Pet1-Cre transgenic mouse line ($hM4Di^{+/-}$/Pet1(210)-$Cre^{+/-}$), the hM4D1 receptor, which is coupled to a Gi protein, will be expressed only in serotonergic neurons expressing the Cre recombinase.

To validate this mouse line, we tested the ability of CNO to silence mCherry-positive serotonergic neurons expressing the hM4Di receptor in the dorsal raphe nucleus. As a control, we analyzed the effect of CNO application on the firing activity of serotonergic neurons of dorsal raphe nucleus from littermate $hM4Di^{+/-}$/Pet1(210)-$Cre^{-/-}$ mice that did not express the Cre recombinase (**Fig. 7**).

Serotonergic neurons of the dorsal raphe nucleus (**Fig. 7 A, left**) were identified on the basis of their morphological and electrical properties (De Kock *et al*., 2006; Kirby *et al*., 2003)(**Fig. 7 B**). For $hM4Di^{+/-}$/Pet1(210)-$Cre^{+/-}$ mice, mCherry positive cells were selected (**Fig. 7 A, right**). Bath application of CNO (5 µM) had no effect on the firing frequency of neurons from $hM4Di^{+/-}$/Pet1(210)-$Cre^{-/-}$ mice not expressing the hM4Di receptor (**Fig. 7 C, top**), while induced a progressive decrease of firing rate in hM4Di expressing neurons of $hM4Di^{+/-}$/Pet1(210)-$Cre^{+/-}$ mice (**Fig. 7 C, bottom**).

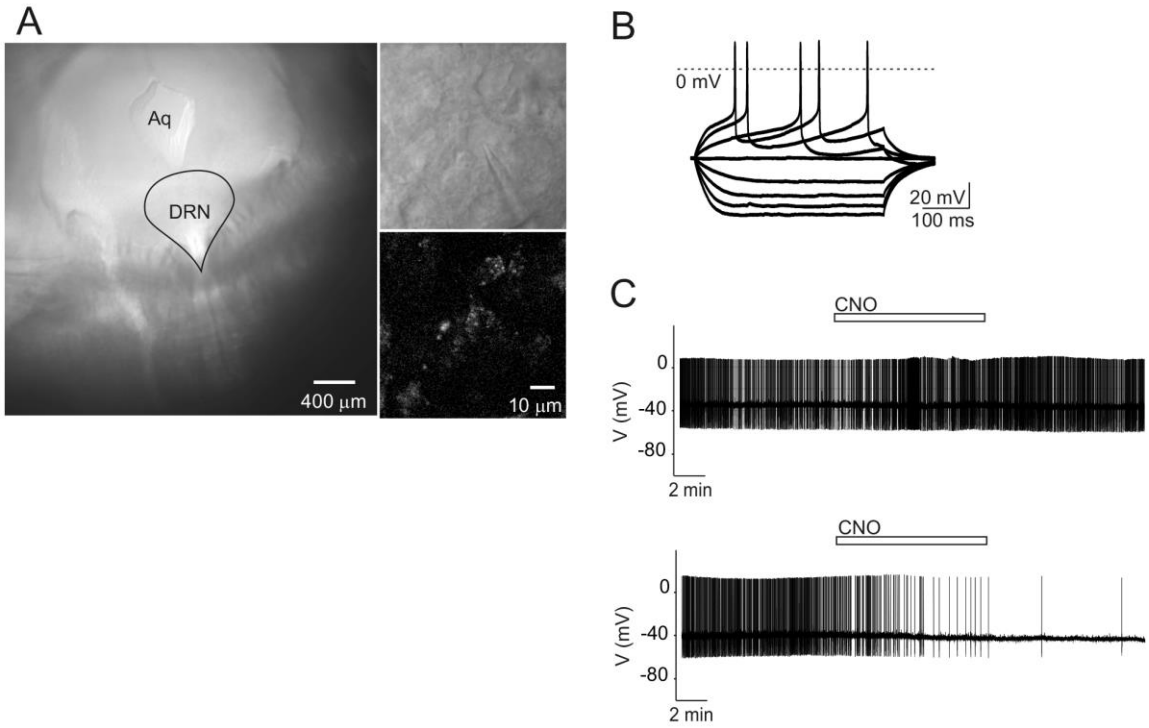


Figure 7. Validation of the animal model for DREADD (designer receptors exclusively activated by designer drugs) pharmacology.

The double transgenic mice expressing the inhibitory receptor hM4Di selectively in the serotonergic neurons were kindly provided by A. Gozzi and M. Pasqualetti. This mouse line has been created by crossing the Pet1(210)-Cre transgenic mouse line with the loxPlox2722-hM4Di-mCherry mouse line; only Pet expressing neurons express the Cre recombinase, necessary for the recombination of the sequence flanked by lox sites (hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$). Only neurons expressing the hM4Di receptor will be sensitive to the Clozapine-N-Oxide (CNO). The hM4Di receptor is linked to a Gi protein, and binding with CNO will lead to cell membrane hyperpolarization and decrease in firing activity. ***A****. Left, Microphotograph of a coronal brain slice (250 μm thickness) containing the Dorsal Raphe Nucleus (DRN), where the majority of serotonergic neurons are located. Neurons arising from DRN send their projections throughout the whole brain, including the dorsolateral striatum. (Aq=Aqueduct; DRN=Dorsal Raphe Nucleus). Right, hM4Di expressing serotonergic neurons of the DRN were identified on the basis of their morphology, fluorescence of the mCherry reporter, and on the basis of their firing properties (****B****). ****C****. Acute application of CNO (5 μM) did not affect the firing of serotonergic neurons of loxPlox2722-hM4Di-mCherry mice negative for the Pet1(210)-Cre expression (hM4Di$^{+/-}$/Pet1(210)-Cre$^{-/-}$)(Top). Conversely, the drug led to a strong inhibition of firing activity in hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ mice, positive for both Pet1(210)-Cre and hM4D1-mCherry gene products (Bottom). In the figure, representative traces of 8 (Top) and 5 (Bottom) experiments are reported.*

Chemo-genetic inhibition of 5-HT release affects synaptic plasticity at excitatory inputs to dMSNs

Once validated, we used the hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ mice as a tool to analyze the synaptic effects of inhibiting 5-HT release at dMSNs synapses of the DLS.

To this purpose, we recorded intrastriatally evoked excitatory post-synaptic potentials (EPSPs) in dMSNs, using horizontal brain slices. Recordings were performed in physiological K^+-based intracellular conditions (high K^+), allowing the activation of dendritic potassium currents in response to synaptic stimulation (**Fig. 8 A, top; Fig. 9 A, top**).

dMSNs were identified on the basis of their negative resting membrane potentials and firing activity (Kreitzer and Malenka, 2007), characterized by a low excitability in response to depolarizing current injection (**Fig. 8 B**). After the recording, the identity of the cell was confirmed by post-hoc immunostaining to analyze the expression of specific markers: dMSNs selectively express substance P (SP) but not A2A receptor, which is present on iMSNs. Only cells showing the correct pattern of expression markers were considered for further analysis (**Fig. 8 C**).

In horizontal brain slices, intrastriatal electrical stimulation not only triggers glutamate release from cortical and thalamic afferents, but also the local release of neuromodulators from dopaminergic and serotonergic terminals (**Fig. 8 A, bottom**)(Wu *et al*., 2015). To isolate excitatory input to dMSNs, recordings were performed in presence of the $GABA_A$ receptor antagonist gabazine (10 µM).

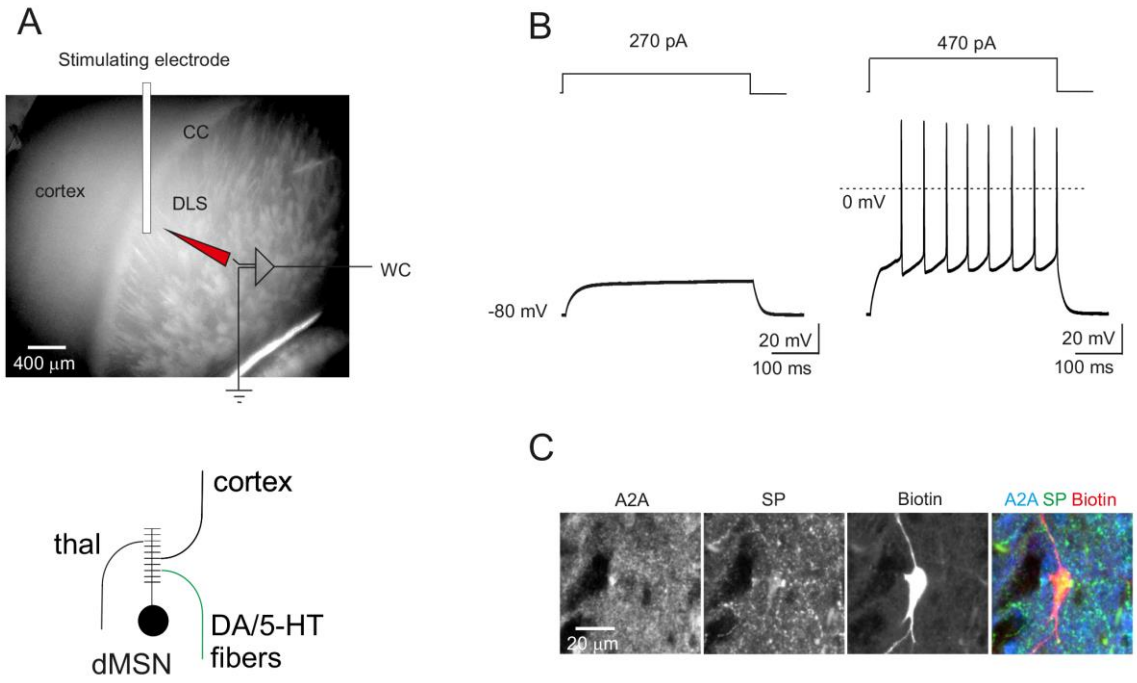


Figure 8. Electrophysiological and immunohistochemical profile of dMSNs in the DLS.

