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DOTTORATO DI RICERCA IN BIOCHIMICA CELLULARE ED ATTIVITA' DEI FARMACI IN ONCOLOGIA (XIX CICLO)

The multiple roles of Dopamine D2 Receptors in health and disease

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INTRODUCTION

1. Dopaminergic System

The neurotransmitter, dopamine (DA), is the most abundant catecholamine in the central nervous system (CNS) where it is involved in a variety of physiological functions including motor control, sexual behaviour, cognition (Blackburn et al., 1992; Jackson and Westlind-Danielsson, 1994; Missale et al., 1998) and control of hormone synthesis and release (Vallone et al., 2000).

The etiology of several diseases has been linked to defective DA neurotransmission. In 1966, Oleh Hornykiewicz described that the content of DA, norepinephrine (NE), and serotonin (SER) was altered in post-mortem brains of patients with Parkinson's disease. In particular DA was the most drastically reduced (Hornykiewicz, 1966). This was the first observation that showed an association between a deficiency in a neurotransmitter and a neurological disease. Parkinson’s disease is characterised by akinesia, rigidity and resting tremors. This neuropathology can be partially alleviated by the administration of L-dopa the biosynthetic precursor of DA, as well as, by DA receptor agonists such as bromocryptine, lergotrile and apomorphine {Hornykiewicz, 1966 #6; Calne, 1978 #7}.

Alterations of the dopaminergic system (Figure1) have also been associated with other dysfunctions in CNS, such as Tourette's syndrome and schizophrenia. According to hypothesis that schizophrenia might directly result from an hyper-activity of the dopaminergic system (Carlsson, 1977;

Bowers et al., 1980; Seeman and Kapur, 2000), the administration of DA antagonists, which decrease the dopaminargic neurotra nsmission, by blocking the activity of DA receptors, are commonly used to hasten remission of acute psychotic illnesses and prevent later exacerbation of chronic psychotic symptoms (Creese et al., 1976).

In addiction, dopaminergic neurotransmission has a key role in the control of lactotrop cell proliferation, indeed loss of D2R mediated signalling leads to the formation of prolactinoma, the most frequent pituitary tumour, in young woman (Iaccarino et al., 2002). In particular Dopamine D2 receptors (D2R) agonists, such as bromocriptine and pergolide, are routinely admistrated in human patients in case of prolactinomas diagnosis.

