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**Dopamine D2 receptors: key regulators of
dopaminergic transmission**

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Abstract

Dopamine (DA) is an important neuromodulator of the central nervous system; an accurate control of DA levels and the resulting interaction with DA receptors is important for a correct brain functioning. Several human neurological disorders such as Parkinson's disease and psychiatry disorders are characterized by DArgic dysfunctions. DA D2 receptor (D2R) occupies a privileged position in the dopaminergic transmission. D2Rs are widely expressed in the brain; importantly, they are expressed presynaptically on dopaminergic neurons as well as postsynaptically on neurons targeted by dopaminergic neurons. To date it has been impossible to discern pre- from post-synaptic D2R-mediated functions. Genetically engineered mice, thus, represent important tools to address this question and identify D2R functions in vivo. Presynaptically, D2Rs (also named autoreceptors) regulate DA synthesis and release from dopaminergic neurons; thereby, their activation (i.e. by agonists/antagonists) affects mice motor behavior and can be used as readout of autoreceptor functions. Importantly, we have recently generated mutants lacking D2Rs either from dopaminergic neurons or from striatal medium spiny neurons (MSNs). D2 autoreceptor mutants present hyperactivity in a novel environment and an increased motor response to cocaine. Unexpectedly, we observed that in the absence of these receptors, the postsynaptic D2Rs (heteroreceptors) exert an inhibitory control on dopaminergic neurons thus affecting the release and the synthesis of DA, a function previously thought to be controlled only by D2 autoreceptors. Conversely, mice lacking D2Rs on the MSNs present a strong impairment of movements and an altered response to drugs such as cocaine, showing their importance in the regulation of the initiation of movement.

1. Background

DA is the most abundant catecholamine of the central nervous system, which is highly conserved among species (Cooper and Neckameyer 1999) (Lints and Emmons 1999). DA is involved in the modulation of a variety of physiological functions ranging from locomotion, motivation, learning and memory to hormone secretion, reward and thermoregulation (Blackburn et al. 1992) (Emilien et al. 1999) (Missale, et al. 1998). Defects in the dopaminergic neurotransmission have been linked to severe pathologies of the central nervous system; decrease in the level of DA in the brain leads to a neurodegenerative pathology known as Parkinson's disease, characterized by severe impairments such as rigidity, inability to direct movements and loss of coordination. This pathology is treated administering L-Dopa (a biosynthetic precursor of dopamine) and dopaminergic agonists (Olanow et al. 2006). Dopamine is also implicated in psychiatric pathologies like schizophrenia (Harrison and Weinberger 2005) (Seeman and Van Tol 1994); one of the current hypothesis for the disease, based on the beneficial therapeutic effects of dopaminergic antagonists, is an over functioning of the system. Also, the Attention Deficit Hyperactive Disorder (ADHD) (Cabib et al. 2002) (Cummins et al. 2011) as well as Tourette's syndrome and other pathologies are apparently caused by increased dopaminergic signaling. The wide variety of human pathologies generated from dysfunctions of the dopaminergic system calls for an in depth analysis to understand the molecular and cellular mechanisms governing the effect of dopamine in the brain.

Dopamine

Arvid Carlsson discovered DA in 1957, at the time other pharmacologists were skeptical about his findings, and believed that DA was merely a metabolite of other transmitters. DA is in fact an intermediate step in the biosynthesis of epinephrine and norepinephrine. However, a clear demonstration of the function of DA as a neuromodulator came from reserpine treatment in rabbits, Carlsson and co-workers made the intriguing observation that the akinetic effects of reserpine could be reversed by an intravenous injection of the DA precursor 3,4-dihydroxyphenylalanine.

Carlsson was awarded the Nobel prize for the discovery of DA in the year 2000.

The biosynthesis of DA in dopaminergic neurons starts from the aminoacid L-tyrosine, which is hydroxylated by the activity of the tyrosine hydroxylase (TH) to 1,3,4-dihydroxyphenylalanine (L-Dopa), TH is the rate-limiting enzyme of DA synthesis. L-Dopa is subsequently decarboxylated by the Dopa decarboxylase leading to the formation of DA (Elsworth and Roth 1997) (Westerink and de Vries 1985) (**Fig. 1**).

DA is then packaged into vesicles, which protects it from enzymatic degradation by the monoamine oxidase (MAO).

Upon nervous stimuli to dopaminergic neurons, vesicles containing DA reach the membrane and fuse with it releasing DA in the synaptic cleft; this mechanism is Ca^{2+} dependent (Meir et al. 1999).

Released DA diffuses also in the extracellular space away from the synapse, thus acting on different neurons (Cragg and Rice 2004) (Garris, et al. 1994). After signaling, DA is metabolized by two different monoamine oxidase (MAO) that converts DA in 3,4-dihydroxyphenylacetic acid and by catechol-o-methyltransferase (COMT) that convert DA in 3-methoxythyramine.

Depending on the extracellular DA concentration at a given time and in a given region, the function of DA is thought to be mainly governed by the quantal amount of amine release in response to the pre-synaptic depolarization (Rizzoli and Betz 2005) (Sara et al. 2005). Reuptake and metabolism are limiting steps of this process. DA released in the synaptic cleft is cleared by the DA transporter (DAT) located on the pre-synaptic terminal. Nevertheless, morphological studies have shown that the reuptake mechanism is clearly distant from the point of release (Mengual and Pickel 2004) implying an important spill-over of the amine released (Rice 2000). It has been also demonstrated that DA released in the extracellular space stimulates inhibitor autoreceptors located on dopaminergic terminals, thus, inhibiting further release (Suaud-Chagny et al. 1991) (Starke et al. 1989).

Tyrosine hydroxylase (TH), a critical enzyme for DA synthesis.

TH, as previously mentioned, is the rate-limiting enzyme in the catecholamine's synthesis; it catalyzes the hydroxylation of tyrosine to L-DOPA (Molinoff et al. 1971) (Molinoff and Axelrod 1971). TH is a tetramer of approximately 240 kDA (Kumer and Vrana 1996) , which is expressed in the brain, gut and retina, in the sympathetic nervous system and the adrenal medulla. This enzyme is predominantly found in the cytoplasm (Haycock et al. 1985). DA bound TH is inactive, while the DA-free form is active. Phosphorylation of TH

by the activity of protein kinase A (PKA) induces the displacement of DA, thus increasing the V_{max} by sixfold (Haavik et al. 1990). The first evidence that TH activity can be regulated by phosphorylation came in the seventies (Lovenberg et al. 1975). However, the direct identification of the sites of phosphorylation took a decade; it was reported that TH is phosphorylated at Ser⁴⁰, through a cAMP and PKA dependent mechanism (Campbell et al. 1986). In vitro also other protein kinases have been reported to be able to phosphorylate TH at Ser⁴⁰ and to increase TH activity. Phosphorylation of TH with MAPKK2 or CamKII increases TH activity proportionally to the extent of phosphorylation of Ser⁴⁰ (Sutherland et al. 1993).

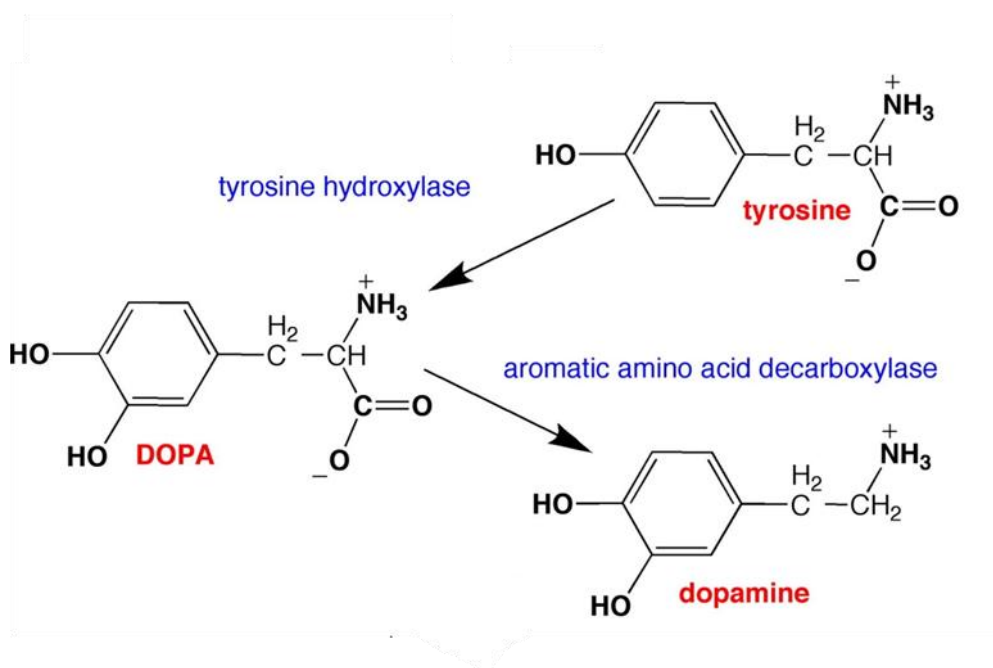


FIG.1) Schematic representation of DA synthesis. DA is synthesized in several brain nuclei of which the predominant are the Substantia Nigra compacta and Ventral tegmental area.

DA release

The dynamics of the release and uptake of DA into and from the synapse as well as from the extracellular space are currently under intense investigation. It is now well known that DA is also an extrasynaptic messenger that functions via volume transmission, escaping the synaptic cleft to bind to extrasynaptic receptors and transporters (Bergstrom et al. 2001). DA is released both in a tonic and phasic manner (Schultz 1998) (Wightman and Robinson

2002) (Justice 1993), which are best measured by different neurochemical techniques. Extensive data indicate that a tonic concentration of DA exists in target nuclei that may play an enabling role in neurotransmission. In the striatum these tonic concentrations of DA are predicted to be 5–20 nmol/L by microdialysis (Justice 1993) and differential normal-pulse voltammetry (Suaud-Chagny, Chergui et al. 1992) and 50–100 nmol/L by theoretical estimations using fast-scan cyclic voltammetry (Kawagoe, Garris et al. 1992) and pharmacologic studies (Ross 1991). In contrast, extracellular DA reaches high concentrations for brief periods (Rebec et al. 1997) (Robinson et al. 2001), during phasic release. These transients are likely to arise from concerted burst firing of DA neurons (Grace 1991) that often occur on presentation of salient sensory input (Overton and Clark 1997) (Schultz 1998). Fast-scan cyclic voltammetry and amperometry have been used to measure DA release after an electrical stimulation of DA neurons that mimics tonic and phasic firing (Kawagoe et al. 1992) (Suaud-Chagny et al. 1995). The amount of DA released is frequency-dependent; as the stimulation approaches frequencies achieved by burst firing, the DAT is saturated and high concentrations are achieved.

Dopaminergic pathways

DA projections originate in the mesencephalon in Nuclei named from A9-A12; from these nuclei four dopaminergic pathways have been identified: 1) The Nigrostriatal; 2) the Mesolimbic; 3) the Mesocortical and 4) the tuberoinfundibular (**Fig. 2**).

The nigrostriatal pathway arises from DA-synthesizing neurons of the midbrain nucleus, the substantia nigra compacta (SNc) which innervates the dorsal striatum (caudate-putamen). The nigrostriatal pathway is involved in the control of movement and its degeneration causes Parkinson's disease, characterized by tremors, rigidity and akinesia (Gerfen 1992) (Lang and Lozano 1998) (Lang and Lozano 1998). The mesocortical pathway originates from the ventral tegmental area (VTA) and innervates different regions of the frontal cortex. This pathway appears involved in some aspects of learning and memory (Le Moal and Simon 1991). The mesolimbic pathway originates from the midbrain VTA and innervates the ventral striatum (nucleus accumbens), the olfactory tubercle (OT) and parts of the limbic system. This pathway has been implicated in influencing motivated behaviour (Koob and Bloom 1988) (Koob 1992). The tuberoinfundibular pathway arises from cells of the periventricular and arcuate nuclei of the hypothalamus. Projections of this pathway reach the median eminence of the

hypothalamus where they release DA into the perivascular spaces of the capillary plexus of the hypothalamic–hypophyseal portal system. Thus, DA is transported to the anterior pituitary where it acts on the lactotrophs to inhibit the release of prolactin. This hormone stimulates milk production from mammary glands and stimulates lactotroph proliferation by an autocrine mechanism in the pituitary gland (Saiardi et al. 1997).

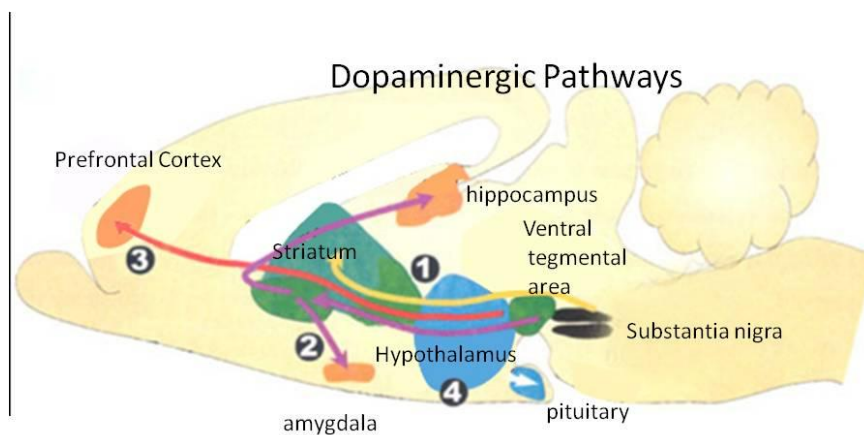


Fig.2) Schematic representation of Dopaminergic pathways.

1) Nigro-striatal pathway; 2) Mesolimbic-pathway; 3) Mesocortical-pathway; 4) tuberoinfundibular-pathway.

DA receptors

DA exerts its functions through binding with specific membrane receptors (Caron and Lefkowitz 1993). These receptors belong to the family of seven transmembrane domain G-protein coupled receptors (GPCR). The first evidence for the existence of DA receptors were biochemical and pharmacological and obtained in the seventies (Caron et al. 1978) (Kebabian 1978). With the increasing advancement of molecular cloning techniques and a great biochemical support, the first GPCR cloned was the β -adrenergic in the eighties; this cloning opened the way to the molecular identification of most GPCR using low stringency cloning strategies and PCR. This way five different genes encoding for five different dopaminergic receptors have been cloned,

and named: D1, D2, D3, D4, D5. Interestingly, the cloning techniques identified more receptors than expected based on pharmacological evidence, which identified only two classes the D1 and D2. However, when these cloned receptors were expressed in cells and their pharmacology studies it was possible to group them into 2 different families: the D1-like and D2-like family. The D1 and D5 receptors belong to the D1-like family and D2, D3, D4 belong to the D2-like family. Importantly, members of the same family share not only pharmacological but also structural and biochemical properties (**Table 1**). The D1-like receptor's family interacts with the stimulatory G-proteins (Gs) and activates the adenylyl cyclase; while the D2-like family is characterized to inhibit this same effector (Gingrich and Caron 1993) (Missale et al. 1998) (Jackson and Westlind-Danielsson 1994). DA receptors share the characteristics of typical 7 transmembrane domains receptor connected by three intracellular and three extracellular loops with an extracellular N-terminus and an intracellular C-terminus.