A. *Top, Infrared microphotograph of a horizontal corticostriatal slice displaying the dorsolateral striatum (DLS), the corpus callosum (CC) and the somatosensory cortex. The stimulating electrode was placed within the striatum. Whole-cell patch-clamp recordings (WC) are performed in the DLS. The stimulation site allows the release of glutamate from cortical and thalamic afferences, as well as the local release of neuromodulators such as dopamine (DA) and serotonin (5-HT) from dopaminergic and serotonergic terminals (Bottom).* ***B.*** *Representative current clamp traces recorded from striatonigral dMSNs.* ***C.*** *Confocal laser scanning microscopy images of triple immunofluorescence for A2A, SP (substance P) and biotin in patched-recorded neurons. (M. Trusel and M. Gritti). Only neurons expressing SP, selective marker for dMSNs and not expressing A2A, marker for iMSNs, were considered for further analysis.*

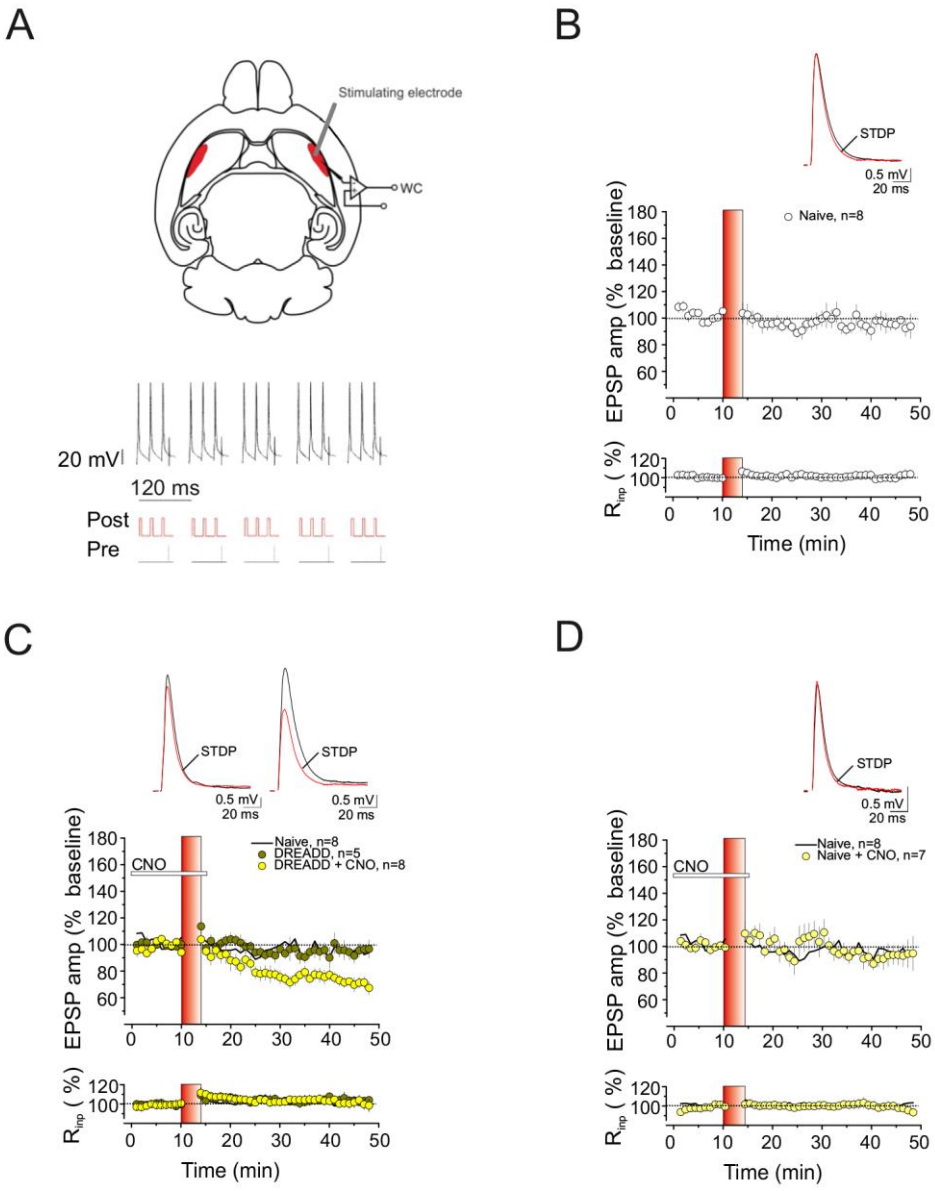
We investigated the role of 5-HT release in modulating the induction of synaptic plasticity at glutamatergic synaptic connections to dMSNs. We induced plasticity by pairing afferent stimulation with postsynaptic spikes, using a protocol which consisted of 20 bouts of EPSPs paired with action potentials, delivered 10 s apart, in cells depolarized from -80 mV to -70 mV. Each bout consisted of five bursts (120 ms apart) each composed of three action potentials at 50 Hz followed by one EPSP (negative timing) (**Fig. 9 A, bottom**). At most synapses, this protocol induces a Hebbian form of spike-timing dependent plasticity (STDP) that results in LTD (Pawlak and Kerr, 2008; Fino *et al*., 2010).

Work from Shen and colleagues, however, demonstrated that delivery of this protocol at dMSNs with an intrastriatal stimulation failed to induce LTD. The absence of LTD was shown to be dependent on the presence of active D1 dopamine receptor, which couples with a Gs signaling that is normally responsible for LTP induction (Shen *et al.*, 2008).

The increasing body of evidence pointing at rewarding action of 5-HT signaling, led to the hypothesis that, similarly to dopamine, also 5-HT could prevent the induction of STDP-LTD in dMSNs.

To investigate this hypothesis, we first confirmed that STDP-LTD was absent at dMSNs connections of naïve mice (97 ± 5% of baseline, n=8; p>0.05, **Fig. 9 B**). In dMSNs of hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ (DREADD) mice, delivery of STDP did not induce synaptic plasticity under control conditions (94 ± 5% of baseline, n=5; p>0.05, **Fig. 9 C**). In contrast, acute application of CNO (5 µM) during plasticity induction gated a form of STDP-LTD (74 ± 4% of baseline, n=8; p<0.05, **Fig. 9 C**) in this DREADD mouse line. Application of CNO in naïve animals failed to induce LTD (92 ± 6% of baseline, n=7; p>0.05, **Fig. 9 D**), thus excluding non-specific effects of CNO.

Collectively, these results indicate that the net effect of suppressing 5-HT release by activating hM4Di receptor at serotonergic terminals is the depression of the excitatory inputs to dMSNs.


A
Stimulating electrode
WC
20 mV
120 ms
Post
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B
STDP
0.5 mV
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Naive, n=8
EPSP amp (% baseline)
R_{inp} (%)
Time (min)
C
STDP
STDP
0.5 mV
20 ms
0.5 mV
20 ms
Naive, n=8
DREADD, n=5
DREADD + CNO, n=8
CNO
EPSP amp (% baseline)
R_{inp} (%)
Time (min)
D
STDP
0.5 mV
20 ms
Naive, n=8
Naive + CNO, n=7
CNO
EPSP amp (% baseline)
R_{inp} (%)
Time (min)

Figure 9. CNO induces LTD in dMSNs of hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ (DREADD) mice.

A. *Top, Schematic representation of a horizontal brain slice displaying the stimulation and recording configuration in the DLS. Bottom, Burst pairing protocol for the induction of STDP-LTD. The protocol consists of 20 bouts of EPSPs paired with action potentials, delivered 10 s apart. Each bout consists of five bursts (120 ms apart) each composed of three action potentials at 50 Hz followed by one EPSP (negative timing).* ***B.*** *In naive animals, STDP failed to induce LTD (RM1W, $F_{16,7}$=0.9, p=0.5682).* ***C.*** *In hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ (DREADD) mice, perfusion of CNO (5 µM) induced LTD in dMSNs, which was absent in control conditions (RM1W, $F_{16,7}$=18.5, p<0.0001; p<0.05 Tukey; RM2W, $F_{2,18}$=9.0, p<0.01; Naïve versus DREADD, p>0.05; Naïve versus DREADD + CNO, p<0.05, Bonferroni).* ***D.*** *In naive animals, CNO (5 µM) did not induce any LTD (RM1W, $F_{16,6}$=1.2, p=0.3; RM2W, $F_{1,13=}$0.3, p=0.6; Naïve versus Naïve + CNO, p>0.05, Bonferroni). Data are presented as time courses (mean ± s.e.m.) of normalized EPSP amplitudes and normalized Rinp. STDP was delivered at the red vertical bar The EPSP time courses were obtained in presence of 10 µM gabazine, and in whole cell current clamp configuration. The Rinp traces show that input resistance was stable throughout the recording. In* ***B.****,* ***C.*** *and* ***D.****, representative traces of the EPSPs recorded 5-10 min before (black) and 20-25 min after (red) the STDP, are showed for every experimental condition. In* ***C.*** *and* ***D.****, the black line shows the time course of plasticity in dMSNs of naive animals from Figure 9 B.*

Pharmacological manipulation of 5-HT4R recapitulates the effect of chemo-genetic inhibition of 5-HT release

5-HT4R antagonism gates STDP-LTD at dMSNs

Results obtained with DREADD pharmacology support the hypothesis that 5-HT release, similarly to dopamine, could shift the strength of dMSNs synapses towards potentiation; indeed, inhibition of 5-HT release enables a form of STDP-LTD in dMSNs.