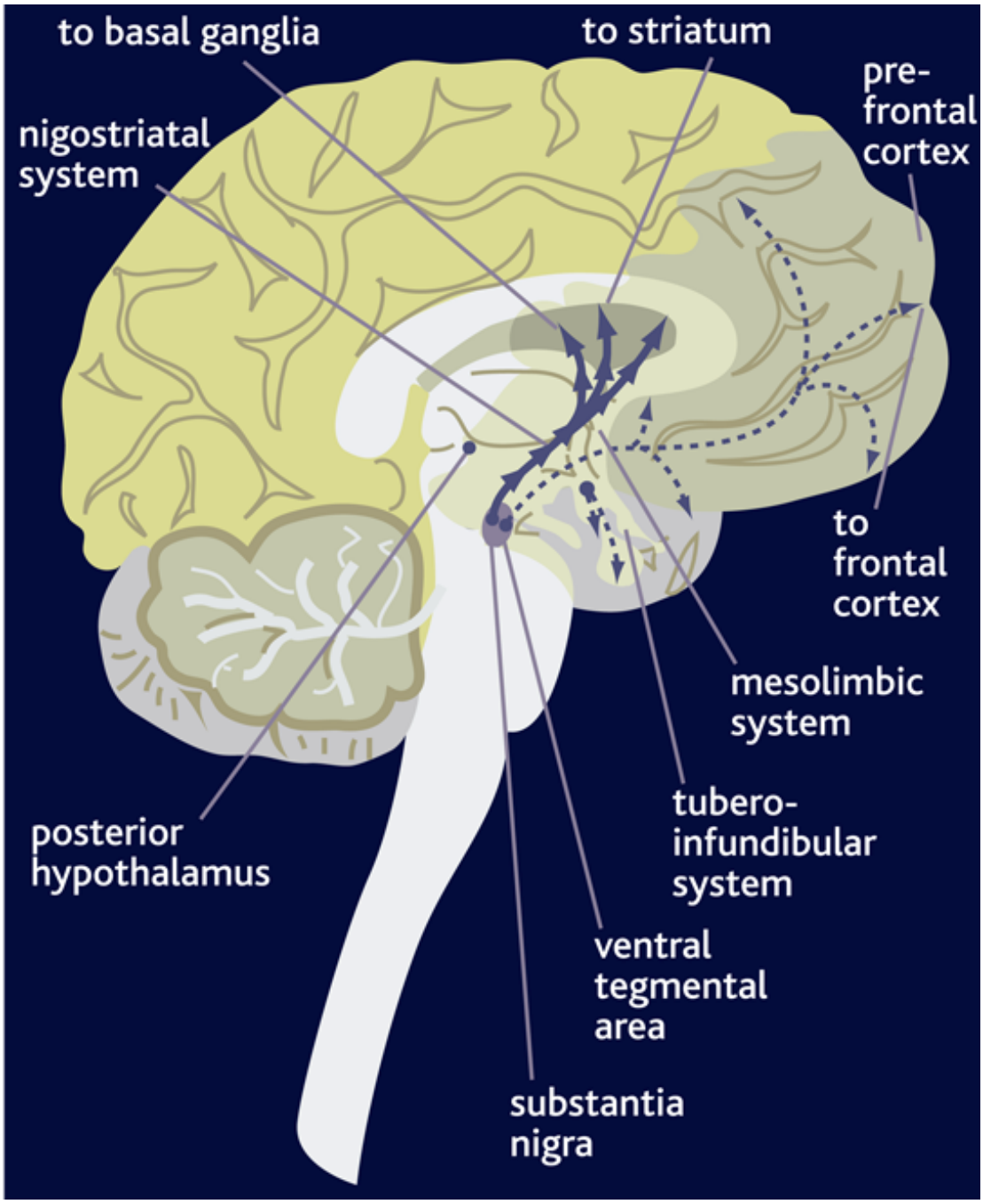

Figure 1. The nigrostriatal and mesolimbic dopamine tracts

2. Dopaminergic Pathways

Although it was established in the 1950s that dopamine was a neurotrasmitter in the CNS, the mechanisms by which DA alteres neuronal signalling in the CNS are still not well understoond (Carlsson et al., 1958). The development of methods for measuring and localizing DA preceded by two decades the development of methods for assaying receptors that responded to DA. One of the most important advances in the ability to define dopaminergic pathways in the brain came with the improvements in fluorescence histochemistry: these techniques have played a crucial role in the modern delineation of the DA circuit in the CNS (Dahlstrom and Fuxe, 1964).

Immunocytochemical studies, together with the application of autoradiographic techniques for visualizing and quantifying the dopaminergic innervation of brain regions have provided new details to assist in the analysis of the distribution of DAergic system (Hokfelt et al., 1984; Joyce et al., 1991).

The dopaminergic (DA) neurons represent a relatively rare neurotransmitter phenotype in the CNS. The majority of dopaminergic neurons in the mammalian CNS are located in the midbrain, where they form the substantia nigra (SN), the ventral tegmental area (VTA) and the retrorubral nuclei. Midbrain DA neurons project to the striatum (or caudate-putamen complex), limbic system and frontal cortex, and recieve afferents

from multiple structures in the diencephalon and telencephalon. Projections originating from brain areas that synthesize this neurotransmitter give rise to four axonal pathways (Figure 2): (1) projections constituting the Nigrostriatal pathway arise from dopamine-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, regulates the extrapyramidal motor system and its degeneration causes Parkinson's disease. 2) 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. It has been demonstrated to influence motivated behavior, such us emotional balance and reward. 3) The Mesocortical pathway arises from the ventral tegmental area (VTA) and projects to neocortex and prefrontal cortex. This pathway seems involved in some aspects of learning and memory; some examples are motivation, attention, planning and social behavior. Hyperactivity of this pathway has been associated with schizophrenia and hallucination. 4) The Tuberoinfundibular pathway arises from the hypothalamus. Projections of this pathway reach the median eminence of the hypothalamus where they release DA into the perivascular spaces of the hypophysial portal vessels. Thus, DA is transported to the anterior pituitary where it acts on the lactotrophs to inhibit prolactin (PRL) synthesis and release as well as plays an antiproliferative action on normal

and PRL-secreting tumors in experimental animals and humans. This hormone stimulates milk production from mammary glands and stimulates lactotroph proliferation by an autocrine mechanism in the pituitary gland.

The first three pathways do not exhibit very clear anatomical boundaries between the neurones of the different cell groups which have been described to appear at the same time during development**.** These observations, coupled with the overlap in some projection fields of the A8, A10 (ventral tegmental area) and A9 (substantia nigra) cell groups, have led to the suggestion that these neurones might be collectively indicated as the mesotelencephalic DA systems (Moore and Bloom, 1978)**.**