	D1-like		D2-like		
	D1	D5	D2	D3	D4
<i>Agonists</i>					
Quinpirole	1900		4.8–576	5.1–24	30–46
Dopamine	0.9–2340	< 0.9–261	2.8–474	4–27	28–450
(–)Apomorphine	0.7–680	122–163	0.7–24	20–32	4
Bromocriptine	440–672	450	5.3–12.6	5–7.4	290–340
7-OH-DPAT	5000		10–103	1–2	650
cis-8-OH-PBZI	21 230	15 060	2470	27	276
SKF-38393	1–150	0.5–100	150–9560	5000	1000–1800
NPA	421	187	0.4–0.9		6.5
<i>Antagonists</i>					
AJ-76			80–270	35–91	
(+)Butaclamol	0.9–3	5–27	0.69–0.8	4.1–11.2	38–51
Clozapine	100–261	194–336	56–230	83–620	9–42
Chlorpromazine	73–90	130–314	0.5–3	1.16–6.1	35
Domperidone			0.3	9.5	
Haloperidol	27–203	33–151	0.6–1.2	2.74–7.8	2.3–5.1
Piperone	99–350	135–4500	0.06–0.37	0.32–0.71	0.05–4
Raclopride	18 000		1–5	1.8–3.5	237–2400
Remoxipride			54–300	969–1600	2800–3690
Risperidone			1.7–5	6.7	7
SCH-23390	0.11–0.35	0.11–0.54	270–1100	314–800	3000–3560
S(–)Sulpiride	20 400–45 000	11 000–77 270	2.5–71	8–206	21–1000
(+)S-14297			297	13	
U-99194A			1572	78	
UH-232			13–40	2.9–9.2	

Table 1) Pharmacological table of dopaminergic receptors: agonists and antagonists.

The D1-like family

The D1 Receptor

The D1 receptor was cloned for the first time in 1990 (Monsma et al. 1990). This receptor is characterized by a short third cytoplasmatic loop and a long C-terminus. These are typical characteristics of receptors linked to Gs and activator of the adenylyl cyclase, such as the β -adrenergic receptor (Dohlman et al. 1991). D₁ mRNA has been found in the striatum, the nucleus accumbens and the olfactory tubercle. In addition, D₁ receptors have been detected in the limbic system, hypothalamus and thalamus (Missale et al. 1998). Pharmacological, biochemical and behavioral studies, of animals knockout for this receptor, have been shown the importance of D1-mediated signaling in locomotion (Xu et al. 1994), learning, and responses to drugs of abuse (Caine et al. 2000) (Tzschentke and Schmidt 1998) (Smith et al. 1998) among several other deficits. D1 agonist overstimulation of this receptor has also been linked to stereotype-like behavior in rats and mice models (Makihara, et al. 2004) (Wilkinson and Nichol 2000).

The D5 receptor

The D5 receptor gene has a more restricted pattern of expression when compared to the D1 receptor; its expression has been detected in the hippocampus, lateral mammillary nucleus, and in the parafascicular nucleus of the thalamus (Jackson and Westlind-Danielsson 1994). This receptor is composed of 475 amino acids and it is not pharmacologically different from the D1 receptor. The only difference is in the affinity of the receptor for DA, D5 receptors have indeed 10 times higher affinity for DA than the D1 receptor (**Table 1**) (Grandy et al. 1991) (Missale et al. 1998). Low levels of D5 expression have been reported in the prefrontal cortex, hippocampus, hypothalamus, in the olfactory bulbs, in the Nucleus Accumbens (NAcc) and the Caudate putamen (Cpu) (particularly in striatal interneurons) (Sealfon and Olanow 2000).

D2-like family

The D2 receptor

The D2 receptor has been the first member of D2-like family to be cloned. The cDNA encoded for a protein of 415 amino acids (Bunzow et al. 1988).

As compared to the D1 receptor, the D2 receptor contains: I) 3 potential consensus sites for glycosylation in the N-terminus; II) a smaller C-terminus; III) a larger third intra-cytoplasmatic loop (Bunzow et al. 1988). These characteristics are typical of many receptors known to inhibit the adenylyl cyclase activity (Dohlmann et al. 1991).

In 1989 a new form of D2 receptor of 444 amino acids (aa) was discovered; this other isoform was then recognized to originate by alternative splicing of the *Drd2* gene (Dal Toso et al. 1989) (Giros, et al. 1989) (Montmayeur, et al. 1991) **(Fig.3)**

This mechanism inserts exon 6, a very small exon of only 87 nucleotides, in the pre-mRNA. This insertion leads to the presence of additional 29 aa in the third intracytoplasmatic loop of the longest isoform of the D2 receptor: D2L. The other isoform is identical to D2L but does not contain the insert and therefore is shorter; from which the name D2S. D2L has higher affinity than D2S for the G-protein $G_{\alpha i2}$ (Montmayeur et al. 1991) (De Mei, et al. 2009) which has led to the hypothesis that the two isoforms by differential coupling might have different functions in vivo.

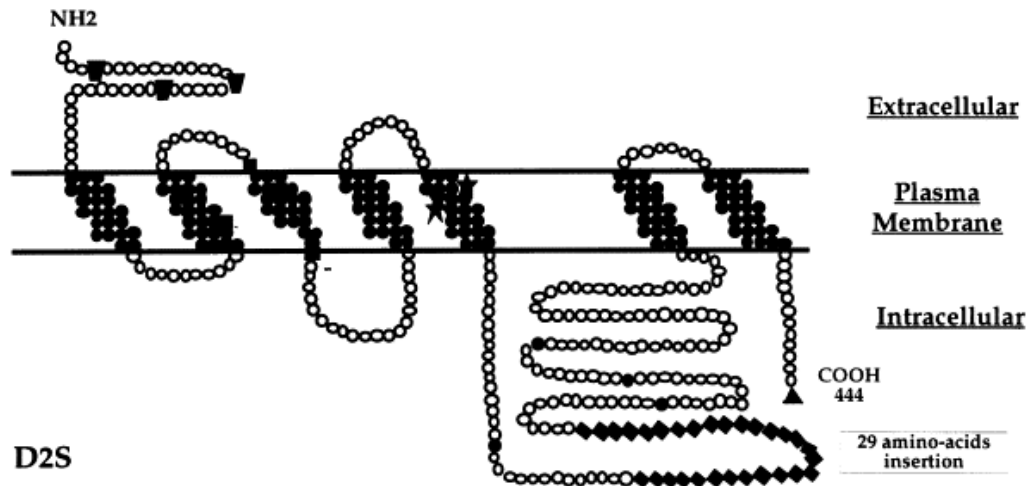
The ratios of D2L/D2S expression favors D2L in all neurons (80/20), with the only exception of the Substantia nigra compacta (SNc) and ventral tegmental area (VTA) where the ratio is 50/50. Thus suggesting a possible DA autoreceptor role for D2S (Montmayeur et al. 1991). (Usiello et al. 2000)

How the splicing of the *Drd2* is regulated starts to be elucidated recently it has been identified a heterogeneous nuclear ribonucleoprotein M (hnRNP M) as a key molecule controlling D2R splicing. It has been shown that binding of hnRNP M to exon 6 inhibited the inclusion of this exon in the mRNA (Park et al. 2011). In the past, a role for steroids in regulating the splicing events was reported (Guivarc'h et al. 1998).

The observation that D2L and D2S have similar pharmacological characteristics and localization led to hypothesize that they might have redundant functions. However, as previously mentioned, biochemical and cellular studies identified slight differences among these isoforms. An important evidence on the different role of the two isoforms came from the analysis of knockout mice for the D2L isoform (Usiello, et al. 2000). Indeed, it showed that absence of D2L removes the postsynaptic response to the typical antagonist haloperidol while preserving D2-

mediated autoreceptor functions (Usiello, et al. 2000) (Wang, Xu et al. 2000).

D2L



D2S

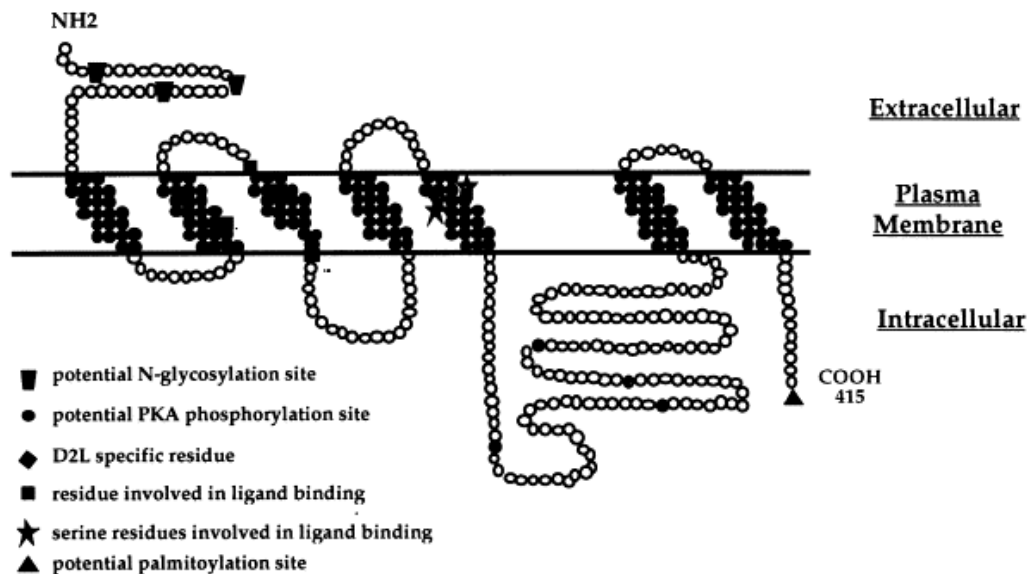


FIG. 3) Schematic representation of the 2 isoforms of D2R.

On the top the D2L isoform; on the bottom the D2S isoform.

Thus supporting that D2L and D2S are not redundant but serve specific roles in vivo.

D2 receptors are highly expressed in the striatal regions and basal ganglia; they are also expressed although to a minor extent in the hippocampus and amygdala (Mansour et al. 1990) (Mengod et al.

1992). In the cerebral cortex DA D2 receptors are expressed in the anterior cingulate cortex (Mansour et al. 1990; Mengod et al. 1992). In the midbrain D2 receptors are highly expressed in the dopaminergic neurons in the VTA and SNc (Mansour, et al. 1990). D2 receptors are also expressed in the anterior and intermedial lobes of the pituitary gland (Mansour. et al. 1990) (Mengod et al. 1992). D2 receptors are expressed in the retina (DA is the most predominant catecholamines in the vertebrates retina (Witkovsky 2004), lungs, intestine and vascular systems. D2 signaling might also be involved in the regulation of circadian rhythms (Doi et al. 2006) (Hood et al. 2010).

The D3 receptor

The second DA receptor cloned of the D2-like family was the D3 (Sokoloff et al. 1990). D3 has higher affinity than D2 for DA and it is glycosylated at the N-terminal as the D2 receptor (Sokoloff et al. 2006).

The more interesting feature is that the *Drd3* gene also undergoes to splicing, however in this case the products are truncated receptors. The function of the different splicing variants has not been studied in depth. D3 receptor has a overall 52% homology with the D2 receptor; the transmembrane regions have however a 75 % homology. This higher structural homology results in the absence of compounds that can pharmacologically discriminate D3 from D2; indeed, to date only few antagonists have been described to be slightly selective for D3, relatively to D2 receptor (Gilbert et al. 2005) (Xi et al. 2006). Agonists have either equal or greater affinity for the D3 receptor (Sokoloff and Schwartz 1995).

DA D3 receptors are slightly expressed in the ventral striatum and very strongly in the island of Calleja; D3 receptors are also expressed presynaptically in dopaminergic neurons of the VTA and SNc (Diaz, et al. 2000) (Hall et al. 1996).

The D4 receptor

The last cloned member of the D2-like family is the D4 receptor (Van Tol, et al. 1991).

The receptor is a protein of 387 amino acids. D4 receptor has 41 % homology with D2 receptor and 39% with D3, and about 56% for both D2 and D3 receptors within the membrane-spanning domains. There is one potential site for N-linked glycosylation in the N-terminus and one consensus cAMP-dependent phosphorylation site in the third cytoplasmatic loop (Van Tol et al. 1991). Ligand binding studies reveal

a pharmacological profile similar to those of D2 and D3 receptors. The D4 receptor display similar or lower affinity for both dopaminergic agonist and antagonist compared to the D2 receptor (Van Tol, et al. 1991). Recently the generation of D4 specific ligands has been reported, thus opening the possibility for a specific pharmacological characterization of this receptor.

The D4 receptor is weakly expressed in different areas of the brain such as the cerebral cortex, amygdala, hypothalamus, and pituitary gland; it is also abundant in the retina (Defagot et al. 2000) (Lidow et al. 1998). Several variants of D4 receptors have been found, particularly in the exon coding for the third cytoplasmatic loop (Van Tol et al. 1992). Clinical studies have reported a correlation between polymorphisms of this gene and the ADHD in the human population (Faraone, et al. 2001)); in addition, an increasing number of D4 receptors have been found in schizophrenic patients, suggesting the possible involvement of this receptor in human psychoses (Ariano, et al. 1997) (Mrzljak, et al. 1996).

The dopamine transporter (DAT)

Dopaminergic neurons express the DAT; this protein is involved in the reuptake of DA from the synapse. DAT is a member of the Na⁺/Cl⁻ transporter family, the protein contains 12 transmembrane domains (Giros and Caron 1993) (Amara and Kuhar 1993).

DAT mediates the recapture of released DA and, together with the D2 autoreceptor, is a key regulator of dopaminergic signaling, by regulating extracellular DA levels. DAT is expressed only in the cells that synthesize DA (Torres, et al. 2003). It is a crucial target for different psycho-active drug such as cocaine (Giros, et al. 1996).

DAT has been shown to interact directly with the D2 receptor, the N-terminal of DAT interacts with the third cytoplasmatic domain of the D2R (Bertolino, et al. 2009). The interaction between DAT and D2R helps the recruitment of DAT reinforcing the DA reuptake. (Lee et al. 2007).

Basal ganglia

The basal ganglia are involved in the performance and selection of the action, they are the center of interaction of different brain areas, in which there is a cohesive functional unit of different information such as voluntary motor control, emotional functions and habits behavior (Groenewegen 2003) (Gurney et al. 2004). The basal

ganglia network is composed of five large subcortical nuclei: the caudate nucleus, the putamen, the globus pallidus, the subthalamic nucleus and the substantia nigra (Groenewegen 2003). These circuits have 3 different functional activity: motor activity, cognition and motivational behavior (Kreitzer and Malenka 2008) (Nambu 2008). Inside the basal ganglia functional circuit, DA has the crucial role to modulate motor functions (Heien and Wightman 2006) (Schultz 2007) through the activation of two pathways contained in this circuit, the direct and indirect pathways. The direct pathway is formed by the striatal projections to the internal segment of the globus pallidus and substantia nigra pars reticulata, which then project to the thalamus. The indirect pathway projects from the striatum to the external segment of the globus pallidus, which then projects to the subthalamic nucleus and from this nucleus to the substantia nigra reticulata. In the classical model of the direct and indirect pathway D1R- and D2R-mediated effect are largely segregated (Gerfen 2000); with D1R regulating the indirect pathway and D2R the direct pathway (Gerfen 2000).

Striatum

Anatomically the striatum is a structure of the forebrain. It is composed by caudate, putamen, ventral striatum and globus pallidum.

Anatomical studies showed that the striatum is the target of the afferences from the cerebral cortex, intralaminar thalamic nuclei and the DAergic neurons.

Striatal neurons have been characterized anatomically and functionally (Kawaguchi, et al. 1995) (Wilson 1993) and can be divided into two different classes: the medium spiny neurons (MSNs), and the interneurons. The MSNs represent the majority of striatal cells (~95%); MSNs can be divided into striatonigral neurons (direct pathway) and striatopallidal neurons (indirect pathway) (Gerfen 2000) (Gether 2000) (Smith et al. 1998). MSNs are gabaergic neurons, but they are also known to synthesize substance P, enkephalin (ENK), dynorphin (dyn) and neurotensin (NT). The interneurons represent only 5% of the striatal neurons, they can be divided in 4 classes: the giant cholinergic interneurons, parvalbumin containing gabaergic interneurons, calretinin containing gabaergic interneurons and somatostatin containing interneurons (Kawaguchi et al. 1995). Interneurons receive glutamatergic afferences from cortical area and thalamus and they innervate MSNs or others interneurons (Tepper and Bolam 2004).