In this MSNs subtype, the effect of dopamine on synaptic plasticity has been extensively studied: activation of Gs coupled D1 dopaminergic receptor strengthens corticostriatal dMSNs connections via NMDAR and ERK signaling activation, thus ultimately driving LTP (Cerovic *et al*., 2013).

On the other hand, the synaptic role of 5-HT at striatal synapses is still largely unexplored. The striatum expresses a multitude of 5-HT receptors subtypes that couple with both Gi and Gq/Gs signaling cascades at both presynaptic and postsynaptic sites. It has been previously reported that MSNs express two Gs coupled 5-HT receptors, the 5-HT6R (Eskenazi *et al.*, 2015) and 5-HT4R (Waeber *et al.*, 1993 and 1996; Patel *et al.*, 1995; Vilaro *et al.*, 2005).

Whilst the behavioral role of 5-HT6 in dMSNs and iMSNs has been recently investigated (Eskenazi *et al.*, 2015), little is known about the potential influence of 5-HT4R on striatal function.

We first confirmed the expression of 5-HT4R transcript in dMSNs using a combined approach of FACS-sorting and qPCR in drd1a-tdTomato^{tg}/drd2-EGFP^{tg} double transgenic mice (Lobo *et al*., 2006). After fluorescent-activated cell sorting from two DLS samples, we performed qPCR analysis to determine the expression level of drd1 mRNA in the tdTomato-positive population relative to EGFP-positive population (**Fig. 10 A**). The amount of drd1 mRNA in the EGFP-positive population was 0.86% of the expression level of drd1 mRNA in the TomatoRD-positive population, confirming that the separation of the distinct drd1- and drd2-positive populations was successful. Next, the cDNA from the tdTomato-population was

used to determine the presence of 5-HT4R transcripts in this population (**Fig. 10 B**). 5-HT4R was present in both samples, suggesting that 5-HT4R is expressed in the drd1-positive dMSNs of the DLS.

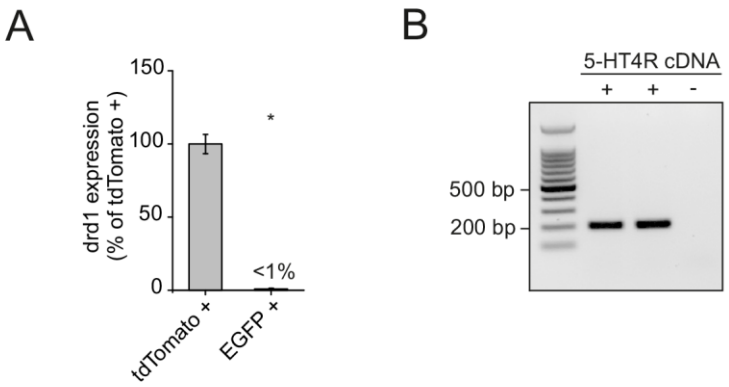

Figure 10. 5-HT4R transcript is present in the drd1-positive dMSN cell population of dr***d1a-tdTomato^{tg}/drd2-EGFP^{tg} double transgenic mice.***

A. *Bar diagram representing the relative expression levels of drd1 mRNA in the sorted tdTomato-positive and EGFP-positive cell populations taken from DLS of drd1a-tdTomato^{tg}/drd2-EGFP^{tg} double transgenic mice. drd1 mRNA expression was more than 100-fold higher in the tdTomato-positive population as compared to the EGFP-positive population (mean ± sd, n=2).* t *test, *p<0.005.* ***B.*** *PCR-products of 196 base pairs from 5-HT4R cDNA of the TomatoRD-positive population (+) and from the MiliQ negative control (-)(A. Boender and M. Gritti).*

We then tested the possibility that pharmacological inhibition of 5-HT4R in naïve animals, which would lead to a decreased Gs-coupled signaling in dMSNs, recapitulates the effect of chemo-genetic 5-HT inhibition of release in hM4Di$^{+/-}$/Pet1(210)-Cre$^{+/-}$ mice.

Indeed, in the presence of the two structurally unrelated 5-HT4R antagonists GR113808 (5 µM) or RS39604 (5 µM) STDP resulted in LTD of postsynaptic responses (GR113808: 80 ± 6% of baseline, n=8; p<0.05; RS39604: 68 ± 4% of baseline, n=10; p<0.0001, **Fig. 11 A)**.

These results suggest that the LTD observed upon chemo-genetic inhibition of 5-HT release during STDP is dependent on the lack of activation of 5-HT4R.

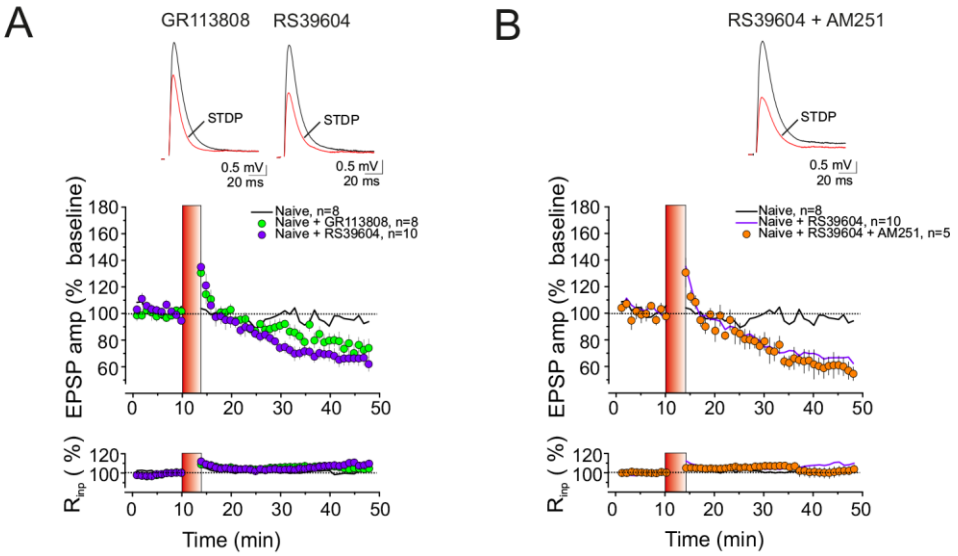

Figure 11. 5HT4R antagonists gate the STDP-LTD in naive animals.

A. *Perfusion of the two structurely unrelated 5HT4R antagonists, GR113808 (5 µM) or RS39604 (5 µM), led to STDP-LTD in naive animals, which was not present in control conditions (GR113808: RM1W, $F_{16,7}$=8.3, p<0.0001; p<0.05 Tukey; RS39604: RM1W, $F_{16,9}$=24.3, p<0.0001; p<0.001 Tukey; RM2W, $F_{2,23}$=9.9, p<0.0001; Naïve versus GR113808, p<0.05; Naïve versus RS39604, p<0.05, Bonferroni).* ***B.*** *The selective CB1 receptor antagonist AM251 (4 µM) failed to block STDP-LTD induced by 5HT4R antagonist RS39604 (RM1W, $F_{16,4}$=12.2, p<0.0001; p<0.05 Tukey; RM2W, $F_{2,20}$=13.4, p<0.0001; Naïve versus RS39604, p<0.05; Naïve versus RS39604 + AM251, p<0.05, Bonferroni). In* ***A.*** *and* ***B.****, the black line shows the time course of plasticity in dMSNs of naive animals from Figure 10 B.*

In dMSNs, dopamine has been shown to suppress the postsynaptic biosynthesis of eCBs and CB1R-mediated STDP-LTD by activating the Gs-coupled D1R (Shen *et al.*, 2008).

This raises the possibility that also 5-HT released during STDP hinders eCBs production via 5-HT4R activation. If this was true, the STDP-LTD occurring in the presence of 5-HT4R antagonists should be CB1R-mediated. Nonetheless, application of the CB1R antagonist AM251 (4 µM) did not abolish the STDP-LTD that occurred in the presence of RS39604 (5 µM)(62 ± 7% of baseline, n=5; p<0.05, **Fig. 11 B**).

To investigate further the mechanism of STDP-LTD induced by 5-HT4R antagonism, we performed a subset of experiments analyzing the synaptic responses generated by twin cortical stimuli delivered 50 ms apart. The rationale of delivering twin cortical stimuli is the evaluation of the locus of expression of the plasticity. If the paired-pulse ratio (PPR), which is

the ratio between the amplitudes of the second and the first peak responses, is increased after STDP, this phenomenon (paired-pulse facilitation, PPF) correlates with a decreased probability of release of neurotransmitter, suggesting a presynaptic locus of LTD expression; if the PPR does not change, plasticity should occur through a postsynaptic mechanism. We observed no change in PPR during the STDP-LTD gated by RS39604 (5 µM)(75 ± 5% of baseline, n=6; $p<0.05$), suggesting a postsynaptic locus of depression (PPR of baseline = 1.31 ± 0.09 versus PPR after STDP = 1.41 ± 0.10, n=6; $p>0.05$, **Fig. 12**). This is consistent with the evidence that this form of STDP-LTD is independent from the activation of presynaptic CB1R.

Together, these observations indicate that STDP-LTD occurring upon 5-HT4R inhibition is not mediated by CB1R and does not show a presynaptic locus of expression.