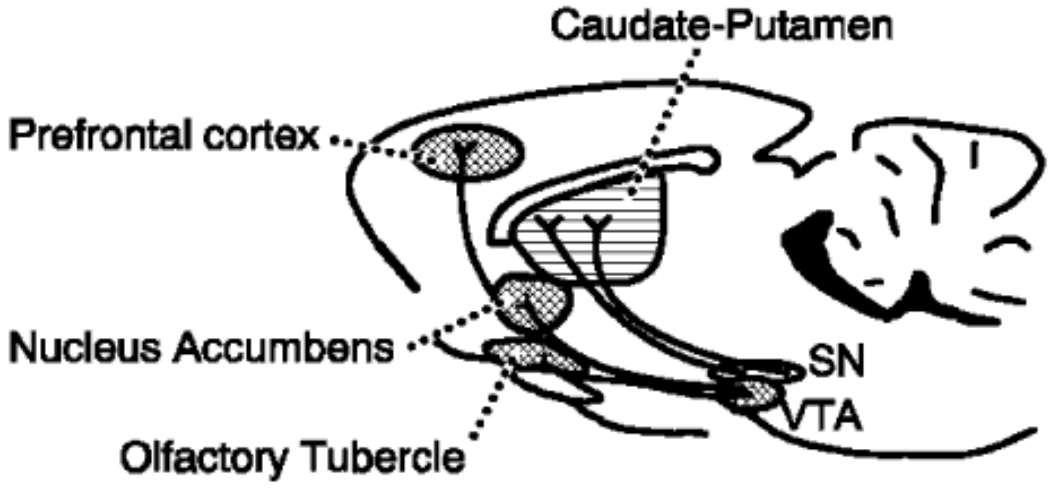

Fig 2: Mouse brain schematic diagram illustrating the ascending dopaminergic pathways arising within the substantia nigra (pars compacta, SNc, A9) and the ventral tegmental area (VTA, A10) depicting main projection areas.

3. Seven-Transmembrane domain receptors

The Seven-Transmembrane domain receptors (7TM) are commonly referred to as G-protein-coupled receptors (GPCR), because they signal through activation of heterotrimeric G-Proteins (Lefkowitz, 2000), also defined as Guanidine-nucleotide regulatory protein complex and composed of α and βγ subunits.

In the absence of agonist, 7TM receptors, such as the β-2 adrenergic receptor β2-AR, are in the low-affinity state. After agonist binding, a transient high-affinity complex of agonist, activated receptor, and G-protein is formed. GDP is released from the G-protein and is replaced by GTP. This leads to dissociation of the G-protein complex into, an α subunits and, a βγ dimer or complex, which both activate several effectors. Gs, for instance, activates adenylyl cyclase, which leads to an increase in cyclic AMP (cAMP). This increase in cAMP in turn activates protein kinase A (PKA), which is a serine/threonine kinase that phosphorylates many different substrates, including 7TM. Actually, each of these subunits is known to be a member of a gene family; 16 α, 5 β and 12 γ proteins have been cloned to date.

G-proteins are generally referred to by their α-subunits. So, the Gs heterotrimeric complex contains α-Gs, α-Gq contains Gq, Gi contains α-Gi and so on. Four distinct subunit subfamilies are recognized: Gs proteins couple to stimulation of adenylyl cyclase; Gi proteins couple to inhibition of adenylyl cyclase as well as to activation of G-protein-coupled inwardly

rectifying potassium (GIRK) channels; Gq proteins couple to the activation of phospholipase C; and G12 proteins couple to the activation of Rho Guanine-nucleotide Exchange Factors (GEFs). However, the combinatorial complexity of αβγ heterotrimers that might form is obviously great and, as yet, relatively little is understood of the specific subunit composition of the G proteins that function in specific pathways (Pierce et al., 2002).

Both, the α subunit and, the βγ dimer signal through the activation, or inhibition, of an ever-expanding list of effectors. Agonist activation of the receptors induces conformational changes which are, yet, poorly understood, but which seem to involve, at minimum, rearrangements of membrane helices 6 and 3. This "activated receptor" can interact with the heterotrimeric G protein, and serves as a guanidine exchange factors (GEF) to promote GDP dissociation, and GTP binding and activation. Receptors vary in their degree of agonist-independent or constitutive activity to couple to G-proteins; receptor mutants resulting in augmented activity have been found in numerous disease. In the current model, the activated heterotrimer dissociates into an α subunit and a βγ dimer, both of which have an independent capacity to regulate separate effectors. Hydrolysis of GTP to GDP, a process that is known to be regulated by regulator of G-protein signalling (RGS) proteins, leads to reassociation of the heterotrimer and termination of the activation cycle (Ross and Wilkie, 2000).

4. Dopamine Receptors

The first evidence for the existence of DA receptors in the CNS came in the early 70s from biochemical studies showing that DA was able to stimulate adenylyl cyclase (AC) (Kebabian and Calne, 1979). Indeed, the DA receptors belong to a large superfamily of neurotrasmitters and hormone receptors which are coupled to their specific effector functions via heterotrimeric GTP-binding proteins (Hepler and Gilman, 1992).

In 1979, Kebabian and Calne found that DA exerts its effects by binding to two receptors types, which were defined as D1 and D2 receptors based on pharmacological and biochemical properties (Kebabian and Calne, 1979). Although, for some time, ample behavioural, biochemical and pharmacological observations had pointed to DA receptor heterogeneity, it was only when it became possible to discriminate between agonist and antagonist action at the level of the cyclic adenosine monophosphate-(c-AMP)-second messenger generating enzyme, adenylyl cyclase (AC), that the dual DA receptor hypothesis became strongly well-established.