D1R and D2R localization

D1R and D2R are the most abundant DA receptors in the brain, particularly in the basal ganglia. Both receptors are segregated in the MSNs of the striatopallidal and striatonigral pathways (Surmeier et al. 2007); however, single cells PCR analyses have shown that D1R and D2R might colocalize in the 20% of striatal neurons (Surmeier and Kitai 1994) (Surmeier et al. 1996). This number was further increased in a report using immunocytochemical and confocal techniques, which reported, surprisingly, that all striatal cells express both D1R and D2R (Aizman et al. 2000). However, recent studies using transgenic mice expressing D1R and D2R proteins tagged with fluorescent markers contradicted these findings and showed that just 5% of MSNs express both receptors (Matamales et al. 2009). These studies have also shown that cholinergic interneurons express only D2R. However between the two receptors, in the basal ganglia circuit the major difference for what concern their localization is that D1R are only expressed by MSNs while D2 receptors are expressed MSNs, interneurons as well as by dopaminergic neurons of the SNc and VTA (Matamales et al. 2009), having the important role of autoreceptor.

Pre- and postsynaptic functions of D2R

As explained in previous paragraphs, D2R is expressed in all dopaminergic neurons as well as in MSNs and interneurons. Studies performed using D2R KO mice have shown that D2R is the only receptor of the dopaminergic system to function as autoreceptor, regulating the synthesis and release of DA (Dickinson et al. 1999) (Rouge-Pont et al. 2002) (Benoit-Marand et al. 2001). Indeed, results obtained using complementary microdialysis and voltammetry analyses show that the autoreceptor function regulating DA release is totally abolished in the absence of D2R in D2R KO mice (Rouge-Pont et al. 2002). D2R has also been shown to have a key role in the auto-inhibition of DA release in response to drugs of abuse; this can be very likely related to the fact that DAT activity might be affected in the absence of D2R (Bertolino et al. 2009). D2R has been reported to regulate the trafficking of DAT at the plasma membrane through activation of the MAPK pathway (Bolan et al. 2007) (Lee et al. 2007). Interestingly, in mice lacking D2L (D2L KO), which still express D2S, the D2R mediated autoreceptor function is still present. This suggested a specific autoreceptor role for the D2S isoform in vivo (Lee et al. 2007) (Usiello et al. 2000).

Dopaminergic neurons originating in the midbrain bring information to the striatum that regulates motor activity and reward; in parallel glutamatergic afferences from cortical areas converge to the striatum, where DA signaling balances the arrival of this information (Wise 2005). At the same time, DA through D2R also controls the activity of the interneurons, whose role is important for a correct regulation of striatal physiological functions (Wang et al. 2006) (Surmeier et al. 2007).

Postsynaptically, D2 receptor is needed for a correct regulation of the different stimuli arising from different area of the brain. D2R on the MSNs working as heteroreceptor modulates the release of other neurotransmitters in striatal and cortical cells; therefore this receptor influences not only DA release but also that of neurotransmitters produced by neurons target of DA afferents.

Pre-synaptic mechanisms mediated by D2R have been implicated in the GABA and glutamate release (Centonze et al. 2004) (Centonze et al. 2002) from striatal and cortical neurons. Altogether, these experimental evidence make D2R a key regulator of dopaminergic transmission not only at the level of dopaminergic neurons but also in heterologous systems.

Signal transduction

The best characterized intracellular effect of DA is on the cAMP pathway (Tan et al. 2003) (Nishi et al. 1997). This pathway is activated by the D1-like receptors and inhibited by the D2-like receptors. In MSNs, elevation of cAMP level leads to the activation of the Protein Kinase A (PKA) (Nishi et al. 1997) and consequently to the phosphorylation of a large series of cellular targets and importantly of the DA- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Bateup et al. 2008). Blockade of D2R stimulates the PKA-dependent phosphorylation of DARPP-32. This effect is most probably mediated via suppression of the inhibition exerted by D2R on adenylyl cyclase. Phosphorylation catalyzed by PKA on Thr34 converts DARPP-32 into a potent inhibitor of Protein Phosphatase 1 (PP-1), thereby amplifying the responses produced by activation of the cAMP/PKA pathway. Importantly, blockade of D2R-mediated signaling produces a motor depressant effect, which is attenuated in DARPP-32 null-mice (Fienberg et al. 1998). Activation of D1Rs increases Thr34 phosphorylation via G_{olf} -mediated stimulation (Herve et al. 2001). Conversely, activation of D2Rs decreases DARPP-32 phosphorylation at Thr34 via G_i -mediated inhibition of cAMP production (Nishi et al. 1997).

In addition, D2R agonists stimulate protein phosphatase-2B activity, thereby increasing dephosphorylation of DARPP-32 at Thr34 (Nishi et al. 1997).

D2 like receptors have also an activity cAMP independent in which AKT is a crucial protein. When DA binds D2R there is the formation of a complex of three proteins: beta-arrestin, AKT and the phosphatase PP2a (Beaulieu et al. 2005). Akt is inactivated through dephosphorylation of thr-308 by PP2A (Beaulieu et al. 2005). AKT inactivation leads to activation of GSK 3 (Glycogen synthetase 3) consequently in this way some dopaminergic behaviors are mediated by signaling pathways different from that of the cAMP (Beaulieu et al. 2007). Importantly, in schizophrenic patients, in which D2R antagonists are the drugs of choice, Akt expression is down-regulated and consequently GSK 3 phosphorylation is decreased (Emamian et al. 2004). Thus, the cAMP independent induction of the AKT pathway by DA through D2Rs might have strong repercussions on human health.

Gene Targeting

Generation of ko mice

The embryonic stem cells were isolated for the first time in the eighties (Evans and Kaufman 1981). These cells are totipotent, thus they have the ability to differentiate in all possible lineages during the development of the animal, including the germ line. These cells can be grown in vitro and they can be manipulated without altering their capacity to be totipotent. Recombinant DNA can be transfected in these cells, which then integrate the genome through the process of homologous recombination. In this way it is possible to introduce mutation in the gene of interest or generate knockin/knockout mice. Indeed, once isolated the desired clone, these cells can be implanted in mouse embryos, where they have the ability to colonize the embryos giving rise to chimeras and through subsequent mating to recombinant mouse lines (Capecchi 1989). The desired cells are obtained by selection on known resistance introduced in the cells by the recombinant vector. Embryonic stem cells for the generation of mutant mice originate from the 129/sv strain which after manipulation will be implanted into a pseudo-pregnant female of the C57BL/6j strain. The continuous backcrossing on a specific background allows for the establishment of animal lines homozygous for the modified gene (a null mutant) (Thomas and Capecchi 1987).

Generation of site-specific knockout mice

In vivo analysis of gene function is often based on the production of animal models with specific genetic modification. This approach has been applied to several areas in biological research. To highlight the importance of the same gene products in different areas, the Cre-loxP system has been one of the best tools used in the last 20 years. The Cre recombinase is a 38 kDA protein encoded by bacteriophage P1 and it can catalyze the specific recombination between DNA specific sites called loxP sites (Sauer 1987; Sauer et al. 1987). When two loxP sites are introduced in the same orientation into a genomic locus, expression of the CRE recombinase results in the deletion of the loxP flanked DNA sequence. Introduction of two loxP sites into the genome flanking an essential part of a target gene without affecting its function and subsequent Cre-mediated deletion will lead to the inactivation of the gene of interest. A specific transgenic Cre mouse expressing the recombinase under the control of a region specific promoter will allow the deletion of the gene in the region of interest (**Fig 4**).

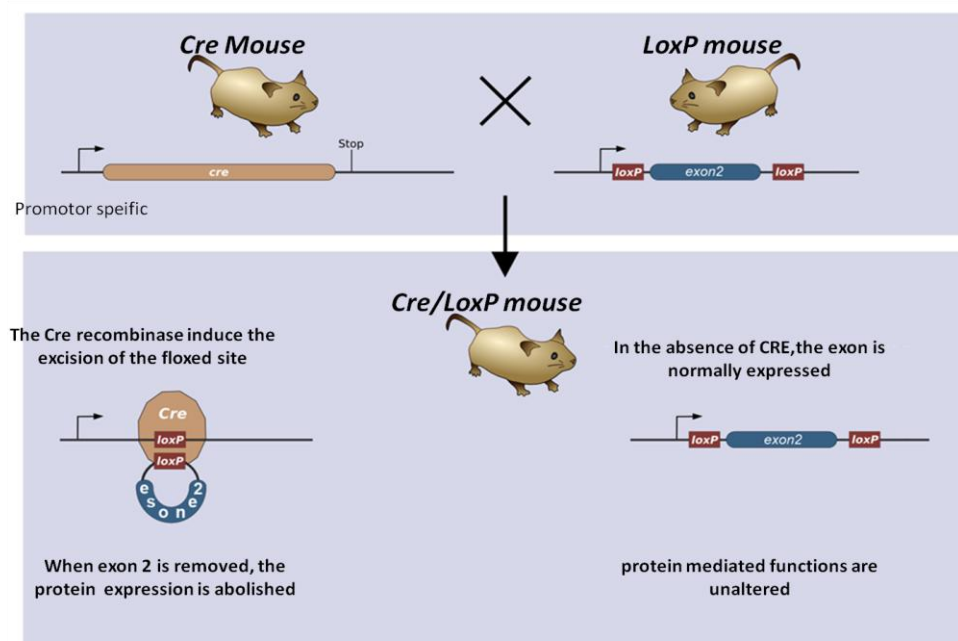


FIG.4) Schematic representation of the Cre-lox system.

The exon 2 of the protein is flanked by two LoxP sites. Mating this mouse with an animal expressing the Cre-recombinase leads to the excision of the exon.

2. Aims of this study

Understanding how DA regulates important physiological functions is a arduous subject due to the existence of five different DA receptors sharing the same ligand and transduction pathways and as in the case of the D2 receptors have two isoforms with similar pharmacology(Usiello et al. 2000; Wang et al. 2000).Therefore, while it is possible to discriminate pharmacologically between the two different sub-families of DA receptors, it is impossible to discriminate pharmacologically between members of the same family. The generation of specific KO mice highlights the value of cell-specific conditional mutant mouse models to determine the importance of the same gene product in different neurons.

During my thesis, I have attempted to elucidate the role of the presynaptic D2R (autoreceptor) and postsynaptic D2R (heteroreceptor) in the physiological control of DA release and their specific involvement in presynaptic (dopaminergic neurons) and post-synaptic (MSNs) functions.

My work has been concentrated on the analysis of two animal models generated in the laboratory before my arrival. These animals are D2R site-specific mutants in which the specific ablation of either the presynaptic D2R (autoreceptor) or the postsynaptic D2R (heteroreceptor) has been achieved. The presentation of my results is articulated in two main parts. In the first part, I will present the pharmacological and behavioral analyses performed in each mutant. In the second part, I will present the consequences of the ablation of D2R at pre- and postsynaptic sites on DA synthesis, release and DA-dependent functions.

3. Materials and Methods

Animals

Animals were housed under standard conditions (12 h light/dark cycles) with food and water ad libitum. All experiments were in accordance to the Institutional Animal Care and Use Committees (IACUC).

Drugs

(-)-Quinpirole Hydrochloride, Haloperidol and Cocaine Hydrochloride were from SIGMA, Baclofen from Tocris. All drugs were dissolved directly in 0.9% NaCl; haloperidol was dissolved as previously described (Usiello et al. 2000).

Generation of mice.

To generate D2R floxed alleles, a LoxP site was first inserted 5' of the 900 bp NcoI genomic fragment of the D2R gene containing exon 2; to allow selection of the recombinant ES cells, a neomycin cassette (pGKneo), flanked by LoxP sites, was inserted at the 3' end of the NcoI genomic fragment (Fig. 5). This generated the LoxP-Exon2-LoxP-pGKneo-LoxP construct, which contained an additional HindIII site allowing discrimination between the WT and floxed alleles (Fig.5). The endogenous NcoI fragment of the D2R gene was then replaced with this construct in the KpnI-SalI genomic fragment of 6.5 Kb (Fig.5). The engineered KpnI-SalI fragment was then used to electroporate ES cells (129/SvEv H1). Chimeras obtained from the injection of positive clones successfully transmitted the mutation to the progeny and homozygous D2R^{flox/flox} mice were obtained. D2R^{flox/flox} mice were then mated with En1Cre mice to generate D2R^{flox/flox/En1Cre/+} animals and D1Cre mice to generate D2R^{flox/flox/D1Cre/+} mice. Mutant and control mice used in this study are in the same genetic background (87.5% C57Bl6 x 12.5% 129 SV). During the establishment of the two mutants lines, we selected by Southern analyses only mice containing the excision of the neomycine cassette.

Genotyping.

Southern blot analyses were performed (Baik et al. 1995) on genomic DNA from tail biopsies of D2R^{flox/flox}, D2R^{flox/flox/En1Cre/+} and D2R^{flox/flox/D1Cre/+} mice. Genomic Hind III digests revealed fragments of 2.5 Kb in D2R^{flox/flox} and 2 Kb in D2R^{flox/flox/En1Cre/+} and D2R^{flox/flox/D1Cre/+} mice after hybridization to the ³²P-labelled NcoI-SalI mouse D2R genomic fragment. A 5.5 Kb fragment corresponding to the CRE

recombinase was obtained in both mutant lines using a 1.12 Kb ^{32}P -labelled probe, containing the CRE coding region.

***In situ* hybridization.**

Brains were rapidly dissected, snap frozen in isopentane-dry ice and stored at -80°C . Coronal cryostat sections ($10\text{ }\mu\text{m}$) were hybridized with ^{35}S -CTP-labelled D2R exon 2-specific probe as previously described (Baik et al. 1995) (Welter et al. 2007). Double in situ hybridizations of sections from $\text{D2R}^{\text{floxflox}}$ and $\text{D2R}^{\text{floxflox/D1Cre/+}}$ were performed using a ^{35}S -labeled D2R-specific exon 2 probe together with the digoxigenin (DIG)-labeled choline acetyl transferase (ChAT) or glutamic acid decarboxylase 1 (Gad1) probes. Radioactive probes were prepared as previously described (Baik et al. 1995) and cold probes as (Clark et al. 2001). Pictures were taken at 20x with a bright microscope (Leica).

D2R and D1R ligand-binding assays.

D2R and D1R ligand-binding assays were carried out on striatal membranes using $[\text{H}^3]$ -Spiperone for D2R (specific activity 91 Ci mmol^{-1} ; Amersham) and $[\text{H}^3]$ -SCH23390 (specific activity 85 Ci mmol^{-1} ; Amersham) for D1R. Binding data were analyzed with GraphPad Prism 4.0 program (GraphPad software). Binding assays were performed as previously described (Baik et al. 1995).

Behavioral analyses.

Behavioral experiments were performed using male mice (>8 week old). 4 days before experiments mice were moved to soundproof behavioral rooms. The open field was a white square box ($30\text{ x }30\text{ cm}$; 70 lux); the home cage was a transparent plastic box ($20\text{ x }30\text{ cm}$). Activity was followed by a video-tracking system (Viewpoint, Lyon, France) for 1 hour. An observer blind to mouse genotype scored rearing activity during the first 5 min of each test. Quinpirole (i.p.) effect was observed in animals not habituated to the testing cage ($20\text{ x }30\text{ cm}$) or ($30\text{ x }30\text{ cm}$) and analyzed for 30 minutes. Cocaine (i.p.) was administered after habituation to a new home cage (1 hr) and activity recorded for 30 min. Catalepsy by haloperidol (i.p.) was evaluated by the bar test (Usiello et al. 2000). Time spent in a cataleptic position was scored 3 h after treatment; cutoff was given at 120 seconds.