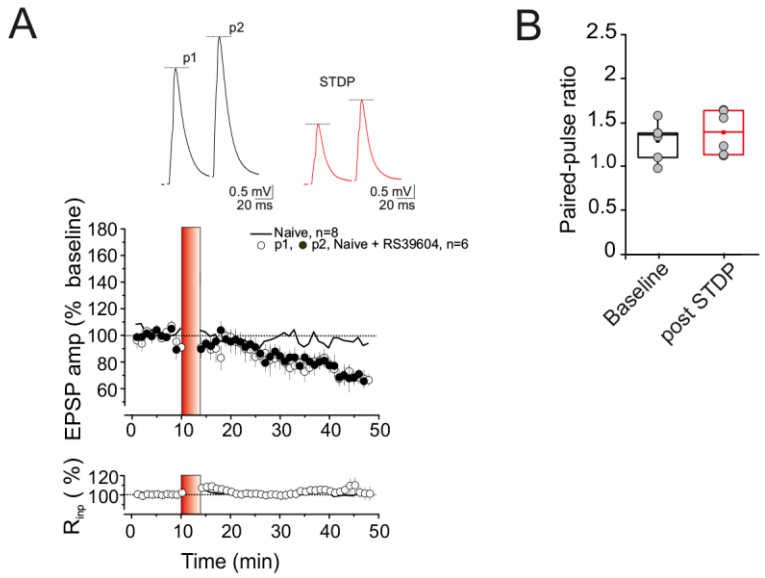

Figure 12. 5HT4R antagonist dependent STDP-LTD is expressed postsynaptically.

A. *Time course of the first peak (p1) and second peak (p2) amplitudes of synaptic responses generated by twin stimuli at a 50 ms interval. The 5-HT4R antagonist RS39604 (5 µM) induced STDP-LTD (RM1W, $F_{16,5}=8.8$, $p<0.0001$; $p<0.05$ Tukey) without changing the PPR. The black line shows the time course of plasticity in dMSNs of naive animals from Figure 10 B.*

B. *Box-chart diagram indicating the paired-pulse ratios of the last 5 min of baseline (black box) and 20-30 min after STDP (red box). PPR was expressed as the ratio between the amplitude of the second and the first EPSP.* t *test, $p>0.05$.*

5-HT4R antagonism influences frequency dependent plasticity at dMSNs connections

STDP is not the only stimulating protocol to induce synaptic plasticity in MSNs of the DLS. A well-established stimulating protocol relies on the high frequency stimulation (HFS) of excitatory afferents to the DLS. The induction protocol consists of 4 × 1-s-long 100-Hz trains repeated every 10 s (Trusel *et al*., 2015). During train stimulation, postsynaptic cell is depolarized from -80 to -50 mV, to mimic cortically-induced “up” states (**Fig. 13 A**).

Similarly to the STDP, we found that intrastriatal HFS failed to induce LTD in dMSNs (98 ± 5% of baseline, n=11; p>0.05, **Fig. 13 B**). This result is consistent with the lack of LTD at dMSN synapses observed in past and recent studies, which applied comparable experimental conditions (Kreitzer and Malenka, 2007; Wu *et al*., 2015). On the other hand, we have recently shown that HFS results in a form of LTD at cortico-dMSN synapses when stimulation occurs in deep cortical layers (Trusel *et al.*, 2015), further supporting the idea that intrastriatal stimulation triggers dopamine/5-HT release from monoaminergic terminals, and the activation of signaling cascades downstream D1R (Wu *et al*., 2015) and 5-HT4R counteracting LTD. Consistent with results gained upon STDP (**Fig. 11**), pharmacological inhibition of 5-HT4R during HFS enabled a form of HFS-LTD in dMSNs (GR113808: 65 ± 6% of baseline, n=6; p<0.001; RS39604: 55 ± 10% of baseline, n=6; p<0.001, **Fig. 13 C**), which was not abolished by the CB1R antagonist AM251 (4 µM)(64 ± 11% of baseline, n=5; p<0.05, **Fig. 13 D**).

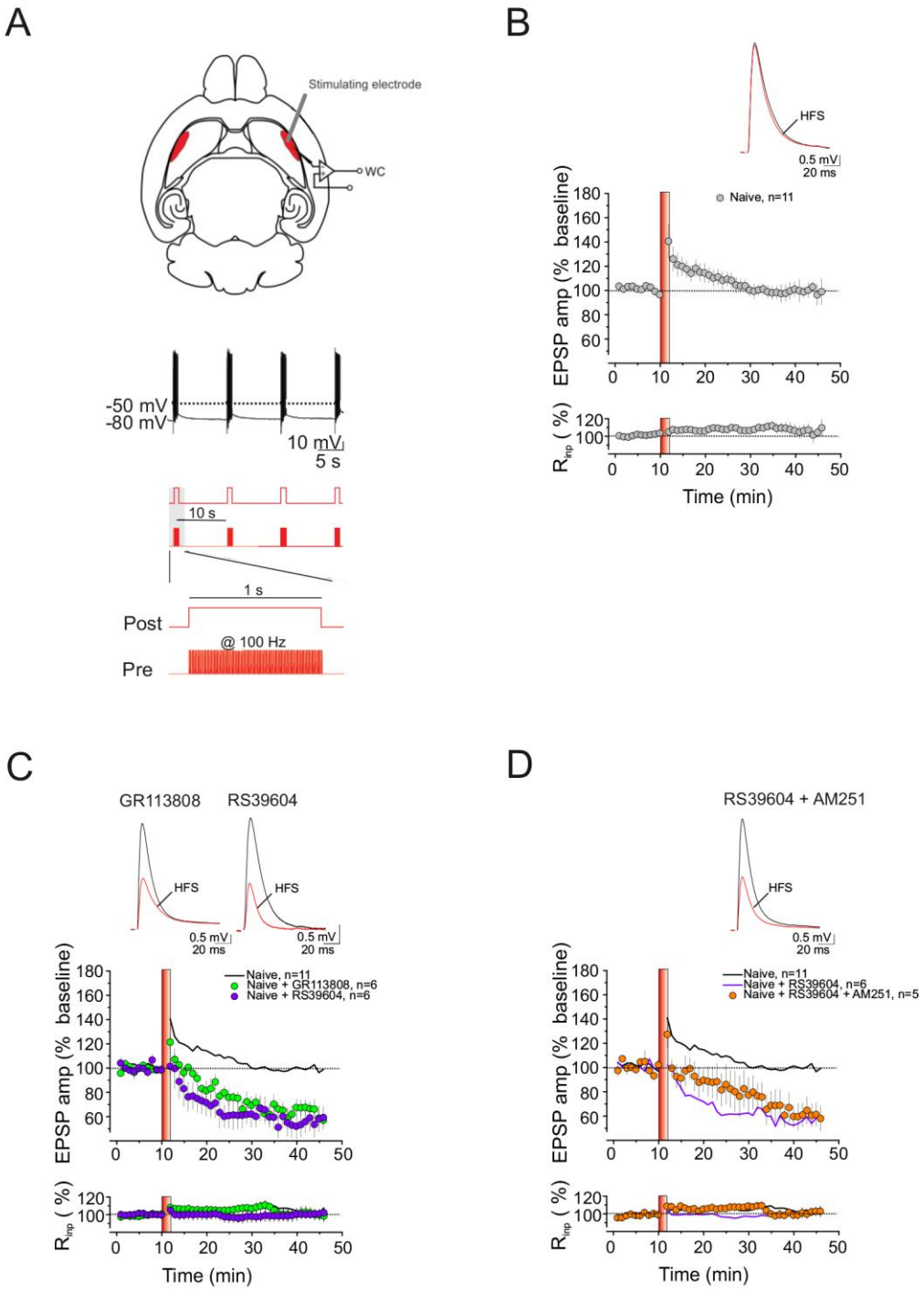
A
Stimulating electrode
WC
-50 mV
-80 mV
10 mV
5 s
10 s
1 s
Post
@ 100 Hz
Pre
B
HFS
0.5 mV
20 ms
Naive, n=11
EPSP amp (% baseline)
R_{inp} (%)
Time (min)
C
GR113808
RS39604
HFS
HFS
0.5 mV
20 ms
0.5 mV
20 ms
Naive, n=11
Naive + GR113808, n=6
Naive + RS39604, n=6
EPSP amp (% baseline)
R_{inp} (%)
Time (min)
D
RS39604 + AM251
HFS
0.5 mV
20 ms
Naive, n=11
Naive + RS39604, n=6
Naive + RS39604 + AM251, n=5
EPSP amp (% baseline)
R_{inp} (%)
Time (min)

Figure 13. 5HT4R antagonists gate the HFS-LTD in naive animals.

A. *Top, Schematic representation of the experimental configuration. Bottom, protocol for the induction of HFS-LTD. The protocol consists of 4 × 1-s-long 100-Hz trains repeated every 10 s. During train stimulation, postsynaptic cell was depolarized from -80 to -50 mV.* ***B.*** *In control conditions, HFS failed to induce LTD (RM1W, $F_{16,10}$=0.25, p=1).* ***C.*** *Perfusion of the two structurally unrelated 5HT4R antagonists, GR113808 (5 µM) or RS39604 (5 µM), led to HFS-LTD (GR113808: RM1W, $F_{16,5}$=13.3, p<0.0001; p<0.001 Tukey; RS39604: RM1W, $F_{16,5}$=14.4, p<0.0001; p<0.001 Tukey; RM2W, $F_{2,20}$=16.8, p<0.0001; Naïve versus GR113808, p<0.05; Naïve versus RS39604, p<0.001, Bonferroni).* ***D.*** *AM251 (4 µM) failed to block HFS-LTD induced by RS39604 (RM1W, $F_{16,4}$=7.3, p<0.0001; p<0.05 Tukey; RM2W, $F_{2,19}$=12.8, p<0.0001; Naïve versus RS39604, p<0.001; Naïve versus RS39604 + AM251, p<0.05, Bonferroni). In* ***C.*** *and* ***D.****, the black line shows the time course of plasticity in dMSNs from Figure 13 B; in* ***D.****, the violet line shows the time course of plasticity in presence of RS39604 from Figure 13 C.*

Taken together, the results obtained so far suggest that 5-HT release negatively modulate time-dependent and frequency-dependent postsynaptic mechanisms of plasticity at excitatory synapses to dMSNs of the DLS (See **Fig. S1** and **S2** for a summary of the results).