Pharmacologically, the hallmark of the D1 receptor is it's high affinity for benzazepine compounds such as SCH 23390, while D2 receptors are recognised for the high affinity for butyrophenones such as spiperone and haloperidol (Seeman and Van Tol, 1994). D1 and D2 receptors exert their biological actions by coupling and activating different G-protein complexes. The D1 receptors interact with the stimulatory G-proteins (Gs) and activate

adenylyl cyclase, whereas the D2R interacts with pertussis toxin sensitive G proteins (Go/i) to inhibit cAMP production (Gilman, 1984; Liu et al., 1994; Picetti et al., 1997)**.**

With the advent of molecular cloning techniques and their application to the neurobiology, it has become clear that the original D1R/D2R classification scheme was too much restrictive (Gingrich and Caron, 1993).

Thus far, five distinct genes encoding for different DA receptor proteins have been defined through molecular cloning. Two of these cloned receptors exhibit the functional and pharmacological properties expected for classical D1 receptors, while the other receptors show the pharmacological characteristics of D2 receptors (Jackson and Westlind-Danielsson, 1994; Missale et al., 1998).

It is now generally recognised that the family of D1R-like and D2R-like receptors exist rather than singular receptor subtypes (fig.3 and fig.4).

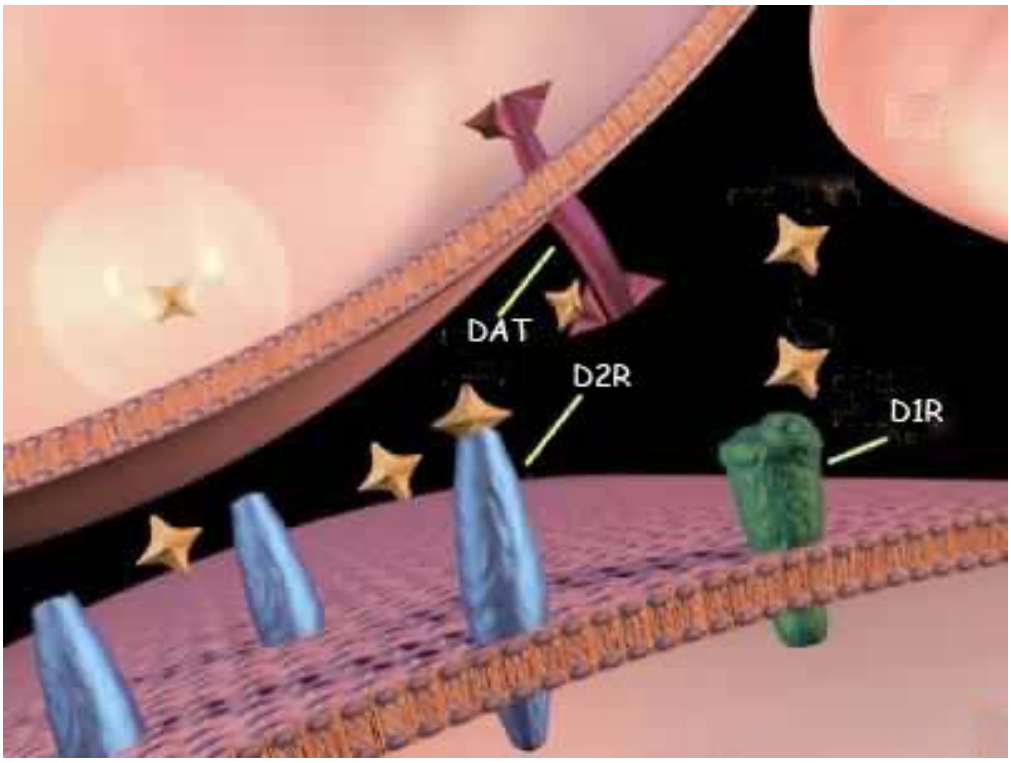

Figure 3: Dopaminergic Synapse and Synaptic Neurotransmission: DA binds D1 and D2R receptors. DAT is implicated in the DA reuptake. Dopamine is synthesized in the nerve terminal and packaged in vesicles. The vesicle fuses with the membrane and releases dopamine. The dopamine molecules can then bind to a dopamine receptor. After the dopamine binds, it comes off the receptor and is removed from the synaptic cleft by uptake pumps (DAT) that reside on the terminal. This process is important so that not too much dopamine is left in the synaptic cleft at any one time.

4.1 D1-like receptor subfamily

4.1.1 Dopamine D1 receptor

The D1 receptor linked to the activation of adenylyl cyclase activity was first cloned in 1990 (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). Overall, both the human and the rat receptors

are 446 residues in length, exhibiting 91% amino acid sequence conserved identity.