Western blots.

Animals treated with saline, quinpirole (0.2 mg/kg; i.p.), were killed 30 min after the administration of the compound. Heads were immediately immersed in liquid nitrogen for 6 s. Brains were frozen, punched in the region of interest and processed as previously described (Svenningsson et al. 2000) to preserve phosphorylations. Protein determination was made by BCA (bicinchoninic acid). Equal amounts of proteins (30 µg) were loaded onto 10% SDS/PAGE and transferred to PVDF membranes (BIO-RAD). Western analyses were performed using antibodies directed against phosphor-Ser⁴⁰-TH (1:1500; Millipore), total TH (1:3000; Millipore), α -tubulin (1:5000; Sigma); Goat anti-rabbit secondary antibodies (1:5000; Millipore) were used and blots revealed with ECL Plus. Quantifications were performed using NIH Image (version 1.42q) software.

Amperometry and cyclic voltammetry.

Adult male D2R^{flox/flox} and D2R^{flox/flox/En1Cre/+} or D2R^{flox/flox/D1Cre/+} were analyzed the same day. Slices were cut at 250 µm and allowed to recover at room temperature for 1.5 hours. Recording temperature was ~30 °C, and carbon fiber electrodes were placed close to corpus callosum (laterodorsal to dorsal section). For amperometry (AMP), a constant voltage of +500 mV was used and traces were digitally filtered using a Gaussian filter (250 Hz cutoff frequency). For cyclic voltammetry (CV), electrodes were calibrated before and after each experiment to estimate DA concentration. Each protocol was performed independently, except the paired pulse experiments performed in CV. Eight prepulses were applied to achieve constant DA release. Single-pulse DA levels and decay parameters were measured using the eighth prepulse. Paired-pulse experiments were performed using AMP (0.5 and 1 s) and CV (5–60 s). Trains of five or ten pulses at 20 Hz were analyzed by CV. Quinpirole was applied for 10 minutes, then removed and washed for additional 10 minutes.

Cell-attached electrophysiological recordings from adult mouse brain slices.

Horizontal midbrain mouse slices (200 µm) preparation, recovery and external solutions were made as described (Kapfhamer, Berger et al. 2010). Cells were visualized with an upright microscope using infrared differential interference contrast (IR-DIC) illumination and cell-attached voltage clamp recordings were made with a Multiclamp 700A amplifier (Axon Instruments). Electrodes (2–4 MΩ)

contained in mM: 117 cesium methanesulfonic acid, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, 0.25 MgGTP (pH 7.2–7.4), 275–285 mOsm. Baclofen (2 μ M) and Quinpirole (2 μ M) were applied by bath superfusion.

HPLC analysis.

Tissues were homogenized by sonication in 30 volumes (w/v) of ice-cold 100 mM HClO₄. The homogenates were centrifuged for 20 min at 15,000 g, 4 °C. The supernatants were recovered and passed through 0.2 μ m filters and monoamines and metabolites quantified in the filtrates by HPLC-EC. The HPLC system consisted of a BASi (West Lafayette, IN) LC-4C detector coupled to a BASi LCEC radial flow cell. Flow was provided by a Shimadzu (Columbia, MD) LC-20AD solvent delivery module, preceded by an online degasser series 1100 from Agilent (Santa Clara, CA). The chromatograms were analyzed using PowerChrom software (eDAQ, Colorado Springs, CO). Monoamines in 10 μ l tissue filtrate were separated on a 1 x 100 mm UniJet microbore 5 μ m C-8 column. The mobile phase consisted of 24 mM Na₂HPO₄, 0.3 mM sodium octyl sulfate, 27.4 mM citric acid, 107 μ M EDTA and 11 % (v/v) MeOH, pH adjusted to pH = 4.5 with NaOH. The flow was set at 100 μ l/min and the potential was set at + 750 mV relative to an Ag/Cl reference electrode. Elution times were as follows (in min): DOPAC = 2.6; Dopamine = 5.1; HVA = 6.4;

Statistical analysis.

All values are given as mean \pm S.E.M. Analysis of variance (ANOVA) was used to determine group effects and interactions followed by the appropriate Post-hoc comparison or Student's t-test ($P < 0.05$ was considered statistically significant).

4. Results and Discussion

Analysis of the D2R site-specific knockout mice

To evaluate the pre- and post-synaptic functions of the D2R receptor, site-specific mutant have been generated. To generate them, the exon 2 of the D2R gene was flanked by loxP sites and D2R^{flox/flox} mice were generated, these mice are indistinguishable from their control wild-type (WT) mice (**Fig.5**).

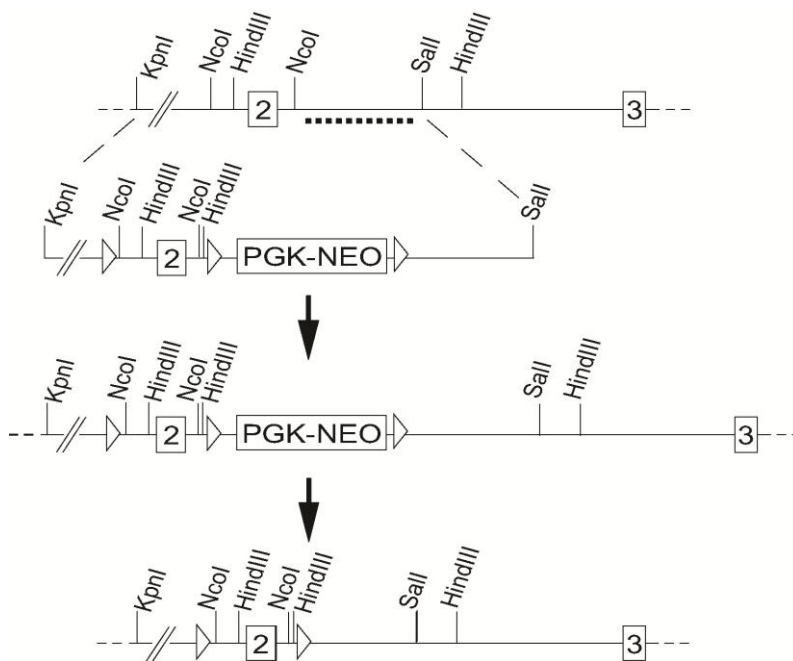


Fig 5) Strategy to generate D2R^{flox/flox} mice.

''' represents the probe used for genotyping.

Presynaptic D2 autoreceptor mutants

To study the presynaptic functions of the D2R, we have mated the D2R^{flox/flox} mice with a mouse carrying the Cre recombinase under the control of the engrailed-1 (En1) gene promoter (Kimmel et al. 2000).

The expression of the engrailed-1 (EN1) gene is restricted to the major mesencephalic dopaminergic nuclei, the SNc and VTA. The En-1 gene is a homeobox gene which is important during embryo development, indeed animals lacking this gene are not viable and do not have a cerebellum in a CD1 mouse background. En-1 is also expressed in the adult although its role at this stage has not been completely elucidated (Gherbassi and Simon 2006). Therefore, mice obtained from the mating of D2R^{flox/flox} mice with En-1CRE mice should not express D2R in the SNc and VTA. Thus, we performed in situ hybridization analyses comparing D2R^{flox/flox} mice and D2R^{flox/flox/En1CRE/+} mice (**Fig 6**). Thus, we prepared an antisense ³⁵S-labeled riboprobe of exon2 of the D2R gene and hybridized this probe to brain cryosections (10 µm) of both D2R^{flox/flox} and D2R^{flox/flox/En1CRE/+} mice. These analyses showed the specific deletion of the D2R in the SNc and VTA in D2R^{flox/flox/En1CRE/+} compared to WT controls; the expression of the D2R mRNA is instead normal in the Caudate putamen (CPu) and Nucleus accumbens (NAcc) of both genotypes (**Fig. 6a**).

To support these experiments at the protein levels, ligand binding analyses were performed on striatal membranes using ³H-spiperone, a D2-like specific antagonist. This experiment showed 20 % reduction of D2R binding sites in D2R^{flox/flox/En1CRE/+} compared to control, imputable to the deletion of D2R from the VTA and SNc dopaminergic projections. However, no difference in the receptor affinity for the ligand were observed (**Fig. 6b**). In addition, we also controlled whether D2R ablation might induce differential expression of D1R or a change in the affinity for D1 ligands, but no differences either in the number of D1 receptors sites or affinity for ³H-SCH 23390 were detected (**Fig 6c**). Together these analyses showed the specific ablation of presynaptic D2 autoreceptor from the dopaminergic neurons, making them a suitable model to study the role of presynaptic D2 autoreceptors in vivo.

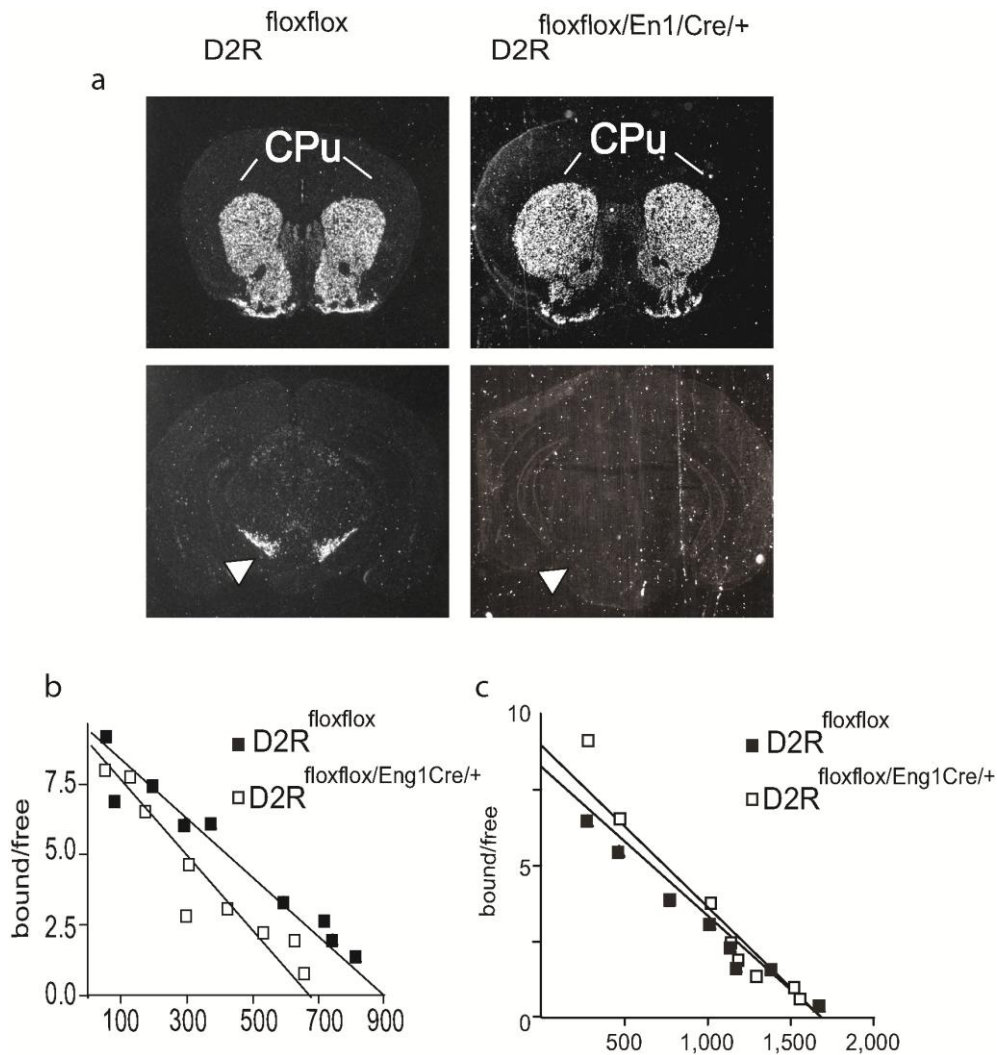


Fig.6) Characterization of the D2R^{flox/flox/En1Cre/+} mice.

a) *In situ* hybridization using the D2R Exon2 probe showing selective ablation of D2R in SN and VTA (\triangleright) for the D2R^{flox/flox/En1Cre/+} no difference in the CPU and Nacc between the genotype. **b)** D2R binding sites on striatal membranes from D2R^{flox/flox} (■) (Bmax = 884 ± 25 fmol/mg protein; Kd = 171 ± 49 pM) and D2R^{flox/flox/En1Cre/+} (□) (Bmax = 690 ± 37 fmol/mg protein; Kd = 132 ± 21 pM). **c)** Pharmacological characteristics of D1R binding sites in D2R^{flox/flox} and D2R^{flox/flox/En1Cre/+} striatal membranes. (■) (Bmax = 1703 ± 204 fmol/mg protein; Kd = 297 ± 93 pM) and D2R^{flox/flox/En1Cre/+} (□) (Bmax = 1682 ± 168 fmol/mg protein; Kd = 354 ± 115 pM). Values are given ± S.E.M. (n=3).

Postsynaptic D2 heteroreceptor mutant

D1R and D2R are highly expressed in the MSNs of the striatum. To generate a mouse in which the expression of the D2R is ablated in MSNs we have mated our D2R^{flox/flox} mice with a mouse in which the Cre recombinase is expressed under the control of D1R receptor (D1-CRE mice) (Lemberger et al. 2007). Mice so generated, named D2R^{flox/flox/D1CRE/+}, should not express any longer the D2R in these neurons. To test the site-specific ablation of D2R in MSNs, in situ hybridization analyses were performed by comparing D2R^{flox/flox} mice and D2R^{flox/flox/D1CRE/+} mice.

Experiments of in situ hybridization were performed as previously described for the presynaptic D2 autoreceptor mutants, using a antisense ³⁵S-labeled riboprobe of exon 2 of the D2R gene. These analyses clearly showed the deletion of the D2R mRNA in CPu and NAcc of these mice (**Fig.7a**). However, D2R are also expressed in striatal cholinergic interneurons. To control that D2R mRNA was still expressed in these cells, we performed double in situ experiments using an antisense digoxigenin (DIG)-labeled riboprobe for the choline acetyltransferase (ChAT) cDNA together with the exon 2 riboprobe. The results of these analyses showed that D2R mRNA was specifically ablated only in the MSNs, while was still expressed in the cholinergic interneurons (**Fig 8**) in D2R^{flox/flox/D1CRE/+} mice.

To support these experiments at the protein level, ligand binding analyses were performed on striatal membranes using ³H-spiperone, as described in the presynaptic D2 autoreceptor mutant mice. These experiments confirmed a 70% reduction of D2R binding sites in D2R^{flox/flox/D1CRE/+} mice as compared to D2R^{flox/flox}, with no changes in the affinity of the remaining receptors for the ligand (**Fig 7b**). Similar to the presynaptic D2 autoreceptor mutants, D2R deletion in MSNs does not result in differences in the number of sites or in the affinity of the D1R (**Fig7c**). Altogether these analyses confirm the suitability of the D2R^{flox/flox/D1CRE/+} mice as a model to study the role of D2R on MSNs.