Serotonergic modulation of dendritic Ca^{2+} dynamics

Next, to elucidate the molecular mechanisms underlying LTD gated by 5-HT4R antagonism, we measured Ca^{2+} dynamics in the proximal dendrites of dMSNs. The activity dependent regulation of intracellular Ca^{2+} concentration, which depends both on NMDARs and back propagating action potentials is crucial for the induction of striatal synaptic plasticity (Adermark and Lovinger, 2007; Kreitzer and Malenka, 2008; Shen *et al*., 2008; Surmeier *et al.,* 2009; Lerner and Kreitzer, 2012; Plotkin *et al*., 2013). Hence, we investigated the effect of 5-HT4R antagonism on dendritic Ca^{2+} transients evoked by plasticity induction protocols.

Effect of 5-HTR antagonism on dendritic Ca^{2+} signaling triggered by spike-timing and high-frequency stimulation protocols

To study intracellular Ca^{2+} dynamics during STDP we combined patch-clamp techniques with 2-photon Ca^{2+} imaging. Briefly, via the patch electrode, dMSNs were filled with Alexa 568, to allow the visualization of the cell and dendrites, and with the specific high affinity Ca^{2+}-sensitive fluorescent dye Fluo4 (**Fig. 14 A**). Ca^{2+} transients were evoked by one bout of the STDP induction protocol, and were recorded in proximal dendrites (~30-60 µm from soma) through a line scan acquisition. The area of the fluorescence transients at the recording site is approximately proportional to the changes of intracellular Ca^{2+} (Yasuda *et al*., 2004)(**Fig. 14 B**).

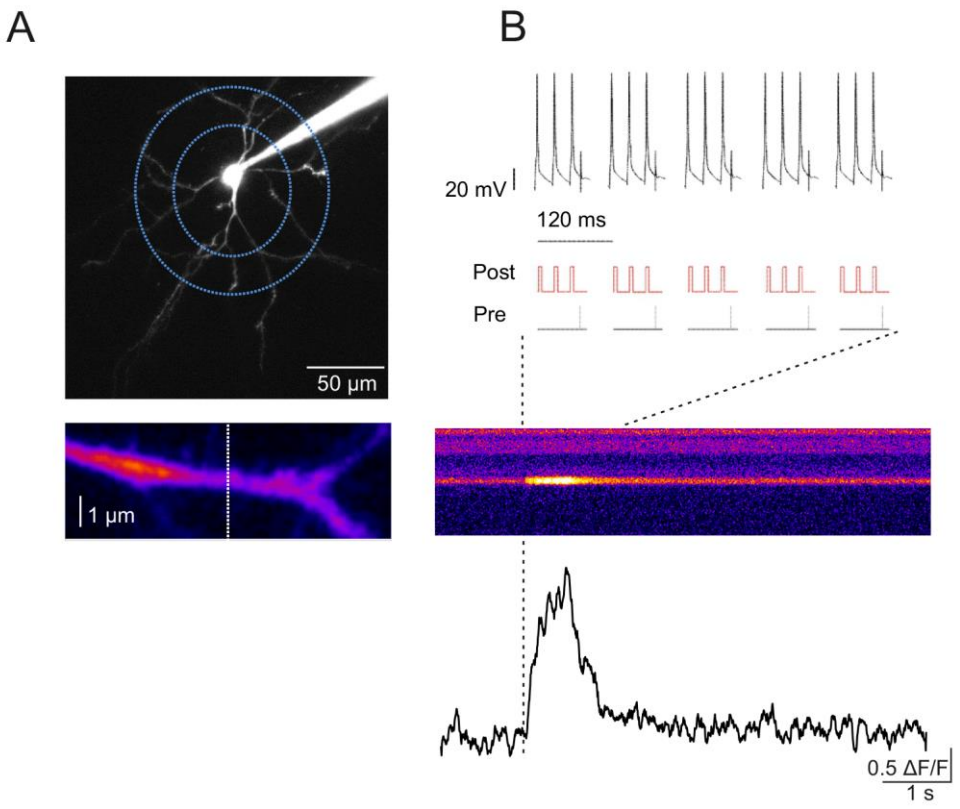

Figure 14. Ca^{2+} transients evoked by a single bout of STDP protocol in the proximal dendrites of dMSNs.

A. *Top, Maximum intensity projection image of a dMSN from the fluorescence of Alexa Fluor 568. Blue dotted circle lines delimit the region of proximal dendrites, 30-60 µm from the soma. Bottom, Example of a proximal dendrite from which the line scan is taken.* ***B.*** *One bout of the STDP conditioning protocol was used to evoke a dendritic Ca^{2+} transient, which is measured as increased fluorescence of the Fluo4 sensitive dye.*

In the absence of pharmacological manipulations, the Ca^{2+} transient evoked by a single bout of STDP was stable over time (107 ± 8% of control at time 0, n=8; p>0.05, **Fig. 15 A, B**). Acute application of either GR113808 (5 µM) or RS39604 (5 µM) caused a slight, albeit significant, increase in the area under the curve of the fluorescent transient, indicating that 5HT4R inhibition induced an increase in the evoked dendritic Ca^{2+} signal (GR113808: 122 ± 6% of control, n=15; p<0.05, **Fig. 16**; RS39604: 131 ± 7% of control, n=14; p<0.05, **Fig. 17**).

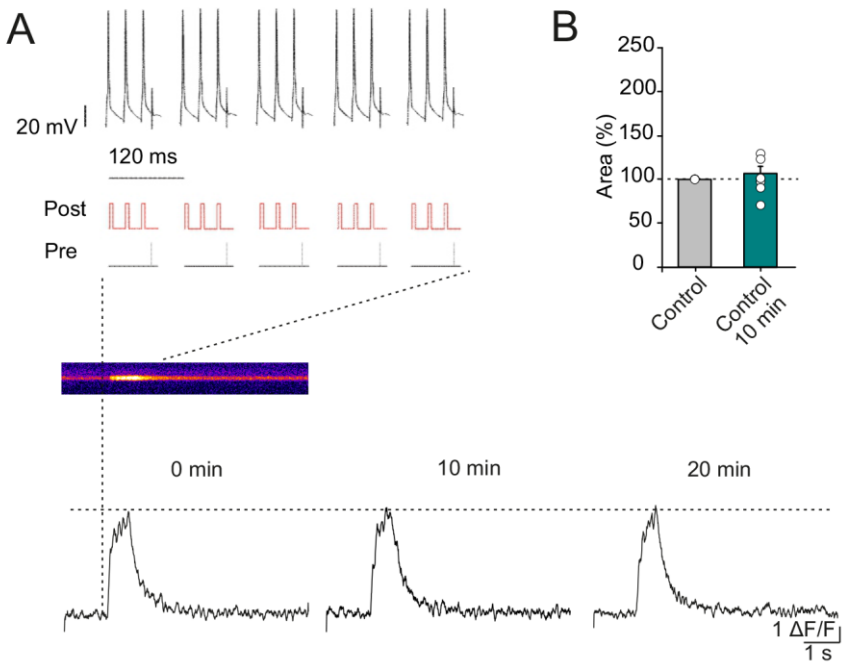

Figure 15. Ca^{2+} transients induced by intrastriatal STDP are stable over time.

A. *Top, schematic representation of one bout of STDP protocol used to evoke Ca^{2+} transients. Bottom, Ca^{2+} transients evoked by STDP were stable over time.* ***B.*** *Normalized area under the curve of the fluorescence transients measured at time 0 and after 10 min.* t *test, p>0.05.*

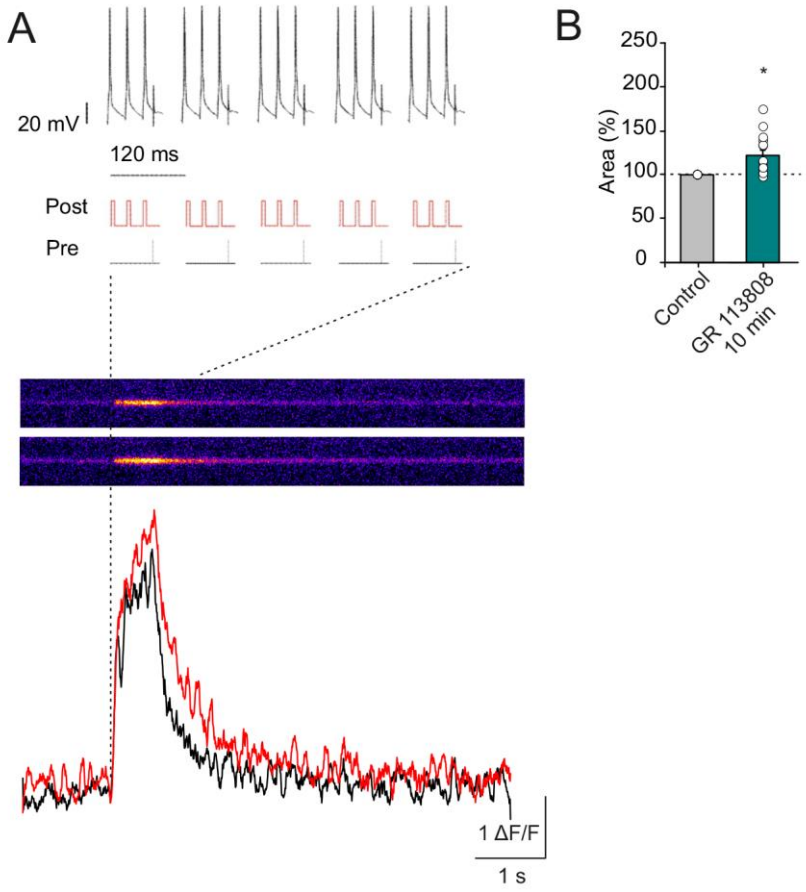

Figure 16. 5-HT4R antagonist GR113808 enhances Ca^{2+} transients induced by STDP in dMSNs.