Hydropathy analysis of this protein predicts the presence of seven transmembrane domains where the N-terminus is localised to the extracellular surface and the C-terminus projects into the cytosol. This overall topography has been suggested for all of the G protein-linked receptors that have been cloned thus far. Several other structural features of the D1 receptor are particularly noteworthy. First, the D1 receptor genes appear to lack intones within the coding regions, there are two potential sites for N-linked glycosylation, one on the N-terminus and another on the second extracellular loop (Sunahara et al., 1990). Moreover, the D1 receptor has a small third cytoplasmic loop and a long C-terminus. These features are characteristic of Gs coupled receptors, and activate adenylyl cyclase such as the β-adrenergic receptor. In addition, there is one consensus site for cAMP-dependent phosphorylation in the third cytoplasmic loop and a conserved Cys at the C-terminus, the latter of which may serve as a site for palmitoylation.

When expressed in various mammalian cells, the cloned rat and human D1R have shown to exhibit the pharmacological and functional characteristics expected for a D1 receptor subtype. Indeed, pharmacologically specific binding for SCH23390 a D1-antagonist ligand, as well as the increase of cAMP induced by administration of SKF 38393 were observed using

transfected cells (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990).

4.1.2 Dopamine D5 receptor

The rat D5 DA receptor is 475 amino acids in length and presents overall 83% identity in comparison with the human D5R, but 95% identical residues in the transmembrane regions (Grandy et al., 1991; Sunahara et al., 1991; Weinshank et al., 1991). So far, it has not been possible to differentiate pharmacologically D1 and D5R receptors. The sensitivities of these two receptor subtypes to antagonists are similar. Nevertheless, these compounds generally show a slightly higher affinity for the D1R than D5R, with (+) butaclamol as the most discriminating. The affinity of agonists at D1R and D5R receptors is similar. The most consistent difference is represented by DA itself, which has around 10 times higher affinity for the D5R than D1R (Grandy et al., 1991; Sunahara et al., 1991; Weinshank et al., 1991).

4.2 D2-like receptors subfamily

4.2.1 Dopamine D2 receptor

The first member of the DA receptor family to be cloned was the D2R. The cDNA encoded a protein of 415 amino acids which, when expressed in

cells exhibited appropriate radioligand binding activity with the pharmacological characteristics expected for a D2 receptor (Bunzow et al., 1988).

Biochemical analysis indicated that the N-terminus contained consensus sequences for three potential N-linked glycosylation. In contrast to the D1 and D5 receptors, the size of the C-terminus of the D2R receptor is rather small while the third intra-cytoplasmic loop appears larger (Bunzow et al., 1988). This feature of having a large third cytoplasmic loop and a short C-terminus is a characteristic of many receptors, which inhibit adenylyl cyclase activity (Dohlman et al., 1991). The human homologue of the rat D2 receptor has been subsequently cloned and shown to be 96% identical to the rat receptor with one amino acid deletion (Grandy et al., 1989).

After the initial cloning of the D2 receptor cDNA a second very similar cDNA was isolated. It was then determined that the D2 receptor exists in two isoforms that differ in length by 87 nucleotides (29 amino acids) and are derived from the same gene by a mechanism of alternative splicing (Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989; O'Malley et al., 1990). The location of this splice variant occurs within the putative third intra-cytoplasmic loop (IL3) of the D2 receptor. Detailed investigation of the D2 receptor gene has revealed the presence of eight exons, seven of which are coding. The alternative splicing of the sixth exon, which encodes a 29 amino acid insertion in the putative IL3 of the receptor, generates the D2L

(444aa) and D2S (415aa) isoforms in rodents (Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989; O'Malley et al., 1990).

The two isoform of the D2R have been studied most extensively: both D2 receptor isoforms, D2RL and D2RS, are present in human, rat, bovine and mouse tissues. In situ hybridization and RNAse protection studies, *in vivo,* suggest that both mRNAs are co-expressed in the same cells and regions where D2R are expressed (Montmayeur et al., 1991; Montmayeur et al., 1993; Guiramand et al., 1995). Interestingly, the D2RL isoform appears to be predominantly expressed in all brain regions and tissue examined with respect to D2S (Montmayeur et al., 1991)**.** The two cloned D2R isoforms from rat and humans expressed in different cell lines reveal have virtually indistinguishable binding characteristics, corresponding to those of the native striatal DA D2 receptor. The similar expression patterns and pharmacology have led for long time to the conclusion that D2L and D2S had redundant functions *in vivo* (Jackson and Westlind-Danielsson, 1994). The recent availability of the antibody for both the isoforms has confirmed, *in vivo*, a different ratio in D2R isoforms expression throughout the forebrain (Khan et al., 1998). These evidences, taken together, have led to the suggestion that cellular mechanisms regulating the rate of splicing and the final ratios of the D2R isoform products may have a physiological and clinical relevance.

4.2.2 Dopamine D3 receptor

The second DA receptor within the D2R-like family cloned and characterised has been the DA D3 receptor (Sokoloff et al., 1990).