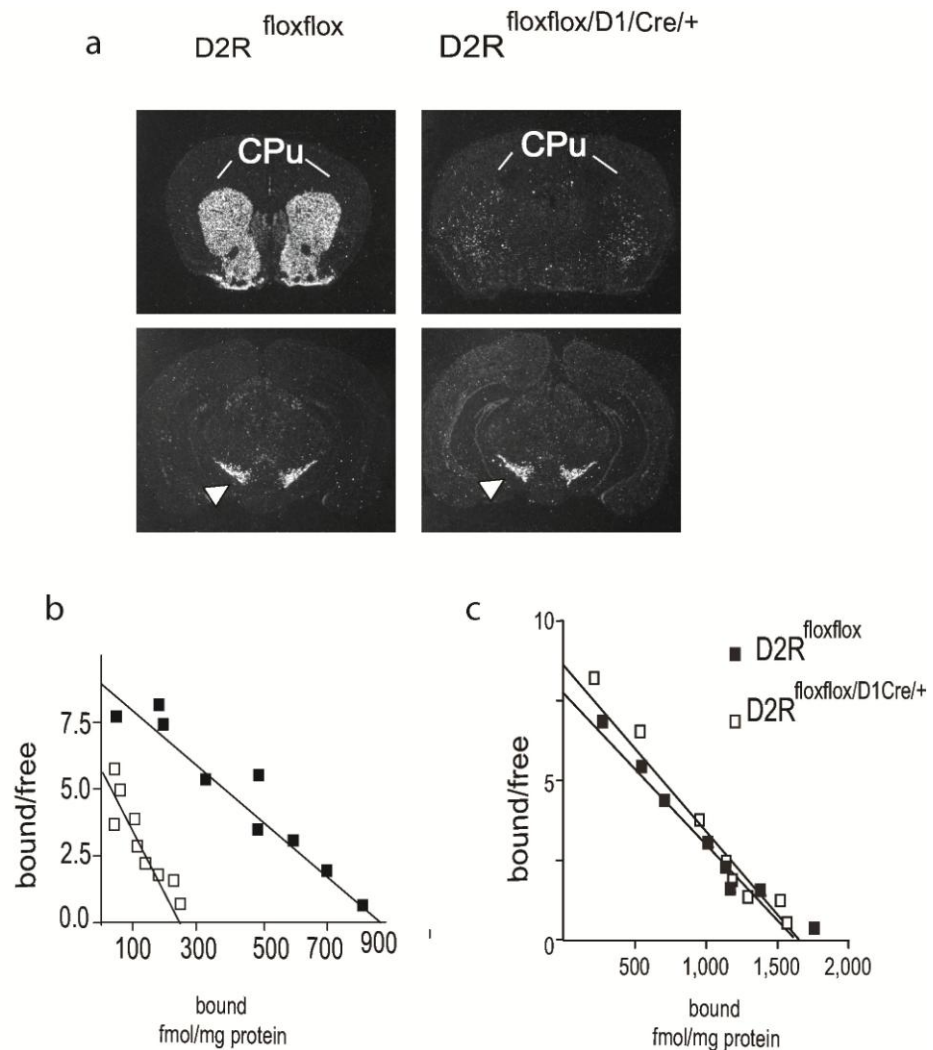


Fig.7) Characterization of the D2R^{flox/flox/D1Cre/+} mice.

a) *In situ* hybridization using the D2R Exon2 probe showing selective ablation of D2R in the CPu and NAcc for the D2R^{flox/flox/D1Cre/+} mice. **b)** D2R binding sites on striatal membranes from D2R^{flox/flox} (■) (Bmax = 875 ± 28 fmol/mg protein; Kd=184±52 pM) and D2R^{flox/flox/D1Cre/+} (□) (Bmax=259 ± 35 fmol/mg protein; Kd= 198 ± 58 pM). **c)** Pharmacological characteristics of D1R binding sites in D2R^{flox/flox} (black bar) and D2R^{flox/flox/D1Cre/+} (white bar) striatal membranes. (■) (Bmax = 1695 ± 269 fmol/mg protein; Kd = 278 ± 85 pM) and D2R^{flox/flox/D1Cre/+} (□) (Bmax = 1686 ± 198 fmol/mg protein; Kd = 354 ± 104 pM). Values are given ± S.E.M. (n=3).

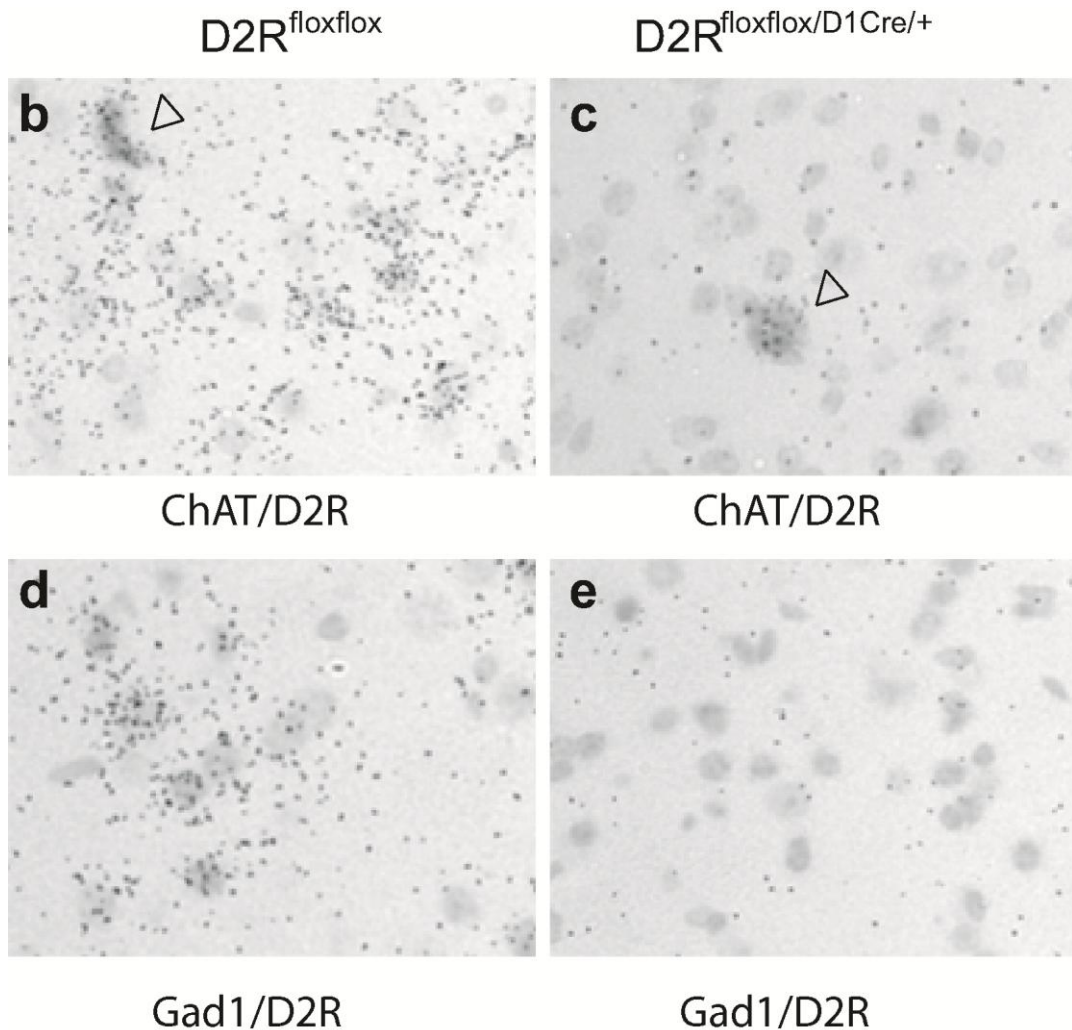


Fig.8) $D2R^{flox/flox}$ and $D2R^{flox/flox/D1Cre/+}$ striatal characterization.

Double in situ hybridizations of sections from $D2R^{flox/flox}$ and $D2R^{flox/flox/D1Cre/+}$ mice using a ^{35}S -labeled D2R-specific exon 2 probe together with the digoxigenin (DIG)-labeled choline acetyl transferase (ChAT) or glutamic acid decarboxylase 1 (Gad1) probes. **b)** $D2R^{flox/flox}$ and **c)** $D2R^{flox/flox/D1Cre/+}$ sections in which the D2R and ChAT probes were used. These analyses showed persistence of D2R in striatal ChAT interneurons; arrows indicate ChAT labelled cells. **d)** Double in situ hybridization using D2R and Gad1 probes in $D2R^{flox/flox}$; **e)** $D2R^{flox/flox/D1Cre/+}$ striatal sections; these analyses showed absence of D2R in MSNs.

D2R and motor activity

Presynaptic D2 autoreceptor mutants.

One of the prominent functions of D2R is the control of motor activity (Baik et al.1995).

To assess if the ablation of the D2R in the SNc and VTA neurons leads to impairments of motor functions in the presynaptic D2 autoreceptor mutants ($D2R^{\text{floxflox/En1CRE/+}}$), these animals and their control littermates ($D2R^{\text{floxflox}}$) were tested for their locomotion in a new home cage and in their performance on the rotarod test (Baik et al. 1995; Usiello et al. 2000) to assess their motor coordination. Interestingly, the results of these analyses (**Fig. 9a and 10a**) show no difference in the motor behavior of these mice as compared to control mice either in the new home cage or on the rotarod.

Then, we tested $D2R^{\text{floxflox/En1CRE/+}}$ and $D2R^{\text{floxflox}}$ mice in the open field (**Fig.9b**). The open field is a behavioral paradigm that contains an anxiogenic component and measures the reactivity of the subject to the novel, mildly stressful environment and how the subject reacts to it by measuring the time to habituate to it. This test allows to have a qualitative and quantitative measures of the animal locomotor's activity; in addition behavior in this test has been shown to depend on responses mediated by the mesolimbic system (Hooks and Kalivas 1995) in contrast to those obtained in the home cage that are dependent on the activation of nigrostriatal system (Hooks and Kalivas 1995). Importantly, when $D2R^{\text{floxflox/En1CRE/+}}$ mice were tested in the open field, they showed an exaggerated response both in horizontal and vertical movements, as compared to controls. Similarly, when motor activity was challenged in response to an acute administration of cocaine (DAT blocker), which also engages responses of the mesolimbic system (Delfs et al. 1990), we also observed an outstanding increase of the motor activity in $D2R^{\text{floxflox/En1CRE/+}}$ as compared to WT siblings (**Fig.9c**). Altogether, these results indicate that loss of presynaptic D2 autoreceptors results into an over-response to a novel environment and increased sensitivity to cocaine, both parameters connectable to an altered presynaptic D2 autoreceptor mediated control of DA. They also suggest that D2 autoreceptor deficiency is more evident in functions dependent from the mesolimbic than the nigrostriatal system.

Postsynaptic D2 heteroreceptor mutants

To analyze the role of the D2R expressed in the MSNs on motor activity, we performed the behavioral tests described in the above paragraph, on $D2R^{\text{floxflox/D1CRE/+}}$ and compared to that of

D2R^{flox/flox} mice. Importantly, the motor phenotype of D2R^{flox/flox/D1Cre/+} mice strongly resembles that of D2R-null mice previously analyzed in the laboratory (Baik et al. 1995). Indeed, D2R^{flox/flox/D1Cre/+} mice are characterized by a strong impairment of horizontal and vertical activity and fail to perform on the rotarod (Fig 9d and 10b). The motor impairment is as outstanding when postsynaptic D2 heteroreceptor mutants were tested in the open field (Fig 9e). In addition, and again in line with results obtained in D2R-null mutants, D2R^{flox/flox/D1Cre/+} mice have a totally blunted response to cocaine (Fig. 9f) (Welter et al. 2007). Altogether these results show that the presence of D2R on striatal MSNs is an absolute requirement to translate inputs into motor activity.

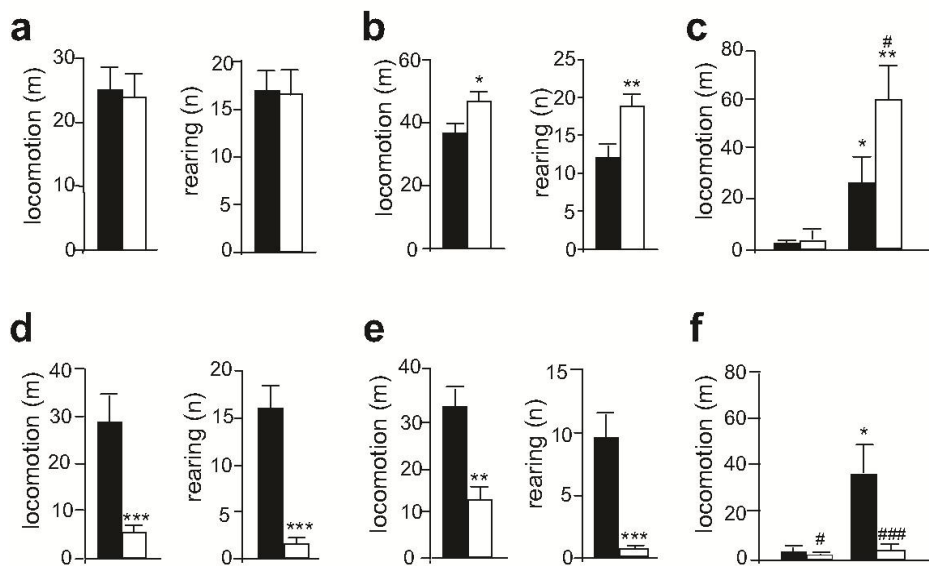


Fig.9) Loss of D2 autoreceptors or heteroreceptors differently affects motor behavior.

a) Locomotion and rearing activity of D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars) mice in the home cage (n = 9-10). No difference was observed between genotypes; Student's t-test: P>0.05. **b)** Locomotion and rearing activity in the open field of D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars) (n = 9-10); Student's t-test: * P < 0.05 and rearing ** P<0.01. **c)** Motor activity after acute cocaine injection (15mg/kg) recorded for 30 min. D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars). Two-way Anova Treatment x Genotype, (F_(1,34) = 5.249), P<0.05. Saline vs treated *: P<0.05, **: P<0.01. D2R^{flox/flox/En1Cre/+} vs D2R^{flox/flox} #: P<0.05. **d)** Locomotion and rearing activity of D2R^{flox/flox} (black bars) and D2R^{flox/flox/D1Cre/+} (white bars) mice in the home cage (n = 9-10). Student's t-test: *** P < 0.001; **e)** Locomotion and rearing activity in the open field of D2R^{flox/flox} (black bars) and

D2R^{flox/flox/D1Cre/+} (white bars) (n = 9-10). Student's t-test: ** P<0.01 *** P<0.001. **f)** Motor activity after acute cocaine injection (15mg/kg) recorded for 30 min. D2R^{flox/flox} (black bars) and D2R^{flox/flox/D1Cre/+} (white bars). Two-way Anova Treatment x Genotype, ($F_{(1,49)}=5.759$), P<0.05. Saline vs treated *: P<0.05, D2R^{flox/flox/D1Cre/+} vs D2R^{flox/flox} #:P<0.05 ,###: P<0.001

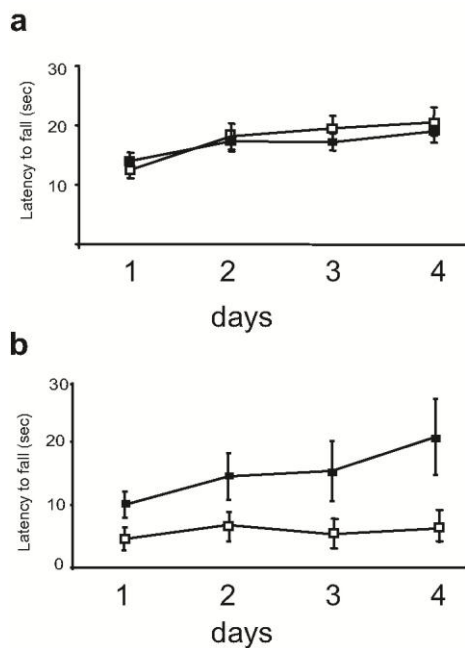


Fig.10) Motor coordination was assessed using the rotarod test.

a) Values represent the average \pm S.E.M. of time (sec) spent on the rotarod across four days by D2R^{flox/flox} (■) and D2R^{flox/flox/En1Cre/+} (□) mice. Both genotypes showed similar latency to fall ($F_{(3,36)} = 1.178$, $P > 0.05$) and improved their performance with time ($F_{(3,36)} = 14.426$, $P < 0.001$). The rotarod test was performed by placing mice on the rotating rod and measuring their fall latencies. Rotations were increased from 4 to 40 rpm over a period of 5 min. Mice were given 3 trials/day on 4 consecutive days; in each day the best performance was used for analysis. Rotarod data were analyzed by ANOVA with repeated measures. **b)** same as in a) for D2R^{flox/flox} (■) and D2R^{flox/flox/D1Cre/+} (□) mice. D2R^{flox/flox/D1Cre/+} showed shorter latencies to fall ($F_{(3,34)} = 6.239$, $P < 0.05$). Values represent the average \pm S.E.M. of time (sec) spent on the rotarod across four days.