A. *Ca^{2+} transients (top) and corresponding relative changes in fluorescence (bottom) evoked by a single bout of STDP protocol, before (black line) and after the application of the 5-HT4R antagonist GR113808 (5μM; red line).* ***B.*** *Normalized area under the curve of the fluorescence transients before and after GR113808 application.* t *test, *p<0.05.*

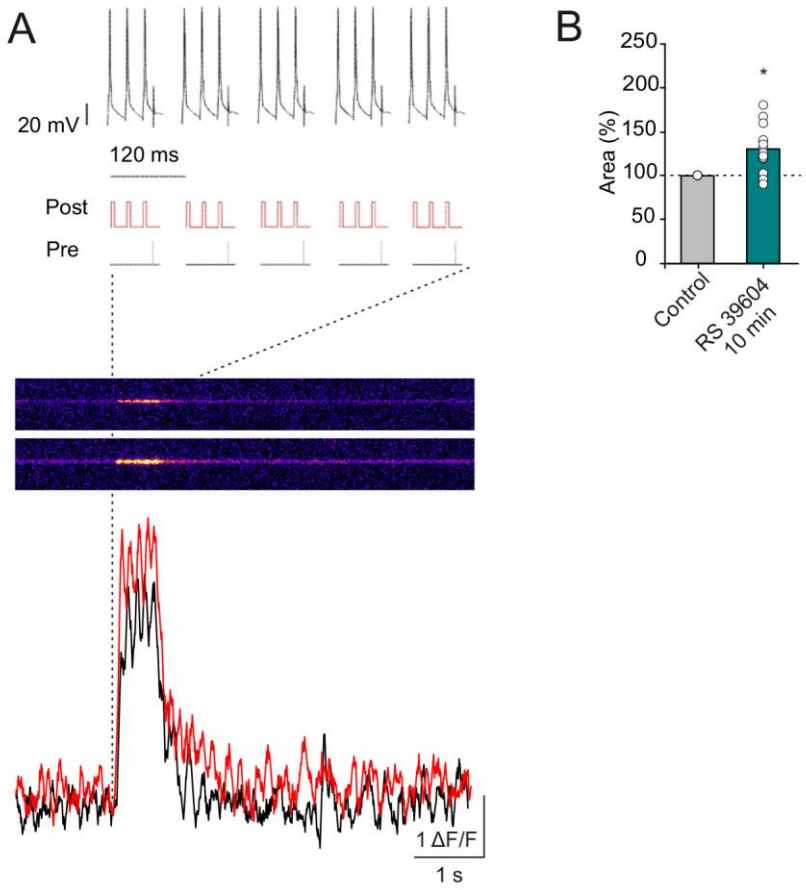

Figure 17. 5-HT4R antagonist RS39604 enhances Ca^{2+} transients induced by STDP in dMSNs.

A. *Ca^{2+} transients (top) and corresponding relative changes in fluorescence (bottom) evoked by a single bout of STDP protocol, before (black line) and after the application of the 5-HT4R antagonist RS39604 (5μM; red line).* ***B.*** *Normalized area under the curve of the fluorescence transients before and after RS39604 application.* t *test, *p<0.05.*

We obtained similar results when we analyzed the Ca^{2+} transients induced by a single HFS train. To avoid saturation of the Ca^{2+} fluorescence upon HFS, we used the low affinity Ca^{2+} indicator, Fluo5F, which provided stable HFS-induced Ca^{2+} transients over time

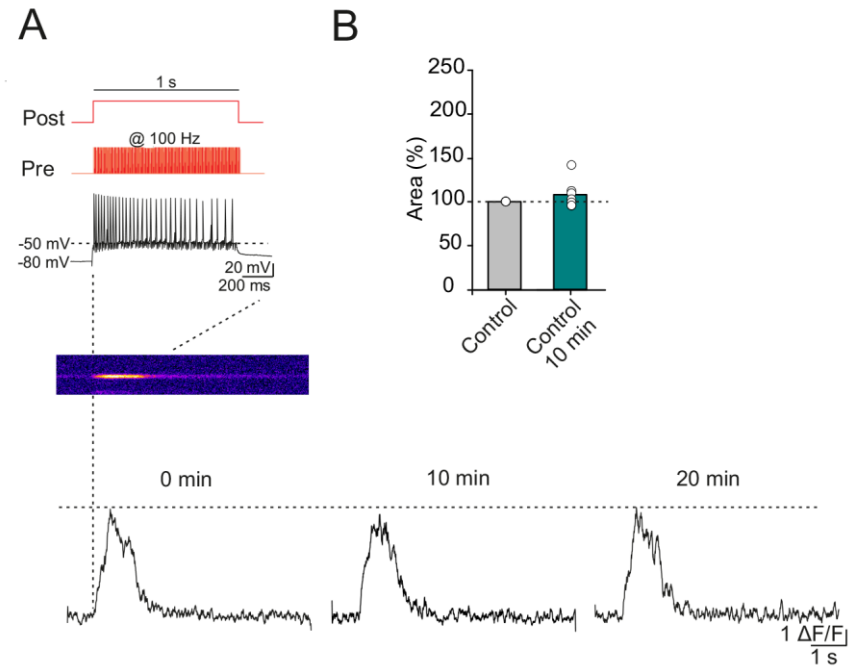

Figure 18. Ca^{2+} transients induced by intrastriatal HFS are stable over time.

A. *Top, postsynaptic response (black trace) to the HFS pairing (red trace) in dMSNs. Bottom, Ca^{2+} transients evoked by a single train of HFS were stable over time.* ***B.*** *Normalized area under the curve of the fluorescence transients quantified at time 0 and time 10 min.* t *test, p>0.05.*

(10 min: 108 ± 6% of control at time 0, n=7; p>0.05, **Fig. 18 A** and **B**). In the presence of the 5-HT4R antagonist GR113808 (5 µM)(**Fig. 19 A**), the area of the transient showed a significant increase compared to control (167 ± 11% of control, n=7; p<0.05, **Fig. 19 A** and **B**).

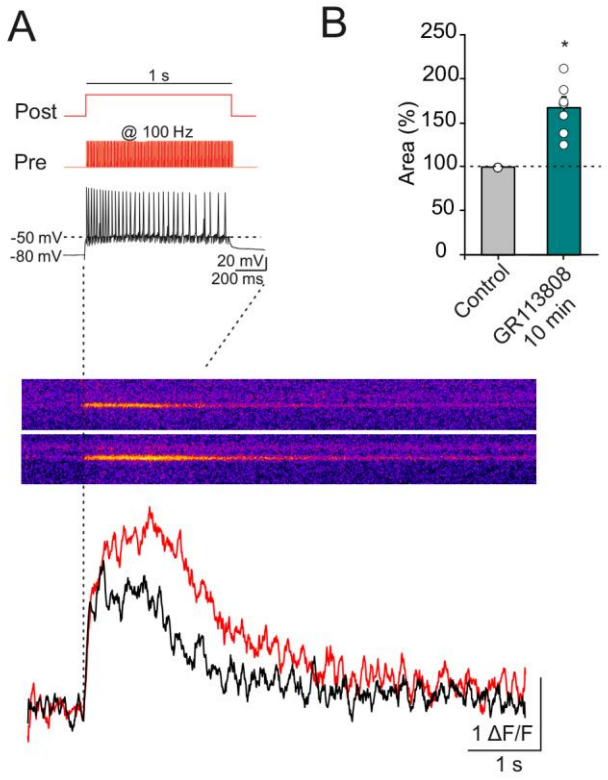

Figure 19. Antagonism of 5-HT4R enhances Ca^{2+} transients induced by HFS in dMSNs.
A. *Ca^{2+} transients (top) and corresponding relative changes in fluorescence (bottom) evoked by a single train of HFS before (black line) and after application of the 5-HT4R antagonist GR113808 (5 µM; red line).* ***B.*** *Normalized area under the curve of the fluorescence transients before and after GR113808 application.* t *test, *p<0.05.*

Collectively, these results indicate that 5-HT4R antagonism enhances the dendritic Ca^{2+} transients evoked by both STDP and HFS.

Discussion

The dorsal striatum plays the fundamental role of processing cortical information to shape motor and cognitive processes such as action selection, motor initiation, procedural learning and reward-guided behaviors. This is actuated by the coordinated activity of dMSNs and iMSNs (Cui *et al*., 2013; Trusel *et al*., 2015; Kravitz and Kreitzer, 2012). The direct pathway involved in selection of appropriate actions on the basis of previous experience, and mediating reinforcement and reward, and the indirect pathway aimed at suppression of all the other competing motor programs, and regulating punishment and aversion.

MSNs integrate the excitatory cortical and thalamic glutamatergic inputs with monoaminergic signals coding for the motivational state of the animal (Kreitzer and Malenka, 2008). Specifically, monoamine neuromodulators such as dopamine and 5-HT transduce salient environmental cues and internal states at the neuronal level. Binding their receptors, these monoamines modulate intracellular signaling cascades that control synaptic efficacy, leading to the remodeling of neuronal circuits on the basis of external environment and internal states.