Overall, the D3 receptor is 52% homologous with the D2 receptor; however, this homology increases to about 75% within the transmembrane regions. As with the D2 receptor, the D3 receptor contains consensus sequences for N-linked glycosylation as well as a cAMP-dependent phosphorylation site in the third cytoplasmic loop (Sokoloff et al., 1990).

The human gene for D3R receptor has also been characterised (Giros et al., 1990). Surprisingly, the human receptor has 46 fewer amino acids in the third cytoplasmic loop, resulting in a protein of 400 residues in length. Excluding this deletion, the human receptor is 88% homologous overall and 97% homologous in the transmembrane domains when compared with the rat receptor.

Expression of the D3 receptor in CHO cells indicates that its pharmacology is similar to that of the D2 receptor. Few antagonists have been described to be slightly selective for D3 receptor, relatively to D2 receptor. Other antagonists examined were between 2 and 30 fold D2R selective. Conversely, agonist ligands demonstrated either equal or greater affinity for the D3 receptor (Sokoloff et al., 1995).

4.3.3 Dopamine D4 receptor

The most recent member in the D2R-like family identified and cloned is D4R receptor (Van Tol et al., 1991). This receptor consists of a protein of 387 residues in length with seven membrane-spanning domains. The membrane topography is similar to that for the D2 and D3 receptors, including the existence of a large third cytoplasmic loop and short C-terminus. The homology of the D4 receptor to the D2 and D3 receptors is 41% and 39% overall, respectively, and about 56% for both D2 and D3 receptors within the membrane-spanning domains. There is one potential site for N-linked glycosilation in the N-terminus and one consensus cAMP-dependent phosphorylation site in the third cytoplasmic loop of the D4 receptor (Van Tol et al., 1991). Characterization of the binding activity revealed a pharmacological profile, which was also similar to those of the D2 and D3 receptor. The D4 receptor display similar or lower affinities for both DAergic antagonists and agonists compared to the D2 receptor (Van Tol et al., 1991).

Importantly, however, the atypical antipsychotic clozapine, exhibit about 10-fold higher affinity for the D4 receptor than for D2 receptor. This has led to the suggestion that clozapine might exert its antipsychotic activity primarily through blocking the D4 receptor (Seeman et al., 1993). Several polymorphic variants of the D4 receptor have been found in humans, which

differ by the number of repeated units (composed of 16 aa), located in the third cytoplasmic loop region of the receptor (Van Tol et al., 1991). In line with the concept of a role of this domain in the activity of 7TM-receptors, the human D4 receptor polymorphic variants seem to have different impact in controlling behavioural properties in the human population (Benjamin et al., 1996; Ebstein et al., 1996). Thus, this region represents a key functional domain of D4 receptor activity. Furthermore, an increased number of D4 receptors have been reported in schizophrenic patients, suggesting a role for these receptors in human psychosis (Seeman et al., 1993).