Behavioral responses of pre- and post-synaptic D2r mutants to pharmacological challenge of D2-like agonists and antagonists.

Presynaptic D2 autoreceptor mutant mice.

D2-like agonist administration.

Presynaptic D2 autoreceptor mutants were then analyzed for their behavioral response to pharmacological challenge using dopaminergic D2-like specific ligands. For this, $D2R^{flox/flox/En1CRE/+}$ and $D2R^{flox/flox}$ mice were treated with quinpirole, a D2-like specific agonist known to induce motor sedation at low doses by activation of D2 autoreceptor (Usiello et al. 2000). Mice of both genotypes were thus treated either with saline or quinpirole at the doses of 0.02 mg/kg and 0.2 mg/kg and then tested for their locomotor activity in a new home cage. The $D2R^{flox/flox/En1CRE/+}$ mice at the dose of 0.02mg/kg did not present any reduction in the locomotor activity compared to the saline treated, while WT mice, as expected showed a reduction of locomotion (**Fig.11a**). Strikingly, however at the dose of 0.2 mg/kg of quinpirole, we observed a reduction of motor activity in $D2R^{flox/flox/En1CRE/+}$ mice as compared to the saline treated, similar to that observed in WT controls although less potent (**Fig 11a**). These results contrast with the expected loss of effect of low doses of quinpirole in the absence of D2 autoreceptors and suggest the involvement of postsynaptic D2R in the motor sedative effect of quinpirole. Importantly, when the same experiments was performed with mice of both genotype exposed to the open field, while the WT equally showed a reduction of motor activity, the $D2R^{flox/flox/En1CRE/+}$ mice at 0.2 mg/kg did not show any reduction of motor activity compared to the saline treated of the same genotype (**Fig. 12**). Thus, in the absence of D2 autoreceptors quinpirole elicits an effect in the new home cage, but not in the open field suggesting that quinpirole affects responses mediated by the nigrostriatal pathway through projections from the SNc to the striatum more than the mesolimbic pathway. These results are in agreement with those obtained in the absence of any pharmacological challenge in the open field versus the home cage, again showing a differential effect of loss of D2 autoreceptors in projections to the NAcc versus the dorsal striatum.

Postsynaptic D2 heteroreceptor mutants.

D2-like agonist administration

As for the $D2R^{flox/flox/En1CRE/+}$ mice, $D2R^{flox/flox/D1CRE/+}$ mice have been tested for their locomotor activity under quinpirole pharmacological challenge.

D2R^{flox/flox/D1CRE/+} mice did not show a decrease in the locomotor activity when administered with 0.02 mg/kg of quinpirole as compared to saline treated mice; while they showed a decrease at 0.2 mg/kg (**Fig. 11d**). We believe that the absence of effect of quinpirole at the dose of 0.02mg/kg is dependent on the already strong impairment of motor activity in D2R^{flox/flox/D1CRE/+} mice as compared to WT littermates. Indeed, at the dose of 0.2mg/kg they showed a reduction as compared to the saline treated of the same genotype. These experiments show that quinpirole still preserves the property to reduce movements in mice lacking D2R in MSNs, through activation of D2 autoreceptors still present in the SNc and VTA of D2R^{flox/flox/D1CRE/+} mice.

Presynaptic D2 autoreceptor mutants;

D2-like antagonist administration.

Haloperidol is a specific D2-like antagonist. This drug is able to induce catalepsy when it is administered systemically through blockade of D2 heteroreceptor located at postsynaptic sites. D2R^{flox/flox/En1CRE/+} mice and the WT siblings were thus challenged with either saline or 0.04 ,0.4 and 4 mg/kg of haloperidol, and the catalepsy has been measured by performing the bar test (**Fig.11b**). Three hours after treatment, mice were positioned with the front paws on a metallic bar situated at 3.5 cm from the floor and the time needed for bringing the paws to the floor scored. D2R^{flox/flox/En1CRE/+} mice showed a stronger degree of catalepsy at the lower doses (0.04mg/kg) of haloperidol as compared to WT controls. While at the dose of 0.4 and 4 mg/kg haloperidol no differences in catalepsy were observed between D2R^{flox/flox/En1CRE/+} mice and WT controls. These results show that in D2R^{flox/flox/En1CRE/+} mice the postsynaptic D2 heteroreceptor mediated signaling is preserved. The higher response of D2R^{flox/flox/En1CRE/+} mice to the lower dose of haloperidol (0.04mg/kg) can be explained by the reduced number of binding sites present in these mice (as showed in the binding experiment), which very likely allow an increase of the available drug in these mutants.

Postsynaptic D2 heteroreceptor mutants; D2-like antagonist administration

D2R^{flox/flox/D1CRE/+} mice were tested for the effect of haloperidol on catalepsy by the bar test as previously described in D2R^{flox/flox/En1CRE/+} mice. In these mice, we used the two highest concentrations of haloperidol (0.4 mg/kg and 4 mg/kg) (**Fig. 11d**). Interestingly, in D2R^{flox/flox/D1CRE/+} mice we did not observe catalepsy at

the dose of 0.4 mg/kg as compared to WT controls; a minimal effect was observed at 4mg/kg which was anyway irrelevant respect to that obtained in WT controls. These data support the role of D2Rs expressed by the MSNs in eliciting the cataleptic effects of haloperidol

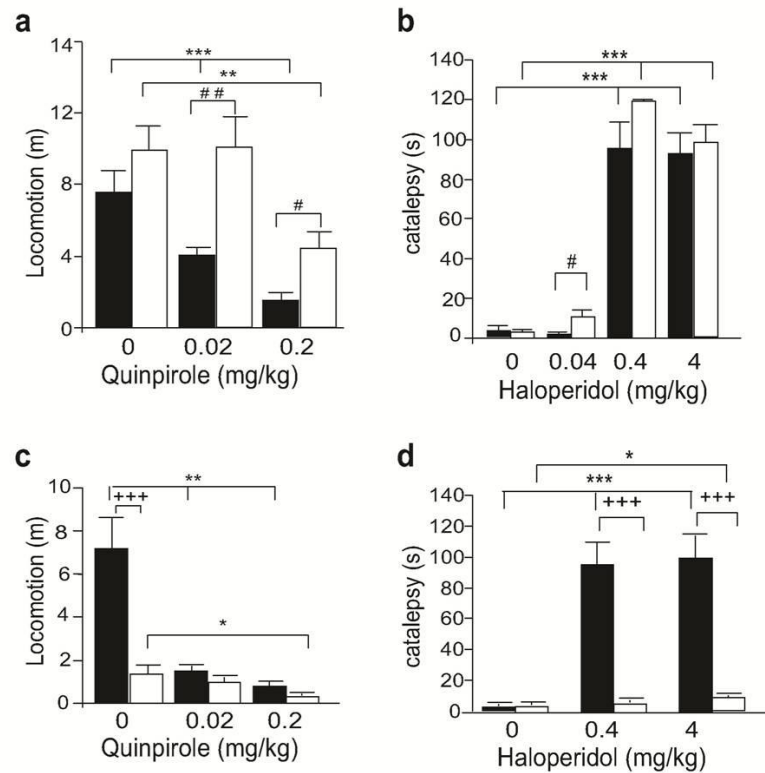


Fig.11) Behavioral effects of D2-like agonists and antagonists in D2R^{flox/flox/En1Cre/+} and D2R^{flox/flox/D1Cre/+} mice.

a) Differential quinpirole induced motor sedation in D2R^{flox/flox} (black bars) vs D2R^{flox/flox/En1Cre/+} (white bars) mice; Two-way Anova significant Genotype ($F_{(1,48)} = 18.967$, $P < 0.001$) and Treatment effects ($F_{(2,48)} = 17.878$, $P < 0.001$). **b)** Haloperidol-induced catalepsy in D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars) mice; Two-way Anova Treatment effect ($F_{(3,81)} = 135.637$, $P < 0.001$) and a trend toward genotype effect ($F_{(1,81)} = 3.906$, $P = 0.052$). **c)** Quinpirole induced sedation in D2R^{flox/flox} (black bars) vs D2R^{flox/flox/D1Cre/+} (white bars) mice; Two-way Anova: Genotype x Treatment ($F_{(2,37)} = 8.284$, $p < 0.01$). **d)** Haloperidol-induced catalepsy in D2R^{flox/flox} (black bars) vs D2R^{flox/flox/D1Cre/+} (white bars) mice. Two-way Anova Genotype x Treatment effect ($F_{(2,57)} = 14.635$, $p < 0.001$) Saline vs treated *: $P < 0.05$; **: $P < 0.01$; *** : $P < 0.001$; D2R^{flox/flox/En1Cre/+} treated vs D2R^{flox/flox} treated #: $P < 0.05$; ##: $P < 0.01$. D2R^{flox/flox/D1Cre/+} treated vs D2R^{flox/flox} treated +++: $P < 0.001$.

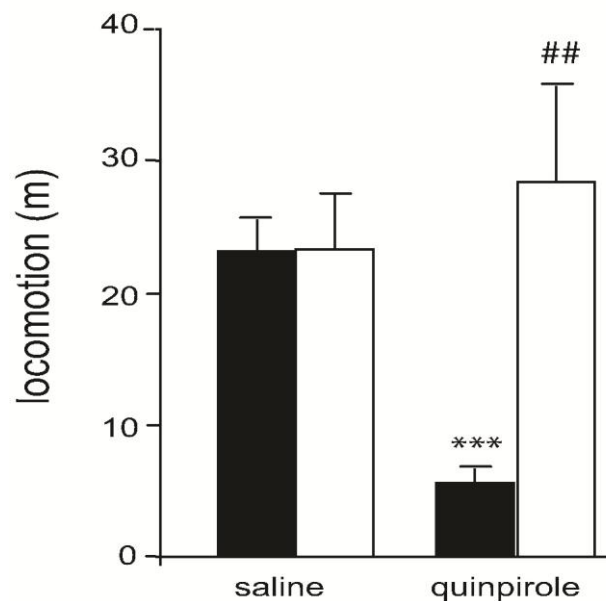


Fig.12) Open field in D2R^{flox/flox/En1Cre/+}

D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars) were treated with quinpirole (0.2mg/kg) and immediately exposed to the open field for 30 min. Motor activity was measured with a videotracking system. While quinpirole induced motor sedation in D2R^{flox/flox} mice, no effect of this drug was observed in D2R^{flox/flox/En1Cre/+} mice. Treatment x Genotype, ($F_{(1,33)}=10.94$). Saline vs quinpirole ***: $P < 0.001$; D2R^{flox/flox} treated vs D2R^{flox/flox/En1Cre/+} treated ##: $P < 0.01$.

Pre- and post-synaptic D2R-mediated effects on DA synthesis and release

Electrophysiological characterization of the response of dopaminergic neurons in D2R^{flox/flox/En1CRE/+} mice.

To assess the firing properties of dopaminergic neurons in D2R^{flox/flox/En1CRE/+} mice, we collaborated with the laboratory of Dr. Antonello Bonci. Ex-vivo brain slice electrophysiology was performed. Recordings were performed in cell-attached mode to preserve the natural intracellular milieu of the neurons. SNc and VTA neurons from D2R^{flox/flox/En1CRE/+} mice and control littermates were tested. SNc and VTA neurons possessed action potential waveforms of ~1.5-1.8 ms under the cell-attached configuration (Ungless et al. 2004), and were I_h (hyperpolarization-activated current) positive when examined

after breaking into whole-cell mode at the end of the experiment. D2R receptors negatively regulate firing in midbrain DA neurons by activating hyperpolarizing inward rectifier K^+ (GIRK) channels (Beckstead et al. 2004). Neurons from WT mice showed the expected suppression of firing by quinpirole (2 μ M) in both SNc and VTA; while neurons from $D2R^{\text{floxflox/En1Cre/+}}$ mice did not show this inhibition. The GABA_B receptor agonist baclofen (2 μ M) was able to strongly inhibit firing in both WT and $D2R^{\text{floxflox/En1Cre/+}}$ neurons, suggesting that activation of GIRK channels by a receptor other than D2R remained intact in neurons lacking presynaptic D2 autoreceptors (**Fig. 13**).

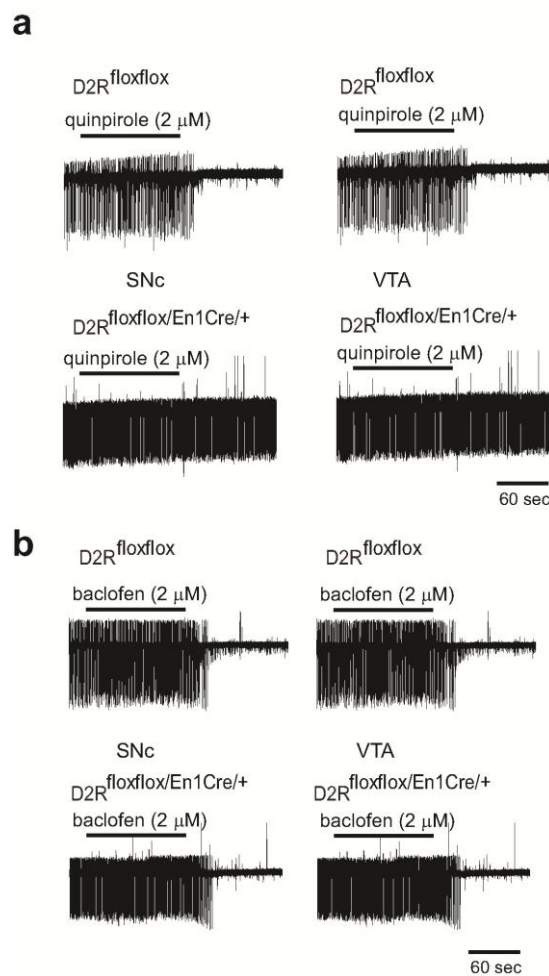


Fig. 13) Electrophysiological characterization of SNc and VTA neurons of $D2R^{\text{floxflox/En1Cre/+}}$ mice.

a) Loss of presynaptic D2 autoreceptor-mediated inhibition of firing in SNc and VTA of $D2R^{\text{floxflox/En1Cre/+}}$ mice as compared to control mice. **b)** Normal GABA_B-mediated inhibition of firing in both genotypes (representative examples).

Tissue content of DA and its metabolites in D2R^{flox/flox/En1CRE/+} mice.

The tissue content of DA and its metabolites 3,4-dihydroxyphenylacetic acid (Dopac) and homovanillic acid (HVA) was determined in homogenates of tissues punches from the Nucleus accumbens and dorsal striatum of D2R presynaptic ko mice and their control littermates.

The content was quantified by high performance liquid chromatography (HPLC) analysis. The total content of DA and HVA was increased in the NAcc and cortical extracts of D2R^{flox/flox/En1CRE/+} mice as compared to the WT littermates. Importantly, no differences either in DA or in its metabolites were observed in extracts from the dorsal striatum between D2R^{flox/flox/En1CRE/+} mice and WT controls (**Fig.14 a,b,c**).

Tissue content of DA and metabolites in D2R^{flox/flox/D1CRE/+} mice.