Whilst dopamine is strictly associated with reward signaling and reward prediction error, the role of 5-HT has historically been related to punishment. Only recently, increasing evidence led to hypothesize 5-HT as a cooperating/synergizing neuromodulator to dopaminergic signal, making it possibly as important as dopamine in coding reward (Kranz *et al.*, 2010). Indeed, there is extensive data supporting an essential modulating role of the serotonergic system in major aspects of reward processing, as well as a change in serotonin transmission accompanied by rewarding activities. Collectively, this evidence points at 5-HT as a fundamental mediator of emotional, motivational and cognitive aspects of reward representation (Kranz *et al.*, 2010). At the level of the striatum, the link between potentiation of direct pathway synaptic connections with reinforcement and reward (Kravitz and Kreitzer, 2012) makes particularly attractive the investigation of the role of 5-HT modulation specifically at dMSNs connections.

During my PhD, I've been investigating the role of serotonergic neuromodulation of glutamatergic synaptic inputs specifically to the direct pathway of the dorsolateral striatum. To investigate the functional role of 5-HT at dMSNs synapses, we first analyzed the effect of the inhibition of 5-HT release on a spike-timing dependent form of plasticity.

Under our experimental conditions, a negative STDP protocol, in which repeated theta burst of postsynaptic MSN spiking precedes presynaptic stimulation, fails to induce LTD when applied intrastriatally. This is consistent with previous observations (Shen *et al*., 2008) that were ascribing the lack of STDP-LTD in dMSNs to the local release of dopamine upon intrastriatal stimulation. In this neuronal subtype, the activation of the Gs-coupled D1R promotes synaptic potentiation (Shen *et al*., 2008). In fact, D1R inhibition is sufficient to gate STDP-LTD (Shen *et al*., 2008).

By using a chemo-genetic approach (DREADD pharmacology) to selectively inhibit 5-HT release during STDP induction, we were able to gate a form of STDP-LTD, unveiling a role for 5-HT in modulating glutamatergic synaptic transmission at dMSNs synapses. These results suggest that 5-HT and dopamine could share common intracellular signaling cascades whose activation prevent the induction of STDP-LTD. Being coupled to Gs signaling, the 5-HT4R subtype is ideally placed at the somato-dendritic compartment of dMSNs (Waeber *et al.*, 1993; Waeber *et al.*, 1996; Patel *et al.*, 1995; Vilaro *et al.*, 2005) to counteract the induction of STDP-LTD, similarly to D1R. Thus, a reduced 5-HT4R activation upon chemo-genetic inhibition of 5-HT release could be responsible for the STDP-LTD observed in dMSNs of DREADD mice. This hypothesis is consistent with the results obtained in naïve mice, in which STDP was induced in the presence of two structurally unrelated 5-HT4R selective antagonists. Inhibition of 5-HT4R during plasticity induction gated a form of STDP-LTD, which was not involving the release of eCBs and the activation of CB1R.

We obtained similar results when plasticity was triggered by high-frequency stimulation (HFS) of cortical afferents to dMSNs. This suggests a negative role for 5-HT, through 5-HT4R activation, in regulating intracellular mechanisms relevant for both timing- and frequency-dependent forms of synaptic plasticity at excitatory dMSN synapses.

Both STDP-LTD and HFS-LTD obtained upon inhibition of 5-HT4R were associated with increased dendritic Ca^{2+} elevations.

5-HT4R activation stimulates the activity of PKA, which is known to positively modulate dendritic Ca^{2+} signal (Higley and Sabatini, 2012). Thus, the evidence that 5-HT4R inhibition enhances dendritic Ca^{2+} levels is counterintuitive. One possible explanation could be that 5-HT4R signaling affects Ca^{2+} levels through the negative regulation of a K^+ conductance. Indeed, it has been previously shown that inhibition of Ca^{2+}-activated K^+ channels boosts dendritic Ca^{2+} signaling in MSN of the DLS (Nazzaro *et al*., 2012; Trusel *et al*., 2015). In other brain regions, 5-HT4R modulates the activity of the slow Ca^{2+}-activated K+ current. Direct evidence that this modulation also occurs at striatal circuits is, however, still missing. 5-HT4R can bind not only to Gs proteins, but also to Gα13 (Ponimaskin *et al*., 2002), that signals to CaMKII. In the hippocampus, CaMKII activation promotes the expression of the A-type K^+ channel Kv4.2 (Varga *et al*., 2004). Furthermore, in medial vestibular nucleus neurons, CaMKII inhibition reduces the activity of the BK Ca^{2+}-activated K^+ currents (Nelson *et al*., 2005). In light of this, 5-HT4R inhibition could decrease either Kv4.2 cell surface expression or BK current, thus potentiating Ca^{2+} signal.

In the striatum, elevations in dendritic Ca^{2+} levels are required for the biosynthesis and release of eCBs from the postsynaptic MSN. eCBs, by acting at presynaptic CB1R, trigger STDP-LTD (Nazzaro *et al*., 2012; Wu *et al*., 2015; Shen *et al*., 2008). Whilst there is a general consensus that eCBs mediate STDP-LTD in iMSNs, this issue remains controversial for dMSNs (Nazzaro *et al*., 2012; Wu *et al*., 2015; Shen *et al*., 2008). Under our experimental settings, the activation of presynaptic CB1R is not required for either STDP-LTD or HFS-LTD gated by 5-HT4R antagonists. eCBs not only act at presynaptic CB1R, but they also activate the postsynaptic TRPV1R (Transient Receptor Potential Vanilloid 1 receptor). We can speculate that the pharmacological inhibition of 5-HT4R, or the lack of its activation upon chemo-genetic inhibition of 5-HT release, may enforce the Ca^{2+} dependent biosynthesis of eCBs in dMSNs. eCBs might then act as an autocrine signal by acting on postsynaptic TRPV1R. Postsynaptic TRPV1R activation leads to a form of postsynaptic LTD that depends

on AMPARs endocytosis (Kauer and Malenka, 2007; Malenka and Bear, 2004, Grueter *et al*., 2010). This raises the possibility that STDP-LTD and HFS-LTD obtained by the negative modulation of 5-HT4R might require the activation of TRPV1R in dMSN. We are currently testing this hypothesis.

Regardless the precise molecular mechanism driving 5-HT4R dependent STDP- (or HFS-) LTD in dMSNs, our results indicate that 5-HT, through 5-HT4R, exerts similar function of dopamine, through D1R, leading to a “pro-active” dMSNs state. Recently, it has been shown how dopamine promotes action reinforcement by acting on a critical time window after the sensorimotor signal. Optogenetic induction of dopamine release resulted in spine enlargement and plasticity enhancement only during a critical time (0.6 s) after STDP protocol. These effects, which require the activation of PKA in the dendritic spines, are independent of Ca^{2+} influx triggered by back-propagating action potentials (Yagishita *et al*., 2014).

We may speculate that dopamine and 5-HT could act through different subcellular mechanisms, one involving spine enlargement and the other Ca^{2+} signaling modulation, to encode reward stimuli at cellular level.

The synaptic effects of 5-HT at striatal circuits might not be exclusively mediated by the activation of 5-HT4R. In unidentified MSNs, it has been previously shown that the acute application of 5-HT on striatal brain slices results in LTD of excitatory inputs. This form of plasticity depends on the activation of the 5-HT1bR subtype (Mathur *et al*., 2011), it is mutually occlusive with presynaptic eCB-LTD, and does not require postsynaptic activity. Additionally, this pharmacological 5-HT1bR-mediated LTD has been measured under voltage-clamp configuration and by using a cesium-based intracellular solution containing the Na^{+} channel blocker QX-314: these experimental settings could mask the postsynaptic contribution of 5-HT4R regulation of synaptic plasticity, isolating only the presynaptic 5-HT1bR contribution. Nonetheless, the chemo-genetic experiments performed in this study suggest that the net effect of inhibiting 5-HT release during associative synaptic plasticity is the depression of cortical inputs to dMSNs.

Cortico-striatal synaptic plasticity could be also indirectly modulated by activity of striatal interneurons through 5-HT-mediated signaling. Bonsi and colleagues showed that rat striatal cholinergic interneurons could be excited by 5-HT by activating the 5-HT2c, 6 and 7 receptor subtypes. This leads to a PLC/adenylyl cyclase-dependent loss of an outward K^+ current, and to firing rate increase (Bonsi *et al*., 2007; Mathur and Lovinger, 2012). 5-HT2cR has been also reported to excite fast-spiking interneurons through the modulation of inwardly rectifying K^+ channels (Blomeley and Bracci, 2009).

The effect of 5-HT on fast-spiking interneurons would theoretically set a high-pass filter for MSN output, while the increase of cholinergic tone would possibly increase the threshold for eCB-LTD to occur at cortico-striatal synapses. Furthermore, It has been proposed that 5-HT1bR-mediated LTD of cortico-striatal glutamate release might act as a high-pass filter for cortical information. Taking these hypotheses together with the evidence of a positive correlation of DRN neuron firing with activity state (Jacobs and Fornal, 1999), 5-HT release in the dorsal striatum could adjust the signal-to-noise ratio of the striatal network to improve action selection performance: a low serotonergic tone would thus decrease this signal-to-noise ratio, resulting in either a paucity of selected actions or a global decrease in action selection threshold, depending on the particular state of the dorsal striatum (Mathur and Lovinger, 2012): in OCD, for instance, where cortico-striatal dysfunction is known to occur, there could be a decrease in action-selection threshold, leading to execution of compulsive or unwanted actions.