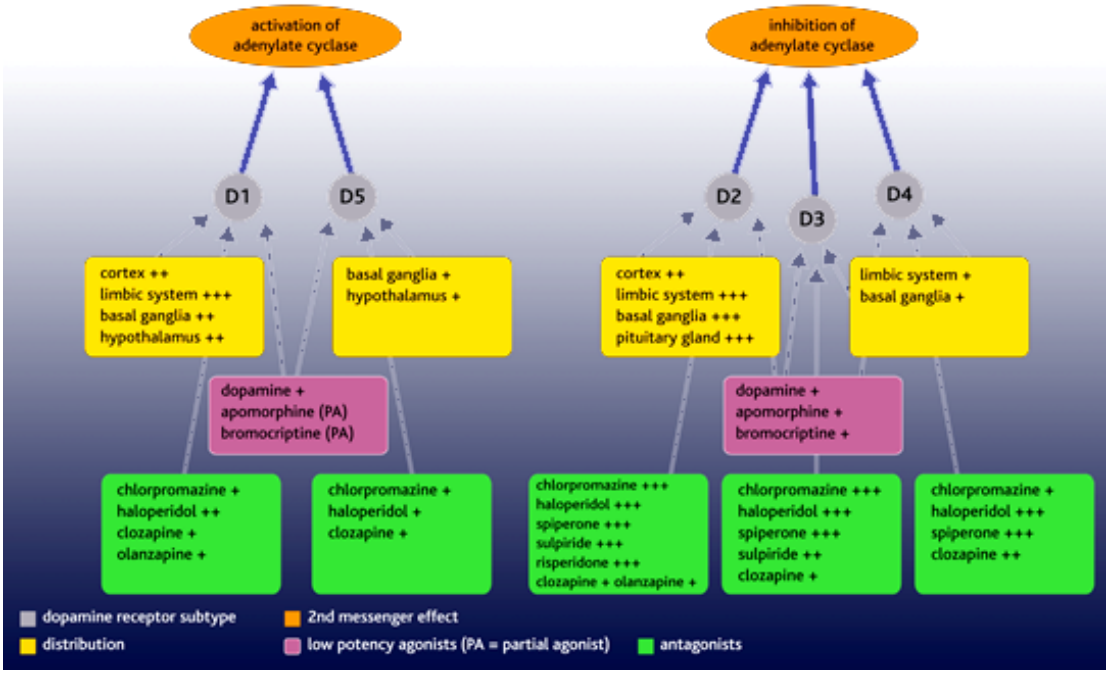

Figure 4. Dopamine receptor subtypes. There are two types of dopamine receptor, D1-like and D2-like receptors. The D1-like receptors comprise D1- and D5-receptor subtypes that are associated with stimulation of adenylate cyclase. The D2-like receptors comprise D2-, D3- and D4-receptor subtypes and these are associated with inhibition of adenylate cyclase. The known functions of dopamine appear to be mediated mainly by D2-like

receptors. All dopamine receptor subtypes are expressed in the brain in distinct but overlapping areas. D1 receptors are the most abundant and widespread in areas receiving dopaminergic innervation (the striatum, limbic system, thalamus and hypothalamus); D2 receptors are widespread in these areas, as well as the pituitary gland. D3 and D4 receptors are present in the limbic system. Schizophrenia is associated with dopaminergic hyperactivity. Dopamine antagonists used as antipsychotic drugs (eg chlorpromazine, haloperidol, risperidone) exert their effects mainly by blocking D2-like receptors. Dopamine agonists, such as apomorphine and bromocriptine, also have greater potency at D2-like receptors. Bromocriptine is used clinically to suppress prolactin secretion arising from tumours of the pituitary gland.

5. Dopamine Transporter

Dopaminergic neurons express the DA Transporter (DAT), a protein involved in the membrane reuptake process (Fig. 4). This protein together with DA autoreceptor is a key element in the control of DA signaling by regulating extracellular DA levels. DAT is a member of substrate-specific, high affinity, Na-dependent membrane transporters family (Amara and Kuhar, 1993). DAT mediates recapture of released dopamine; this event is generally thought to be the primary mechanism for limiting the extent, duration, and area of dopamine receptor activation. Furthermore, DAT represents a crucial target for a variety of psychostimulants, for

catecholamines, and selective neurotoxins (Javitch et al., 1985; Ritz et al., 1987).

The anatomical characterization of DAT expression has been greatly facilitated by radiolabeled ligands, including cocaine, mazindol, methylphenidate, GBR-12935 and nomifensine (Berger et al., 1985; Dubocovich and Zahniser, 1985; Janowsky et al., 1985; Javitch et al., 1985).

An oversimplified scheme of dopamine transporter function would predict that 2Na+, 1Cl-, and 1dopamine molecule bind sequentially to the dopamine transporter, and are transferred across the plasma membrane.

The cloning of DAT cDNA, has been made possible by the high sequence homology with previously cloned transporters (i.e. GABA transporter). Indeed, using a PCR strategy has been possible to obtained cDNA clones of DAT from rat, bovine and human cDNA libraries (Kilty et al., 1991; Usdin et al., 1991; Bannon et al., 1992).

The distribution of DAT mRNA determined by in situ hybridization is consistent with its exclusive expression in dopamine cell dendrites and soma. The highest DAT mRNA levels, as expected have been observed in the substantia nigra and in the ventral tegmental area. In contrast, DAT mRNA is not detected in DA terminal fields or other brain regions (e.g. striatum, nucleus accumbens and cortex).

Since its discovery, DAT has served as a neurochemical index of the density and structural integrity of nigrostriatal dopaminergic nerve terminals (Amara

and Kuhar, 1993). In fact, several studies have shown that lesions of the nigrostriatal pathway produce parallel losses of dopaminergic neurons and DAT expression (Seeman and Niznik, 1990). Nevertheless, it has been observed that the level of DAT expression varies significantly among different dopaminergic cell populations. In addition even in the same cell group, the level of dopamine transporter expression may be subject to regulation.

6. Distribution of dopamine receptors in SNC

In the last decade, the cloning and the identification of multiple DA receptors (DA-Rs) have profoundly changed the understanding of DA-Rs anatomy and pharmacology (Gingrich and Caron, 1993). At present, the function of DA-Rs in different brain regions has not been clearly established and still represents a matter of debate.

Since the cloning of DA-Rs pharmacological, immunocytochemical, in situ hybridization and in situ binding approach have been extensively used to define the function and the distribution of each receptor subtype in the brain areas. This, together with the knockout approach for the different DA-Rs is starting to give a better idea of the possible function of each DA-Rs (Altar

and Marien, 1987; Mansour et al., 1990; Mengod et al., 1992; Surmeier et al., 1996; Aizman et al., 2000; Diaz et al., 2000).