The content of DA and its metabolites was similarly analyzed D2R^{flox/flox/D1CRE/+} mice and WT littermates. In this case, no differences were observed between genotypes in all the regions tested (**Fig.14 c,d,e**).

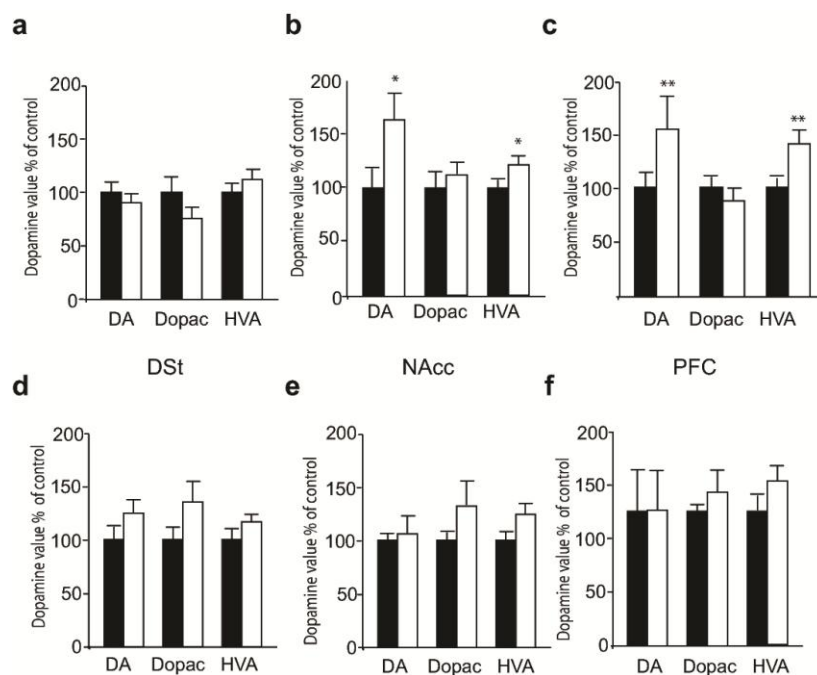


Fig.14) Percentage of DA, DOPAC and HVA in D2R^{flox/flox/En1Cre/+} and, D2R^{flox/flox/D1Cre/+} .

D2R^{flox/flox} levels, were arbitrarily set at 100%. Determination were made by HPLC on extracts from tissue punches of **a)** DSt; **b)** NAcc and **c)**

PFC from D2R^{flox/flox} (black bars), D2R^{flox/flox/En1Cre/+} (white bars). **d)** DSt; **e)** NAcc and **f)** PFC from D2R^{flox/flox} (black bars), D2R^{flox/flox/D1Cre/+} (white bars). *P<0.05, **P<0.01. Student's was used for statistical analysis.

DA release

DA release in D2R^{flox/flox/En1CRE/+} mice.

To assess the role of D2 autoreceptors on the DA release from neuronal terminals, we performed in D2R^{flox/flox/En1CRE/+} mice (collaboration with Dr. David Sulzer's laboratory) cyclic voltammetry experiments in acute brain slices of dorsal striatum and NAcc, under normal conditions or in the presence of quinpirole (D2-like agonist). In the dorsal striatum, D2R^{flox/flox/En1CRE/+} mice compared to the WT mice showed a reduction in DA overflow close to 70% when they were challenged by a single pulse. However, when the same area was challenged with a train of 5 or 10 pulses at 20 Hz, D2R^{flox/flox/En1CRE/+} mice showed an increase of DA overflow of 1.1 and 1.2 fold, respectively as compared to the control mice (**Fig.15**).

D2R^{flox/flox/En1CRE/+} mice and WT littermates were then tested for DA release in the presence of quinpirole. In these conditions, unexpectedly, D2R^{flox/flox/En1CRE/+} mice showed a reduction of DA release of around 80% as compared to the WT controls (**Fig.15b**).

Then we performed the similar analyses in the NAcc of D2R^{flox/flox/En1CRE/+} mice and WT littermates by cyclic voltammetry. In this case we could only perform experiments in the absence of quinpirole due to the low baseline DA level in this area, which makes the amount of DA detectable close to background levels.

D2R^{flox/flox/En1CRE/+} mice compared to WT controls showed a decrease of DA overflow close to 41% by the single pulse in cyclic voltammetry. The 20 Hz train of pulse at 5 and 10 showed an increase in the DA release of 1.3 and 1.4 fold respectively, as compared to the WT controls (**Fig.16**).

The results obtained by single pulses in D2R^{flox/flox/En1CRE/+} mice are similar to those previously described in the D2R-null mouse model (Schmitz et al. 2002). The effect of quinpirole was however completely unexpected, since this drug continued to inhibit DA overflow in the absence of D2 autoreceptors in marked contrast with the complete loss of quinpirole inhibition in D2R-null mice (Benoit-Marand et al. 2001; Schmitz et al. 2002). These results contradict the "assumption" that the inhibition of DA release by quinpirole is selectively due to the activity of presynaptic D2 autoreceptors and strongly point to a postsynaptic D2R-mediated intervention of D2 heteroreceptors in the control of DA release in the absence of autoreceptors.

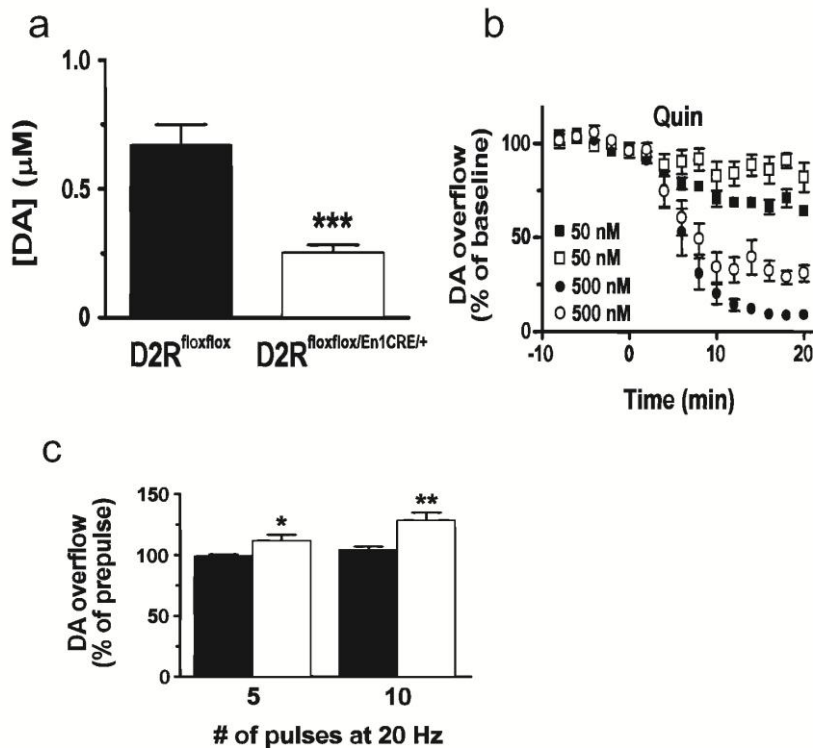


FIG.15) DA release in the dorsal striatum of D2R^{flox/flox/En1Cre/+}

a) Single-pulse stimulation in D2R^{flox/flox/En1Cre/+} mice (n = 19–20) **b)** D2R^{flox/flox/En1Cre/+} mice DA overflow in the presence of quinpirole. (50 nM: n = 7, $F_{(1,261)} = 8.46$, $P = 0.0039$; 500 nM: n = 7–8, $F_{(1,204)} = 23.52$, $P < 0.001$). **c)** Train of pulses at 20 Hz displayed enhanced DA overflow in D2R^{flox/flox/En1Cre/+} mice (CV; n = 6–12; genotype: $F_{(1,32)} = 18.98$, $P = 0.0001$).

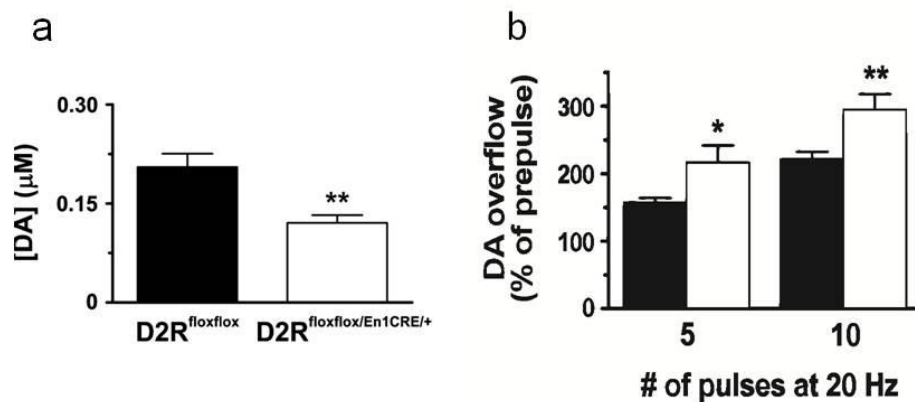


Fig.16) Voltammetry NAcc of D2R^{flox/flox/En1Cre/+}

a) Cyclic voltammetry experiments in the shell of the NAcc of D2R^{flox/flox/En1Cre/+} mice. **b)** Train of pulses at 20 Hz displayed enhanced DA overflow in the shell of the NAcc of D2R^{flox/flox/En1Cre/+} (n = 6–9; $F_{(1,29)} = 21.37$, $P < 0.0001$).

DA release, in D2R^{flox/flox/D1Cre/+} mice.

Cyclic voltammetry in presence and absence of quinpirole was also performed in the dorsal striatum of D2R^{flox/flox/D1Cre/+} mice and WT littermates.

Importantly, when the D2R^{flox/flox/D1Cre/+} mice were tested by a single pulse, they showed a slight decrease of DA overflow of around 20% in comparison to WT littermates. In the train of 5 or 10 pulses at 20 Hz no difference in DA release were noted with respect to WT controls (**Fig.17**). When D2R^{flox/flox/D1Cre/+} mice were analyzed in the presence of quinpirole, interestingly they showed an inhibition of DA overflow, which did not reach the level of WT (79%).

These data strongly support a role of the postsynaptic D2 heteroreceptors in the regulation of DA release from the presynaptic dopaminergic terminals. Thus, for the first time, we were able to show that DA release is not only controlled by autoreceptors, but that in their absence an inhibitory feedback is established which is activated by postsynaptic D2R.

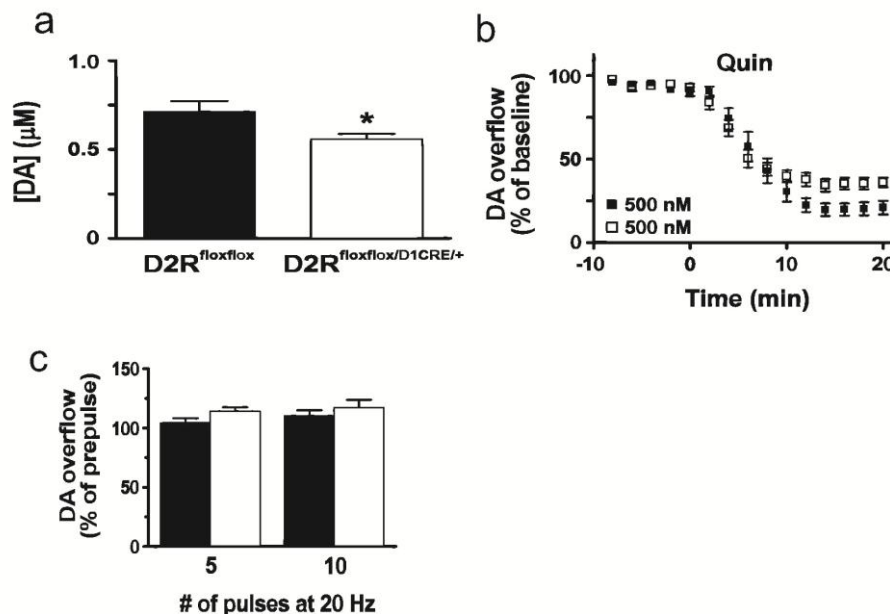


Fig.17) DA release in the dorsal striatum of D2R^{flox/flox/D1Cre/+}

a) Single-pulse stimulation in D2R^{flox/flox/D1Cre/+} mice (n = 35–37). **b)** D2R^{flox/flox/D1Cre/+} mice Da overflow in the presence of quinpirole (500 nM (n = 9–10, $F_{(1,274)} = 33.23$, $P < 0.0001$). **c)** Train of pulses at 20 Hz displayed no changes in DA overflow in D2R^{flox/flox/D1Cre/+} mice (CV; n = 8–12; genotype: $F_{(1,36)} = 2.76$, $P = 0.11$).

DA reuptake

DA reuptake can be estimated with CV from the kinetic parameters of falling phase of the signal: of these, the duration at half-height is a particularly reliable indicator of reuptake. We noted a significant increase in DA reuptake in D2R^{flox/flox/En1Cre/+} mice, whereas reuptake by D2R^{flox/flox/D1Cre/+} mice was identical to WT littermates (**Fig.18**). In addition, we observed that while the DAT inhibitor, nomifensine (5 μ M), did not increase the amplitude of evoked DA overflow of D2R^{flox/flox/En1Cre/+} mice to levels of the WT (0.92 vs 2.36 μ M, respectively; **Fig.18c**) the normalized increase of peak amplitude was higher in the mutant (2.8 vs 2.0-fold increase, respectively; **Fig.18d**). This enhanced response to nomifensine is consistent with a greater relative contribution of DAT activity to peak amplitude in the presynaptic D2 autoreceptor mutant, and is consistent with D2 autoreceptor inhibition of reuptake. Only the D2 autoreceptor, however, appears to inhibit reuptake by DAT even if the postsynaptic D2R showed an effect when the system is challenged with quinpirole.

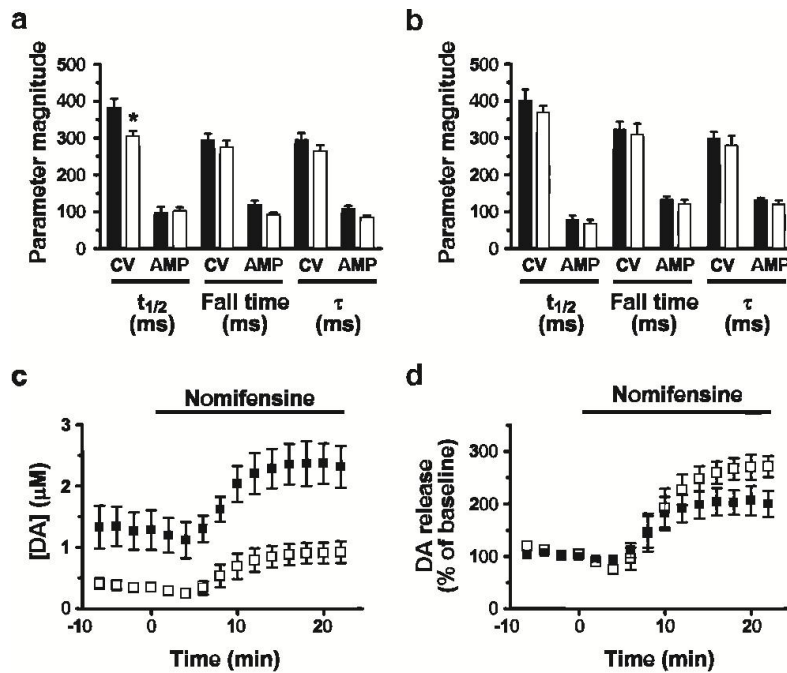


Fig.18) DA reuptake

D2R^{flox/flox}: (black bars), D2R^{flox/flox/En1Cre/+} or D2R^{flox/flox/D1Cre/+}: (white bars). **a)** Decay parameters calculated from the DA overflow spikes obtained from CV experiments showing that D2R^{flox/flox/En1Cre/+} mice exhibit a significant change in spike width ($t_{1/2}$). The decay time from the 75 to 25% of the spike (fall time) and the decay constant (τ) presented no changes ($n = 16-17$). **b)** Decay parameters calculated from the DA overflow spikes in D2R^{flox/flox/D1Cre/+} mice showed no significant changes in spike width, decay time, and decay constant (CV: $n = 13-14$; AMP: $n = 10$). **c)** DA overflow measured in D2R^{flox/flox/En1Cre/+} mice during nomifensine-mediated blockade of DAT does not reach the same levels of WT mice (nomifensine: 5 μ M; $n = 4$; increase in [DA], D2R^{flox/flox}: 2.36 μ M; D2R^{flox/flox/En1Cre/+}: 0.92). **d)** Nomifensine-mediated blockade of DAT induces a significant increase in DA overflow in D2R^{flox/flox/En1Cre/+} mice when compared to the baseline-normalized response with respect to WT mice (nomifensine: 5 μ M; $n = 4$; increase in [DA], D2R^{flox/flox}: ~2-fold; D2R^{flox/flox/En1Cre/+}: ~2.7-fold; $F_{(1,89)} = 8.61$, $P = 0.004$). Maximal DA-overflow concentration and normalized response were calculated using a nonlinear regression which takes in consideration the plateau and the rising independently. Two-tailed unpaired t-test with Welch's correction was used in panel a) and b).