In this framework, 5-HT could act on multiple levels and on multiple receptors and cell-types, and the integration of different signaling pathways at different loci might lead to the overall regulatory effect of this neuromodulator. Data presented in this thesis could provide an additional mechanism for 5-HT, via postsynaptic 5-HT4R, to modulate the activity of direct pathway dMSNs, which are associated with motor activation, reinforcement and reward. These results, hence, could represent a further step towards the understanding of the role of 5-HT in the striatum, which is particularly intriguing given the diversity of 5-HT effects on various cognate receptors.

Conclusions and Perspectives

In conclusion, these data provide evidence for the involvement of 5-HT, through 5-HT4R, in time- and frequency-dependent forms of synaptic plasticity at glutamatergic connections to dMSNs, identifying Ca^{2+} signaling as a putative trigger for a form of postsynaptic form of LTD.

Thus, 5HT-dependent signaling cascades might provide an additional mean to fine tune glutamatergic input to the striatum, enabling proper action selection, and may represent a target for pharmacological and cognitive-behavioral approaches in a therapeutic perspective.

To further dissect the 5-HT role in the regulation of glutamatergic transmission at dMSNs, it will be important to analyze if 5-HT neuromodulation can differently or selectively affect cortico-striatal or thalamo-striatal connections.

Because of the pathophysiology of specific aspects of compulsive behaviors involves the DLS (Nazzaro *et al*., 2012), and serotonergic neuronal activity correlates with repetitive or sustained behavior typical of OCD (Kane *et al*., 2012), we also plan to investigate the behavioral impact of serotonergic modulation of striatal synaptic plasticity.

We will pharmacologically manipulate 5HT4R of the DLS *in vivo*, to test the effect of 5HT on instrumental conditioning of nose poking for food (Nazzaro *et al*., 2012). After a short-training paradigm, mice learn the task and behavior is defined as goal-directed as it is flexible and sensitive to change in the contingency between the action and the outcome. We will test the hypothesis that injection of 5HT4R antagonists in the DLS promotes a shift towards a repetitive and inflexible habitual behavior. To further validate the behavioral relevance of 5HT in shaping instrumental learning we will test the impact of *in-vivo* DREADD pharmacology and optogenetic approaches to inhibit 5HT terminals in the DLS during behavior.

In conclusion, providing molecular and synaptic insights on the neuromodulatory role of 5-HT at striatal circuits, and establishing how this is causally relevant for defined cognitive process implicated in action control, could facilitate the development of new pharmacological approaches to treat symptoms of OCD.

Supplementary Figures

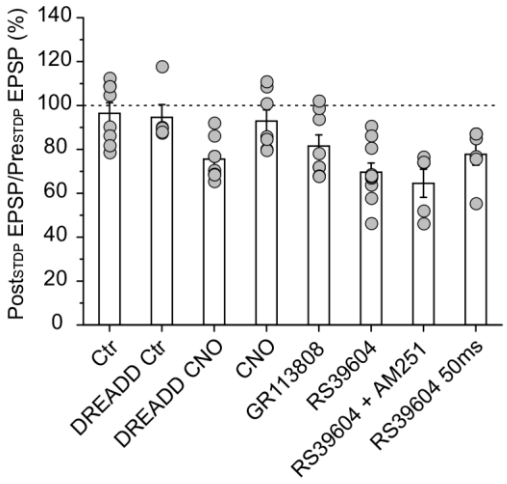

Figure S1. Summary of STDP-LTD experiments.

Bar diagrams summarizing the ratios of post-STDP and pre-STDP synaptic responses of all the experimental conditions described in the Results sections.

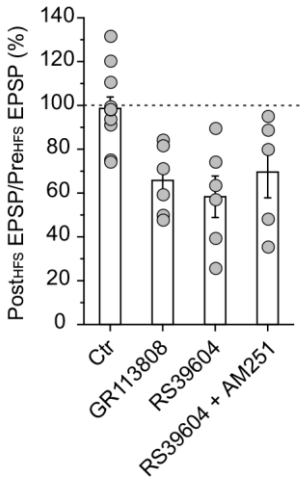

Figure S2. Summary of HFS-LTD experiments.

Bar diagrams summarizing the ratios of post-HFS and pre-HFS synaptic responses of all the experimental conditions described in the Results sections.

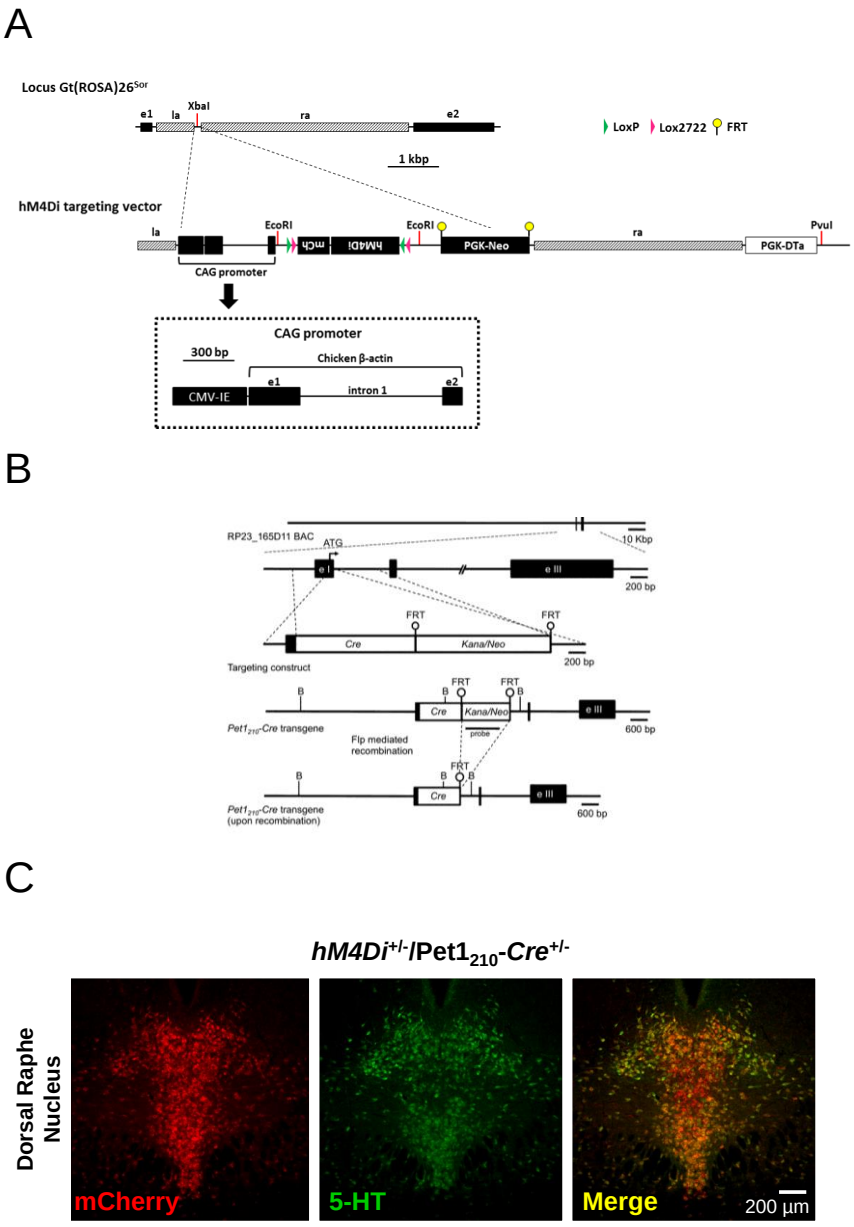

Figure S3. Generation of the hM4Di$^{+/-}$/Pet1$_{210}$-Cre$^{+/-}$ double transgenic mouse.

*The double transgenic mice expressing the inhibitory receptor hM4Di selectively in the serotonergic neurons originate from the match of a loxPlox2722-hM4Di-mCherryknock-in mouse line with a Pet1(210)-Cre transgenic mouse line; constructs of the hM4Di-mCherry transgene (**A**) and Pet1(210)-Cre transgene (**B**) are shown. **C**. Immunostaining for mCherry reporter (red) and serotonin (5-HT, green) in the dorsal raphe nucleus of double transgenic mice shows a colocalization of the two signals, confirming that those mice express the hM4Di-mCherry receptor selectively in serotonergic, Pet-expressing neurons. The figure was kindly provided from M. Pasqualetti, A. Gozzi and A. Giorgi.*

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Addendum: Publications

“Metformin repositioning as antitumoral agent: selective antiproliferative effects in human glioblastoma stem cells, via inhibition of CLIC1-mediated ion current”
Gritti M, Würth R, Angelini M, Barbieri F, Peretti M, Pizzi E, Pattarozzi A, Carra E, Sirito R, Daga A, Curmi PM, Mazzanti M, Florio T; Oncotarget 2014 Nov 30;5(22):11252-68.

“CLIC1 functional expression is required for cAMP-induced neurite elongation in post-natal mouse retinal ganglion cells”
Averaimo S, **Gritti M**, Barini E, Gasparini L, Mazzanti M; J. Neurochem 2014 Nov;131(4):444-56.

“Lamin B1 overexpression increases nuclear rigidity in autosomal dominant leukodystrophy fibroblasts”
Ferrera D, Canale C, Marotta R, Mazzaro N, **Gritti M**, Mazzanti M, Capellari S, Cortelli P, Gasparini L; FASEB J. 2014 Sep;28(9):3906-18.

“Coordinated regulation of synaptic plasticity at striatopallidal and striatonigral neurons orchestrates motor control”
Trusel M, Cavaccini A, **Gritti M**, Greco B, Saintot PP, Nazzaro C, Cerovic M, Morella I, Brambilla R, Tonini R; Cell Rep. Nov. 17, 2015 13, 1-13.