DA synthesis

DA synthesis in D2R presynaptic ko

In addition to modulate DA release, D2 receptor has also the important function to inhibit DA synthesis.

Tyrosine hydroxylase (TH) is the rate limiting enzyme of DA synthesis; its activity is increased by phosphorylation at Ser 40 (Lindgren et al. 2000). To assess the role of pre- and post-synaptic D2R on TH activity we prepared protein extracts from tissue punches of dorsal striatum and NAcc and analyze them by western blot using specific antibodies raised against TH phosphorylated at Ser 40. Quantifications of the bands obtained this way were then expressed as ratio over total TH, to control for the amount of protein present in the extracts. It is important to point out that all extracts showed an equal amount of total TH, indicating that TH expression is not altered between mutants and control mice.

Interestingly, we found that absence of D2 autoreceptors in D2R^{flox/flox/En1CRE/+} mice does not alter the TH phosphorylation level on Ser 40 in the striatum as compared to WT controls. However, in the NAcc we found an important increase of this TH modification as compared to WT extracts. These data are in agreement with those obtained by the HPLC analyses. D2R^{flox/flox/En1CRE/+} mice and controls were then administered with quinpirole at the concentration of 0.2 mg/kg and TH phosphorylation was analyzed. Interestingly, while in the dorsal striatum of both genotypes, quinpirole was still able to reduce TH phosphorylation at Ser 40; it did not affect this parameter in the NAcc of D2R^{flox/flox/En1CRE/+} mice (**Fig.19**).

These results indicate the presence of a postsynaptic D2R activated feedback mechanism in the striatum that control TH activity; this mechanism becomes evident in the absence of D2 autoreceptors.

In the NAcc this mechanism seems to not be present, in agreement with the hyper-activity showed by D2R^{flox/flox/En1CRE/+} mice when tested in a novel environment (open field) or when administered with cocaine; both conditions activating the VTA-NAcc pathway.

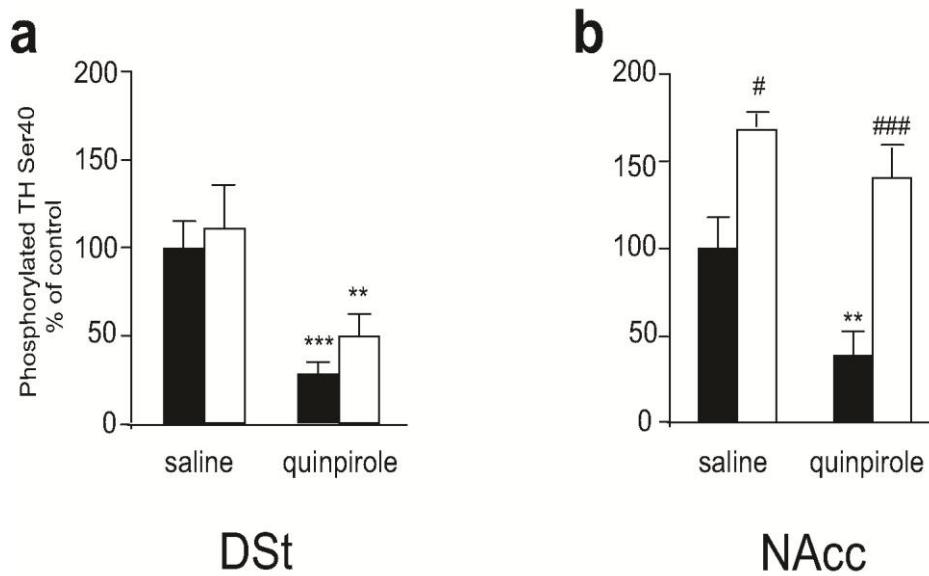


Fig.19) pTH-Ser⁴⁰ levels in DSt and NAcc extracts of D2R^{flox/flox/En1Cre/+}.

Western-blot analyses of pTH-Ser⁴⁰ levels in DSt and NAcc extracts (30 µg/sample) of D2R^{flox/flox/En1Cre/+}, D2R^{flox/flox/D1Cre/+} and D2R^{flox/flox} mice treated either with saline or with quinpirole (0.2mg/kg), as indicated. Values represent the ratio of pTH-Ser⁴⁰/total TH; the values of the ratio from saline treated D2R^{flox/flox} mice were arbitrarily set at 100%. **a)** Ratios obtained from the analyses of DSt extracts from D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars). Two-way Anova shows no genotype effect: $F_{(1,17)}=1.848$ $p>0.05$. **b)** Same as in a) for NAcc extracts, D2R^{flox/flox} (black bars) and D2R^{flox/flox/En1Cre/+} (white bars). Two-way Anova shows Genotype effect: $F_{(1,17)}=33.126$ $P<0.001$. Values are mean \pm s.e.m. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs saline control. D2R^{flox/flox} vs D2R^{flox/flox/En1Cre/+} #: $P < 0.05$, ##: $P < 0.01$.

DA synthesis in the postsynaptic ko mice.

TH phosphorylation at Ser 40 was then analyzed in D2R^{flox/flox/D1CRE/+} mice using the same approach used for the analysis of D2R^{flox/flox/En1CRE/+} mice. Extracts from dorsal striatum and NAcc of D2R^{flox/flox/D1CRE/+} mice and WT littermates were analyzed by western blot analysis. Under saline conditions, D2R^{flox/flox/D1CRE/+} mice did not differ from WT littermates in the amount of TH phosphorylation at Ser 40, both in the dorsal striatum and NAcc. Also, both genotypes equally showed a reduction of TH phosphorylation at the Ser40 residue when challenged with 0.2mg/kg of quinpirole (**Fig.20**).

These results clearly show the presence of D2 autoreceptors in $D2R^{flox/flox/D1Cre/+}$ mice is sufficient to ensure the appropriate control over DA synthesis, suggesting that the D2-mediated postsynaptic feedback on DA synthesis becomes evident only when the D2 autoreceptors are absent.

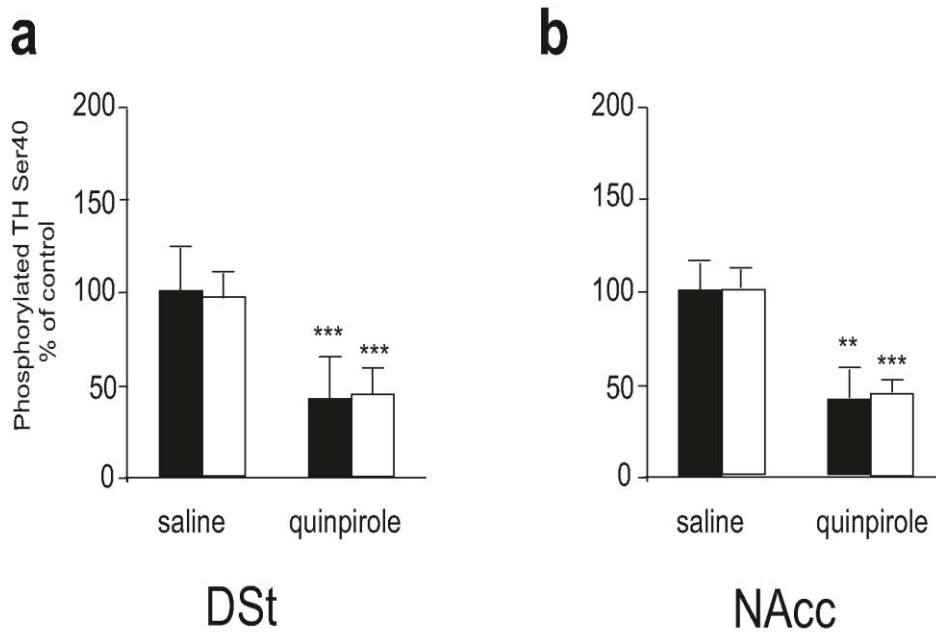


Fig.20) pTH-Ser⁴⁰ levels in DSt and NAcc extracts of $D2R^{flox/flox/D1Cre/+}$

$D2R^{flox/flox/D1Cre/+}$ (white bars) and $D2R^{flox/flox}$ (Black bars) mice treated either with saline or with quinpirole (0.2mg/kg), as indicated. Values represent the ratio of pTH-Ser⁴⁰/total TH; the values of the ratio from saline treated $D2R^{flox/flox}$ mice were arbitrarily set at 100%. **a)** Ratios obtained from the analyses of DSt extracts from $D2R^{flox/flox}$ (black bars) and $D2R^{flox/flox/D1Cre/+}$ (white bars) Two-way Anova no Genotype effect: $F_{(1,18)} = 1.984$ $P > 0.05$. **b)** Same analysis as in a) but using NAcc extracts from $D2R^{flox/flox}$ (black bars) and $D2R^{flox/flox/D1Cre/+}$ (white bars).

Discussion

DA is a key neurotransmitter in the basal ganglia. The understanding and the study of dopaminergic system is difficult due to the presence of dopaminergic receptors in different areas of the brain with similar pharmacological pattern.

The study of the D2 receptor is made difficult due to the presynaptic and postsynaptic localization, this makes hard to discern the functions based on pharmacological treatment.

During my thesis I have used mouse models previously generated in the laboratory; in these models the D2 receptor has been removed in a site-specific manner either from the dopaminergic neurons or in striatal MSNs.

DA functions are implicated in a broad range of neurological, neuropsychiatric and endocrine disorders (Grace et al. 2008). The analysis of D2R-null mice has been critical to assess the importance of this receptor in dopaminergic functions (Baik et al. 1995). However the full ablation of D2R from every site expressing it did not allow the analysis of the presynaptic (autoreceptor) versus the postsynaptic (heteroreceptor) mediated functions in vivo.

Interestingly, the comparison of pre- and post-synaptic D2R mutants has shown unexpected results. Indeed, D2R^{flox/flox/En1CRE/+} mice show normal motor activity and coordination with respect to D2R^{flox/flox/D1CRE/+} mice, in which the motor activity is highly depressed. Thus, loss of D2 autoreceptors does not seem to have a strong impact on locomotion under basal conditions; instead, these animals are hyperactive when exposed to a novel environment and they show an increased motor response when administered with cocaine (DAT blocker). On the contrary, the analysis of D2R^{flox/flox/D1CRE/+} mice shows the importance of postsynaptic D2 receptor on MSNs for a normal locomotion; the absence of D2 receptor in these neurons prevents a normal motor activity and coordination. In addition, D2R^{flox/flox/D1CRE/+} mice show a minimal response to cocaine administration, which closely resemble the phenotype of D2R-null mice.

Loss of D2 autoreceptors in D2R^{flox/flox/En1CRE/+} mice results in the inability by D2 agonists to inhibit the firing properties of dopaminergic neurons. Cyclic Voltammetry analyses performed in pre-synaptic D2 autoreceptor mutants have shown a reduced DA overflow in single pulse experiments in the dorsal striatum, similar to that observed in D2R-null mice (Schmitz et al. 2002). However, the same experiment performed in the NAcc showed a less efficient reduction of DA overflow, these findings suggest a different control over DA release in the dorsal or ventral striatum with important physiological implications. In addition, we were able to show for the first time the presence of a postsynaptic D2R-mediated feedback mechanism controlling DA release. This role has

been further demonstrated at the level of DA synthesis, by the ability of quinpirole to decrease TH phosphorylation at Ser 40 in the dorsal striatum of D2R^{flox/flox/En1CRE/+} mice. This inhibition is very likely not due to the D2R-mediated inhibition of the cAMP levels, but dependent of the activation of postsynaptic circuits regulating the activity of dopaminergic neurons. This postsynaptic effect appears to have a minor role in the NAcc in which the single pulse voltammetry experiment showed a smallest reduction of DA overflow than in the striatum and in which TH phosphorylation is not inhibited by quinpirole.

The differences between dorsal striatum and NAcc are further confirmed by behavioral analyses showing that the ability of quinpirole to reduce motor behavior was present only when D2R^{flox/flox/En1CRE/+} mice were tested in the new home cage, while it did not affect the locomotion in the open field. The lowest amount of DA released from the NAcc, did not allow to measure the loss of inhibition in this region in the presence of quinpirole; nevertheless in the dorsal striatum of D2R^{flox/flox/En1CRE/+} mice quinpirole is able to decrease DA release with less potency than in control mice mirroring the behavioral finding of the new home cage.

In D2R^{flox/flox/D1CRE/+} mice mice cyclic voltammetry showed that the inhibition by quinpirole of DA release is 20% less efficient than in the WT mice, despite to the presence of D2 autoreceptors on the dopaminergic neurons. This finding confirms behavioral analyses, in which despite of the presence of presynaptic D2 autoreceptors, quinpirole affected motor behavior only at the higher dose tested.

D2Rs on dopaminergic neurons are key effectors in the control of DA release, nevertheless in their absence a postsynaptic D2R mediated feedback controls DA release. This control might be executed through regulation of circuits that converge onto dopaminergic neurons and could be mediated by other neurotransmitters such as glutamate, GABA, acetylcholine.

5. Conclusions

The findings of these studies bring us to propose that postsynaptic D2 heteroreceptors, in condition in which the autoreceptor is not functional, activate a feedback mechanism. This can be possible through increase/decrease of other neurotransmitters which could affect DA synthesis and release from the dopaminergic neurons (Bamford et al. 2004) (Surmeier et al. 2007). Importantly, the impact of absence of D2 autoreceptors on SNc and VTA neurons seems to be different. Synthesis is affected only in the projections to NAcc, but not in the projections to dorsal striatum in agreement with the DA tissue content and TH activity. The D2R-mediated feedback mechanisms controlling DA release, in the absence of D2 autoreceptors, seem to be confined to dorsal striatum. Dopaminergic neurons originating from VTA and SNc have been reported to have different properties (Lammel et al. 2008) (Lammel et al. 2011) (Cragg 2005); these properties seems to be mediated not only from the different area of origin of dopaminergic neurons, but particularly from a D2R-mediated effect activated in postsynaptic neurons.

Thus in conclusion, my studies show that DA synthesis and release are functions that can be assigned not only to the presynaptic D2 autoreceptor, but also to postsynaptic D2 heteroreceptors. Indeed, when D2 autoreceptors are absent, DA stimulation of D2 heteroreceptors reveals feedback mechanisms which become dominant in the control of dopaminergic neurons activity.

Future studies might identify which other neurotransmitter could be responsible of these regulations and where these interactions take place.

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