The distribution and abundance of the five DA-Rs is very different depending on which subtype is analyzed. Among the DA-Rs, D1- and D2-R are by far the most robust and widely expressed. Conversely, D3-, D4- and D5-R have a more restricted pattern of expression. Whether there is a physiological significance for the expression of these receptors in particular areas is still under investigation. However, the restricted expression of some DA-Rs in the limbic system as well as the higher affinity for anti-psychotic drugs has suggested a potential role of these receptors in neurological diseases.

D1-R is mainly expressed in the caudate putamen (CP), nucleus accubens (Acb), olfactory tubercle (OT), cerebral cortex (Cx) and amygdala. A high abundance of D1-R has been shown also in the island of Calleja and in the subthalamic nucleus. The binding of D1-R specific ligands could be observed in the substantia nigra (SN), in spite of the lack of mRNA expression. This seems to suggest that D1-R is synthetised in striatal neurons that send their projections to the SN via the direct striato-nigral pathway.

D1-R and D2-R mRNA expression is high within the striatum and nucleus accumbens.

D2-R has a very similar distribution with respect to D1-R, especially in areas like the CP, Acb, OT and SN. Low quantities of D2-R are also present in Cx and in the ventral tegmental area (VTA).

D1-R and D2-R mRNA expression is high within the striatum and nucleus accumbens. Cells expressing D2-R mRNA are a more widely distribution in the midbrain and hindbrain, and may be involved in a host of autonomic functions, as well as in the regulation of dopamine release. Cells expressing D2-R mRNA are abundant in the dopaminergic cells of the substantia nigra and ventral tegmental area. Within the substantia nigra the D2-R mRNA is primarily expressed in pars compacta, with a few cells in the pars reticulata. In contrast, while there are high levels of D1 receptor binding in the substantia nigra, pars reticulata,no cells expressing D1 receptor mRNA could be detected in the substantia nigra and ventral tegmental area (Mansour et al., 1990; Meador-Woodruff et al., 1992).

Cells expressing D2 receptor mRNA are also observed in the superior and inferior colliculus , and in the periacqueductal gray.

In the hypothalamus, cells expressing D1 receptor mRNA have a more limited distribution and are localised in the supraoptic, suprachiasmatic, paraventricular, and rostral arcuate nuclei. In contrast, cells expressing D2R receptor mRNA are more widely scattered in the hypothalamus and are found in the lateral preoptic area, anterior hypothalamic area, lateral hypothalamus, arcuate nucleus and dorsal mammillary nuclei. In the

anterior and intermediate lobe of the pituitary gland only the D2 receptor mRNA is detected at high levels (Mansour et al., 1990; Meador-Woodruff et al., 1992). D2-R is also localized in the retina (Doi et al., 2006), kidney and vascular system.

Examination of the neuro-anatomic distribution of D3-R mRNA in rat brain indicates that it is distinct from that of D2-R mRNA and restricted to few brain regions such as the islands of Calleja, a few septal nuclei, hypothalamus, and distinct regions of the thalamus and cerebellum. Both mRNAs and proteins of D2- and D3-R are expressed by both dopaminergic and dopaminoceptive cells.

Northern blot analysis revealed the presence of D4-R mRNA in the olfactory bulb, frontal cortex and hypothalamus in both rat and monkey brain.

In rat brain, the expression of D5-R mRNA is very restricted to the hippocampus and thalamus and does not seem to overlap significantly with the distribution of mRNA of D1-R. Interestingly, D5-R mRNA seems to be much more widely distributed in the primate brain as compared to rodents and in particular in the Cx where it overlaps with the mRNA of D1- and D2-R. However, the D5-R protein seems to be present in the Cx, CP, OT, VTA, and SN in rat brain.

7. Basal Ganglia

The basal ganglia represent key neural substrates through which the cerebral cortex affects the sensory-motor systems. The basal ganglia network is composed of five large subcortical nuclei: caudate nucleus, putamen, globus pallidus, subthalamic nucleus and substantia nigra (Alexander and Crutcher, 1990; Graybiel, 1990) (Fig. 5).

The complex processes arising from the cortical input in the striatal portion of the basal ganglia are modulated by dopaminergic input from the substantia nigra pars compacta. There are two major pathways through the basal ganglia. The direct pathway is the striatal projection to the internal segment of the globus pallidus and substantia nigra pars reticulate, which then project to the thalamus. The indirect pathway is the circuit from the striatum to the external segment of the globus pallidus (endopeduncolar nuclei in rodents), which projects to the subthalamic nucleus. The subthalamic nucleus in turns projects back to both pallidal segments and the substantia nigra.

In the classical model of the basal ganglia D1R and D2R were thought to be largely segregated (Gerfen, 2000). Thus, neurons bearing mainly D1 receptors constituted the direct striato-nigral output pathway. In contrast, neurons richer in D2 receptors constituted the indirect striato-pallidal pathway. The significance of D1 and D2 receptor-specific regulation of

striato-nigral and striato-pallidal pathways is related to their opposite effect on GABAergic neuron (Gerfen, 2000). Normal movements result from a coordinated balance of cortical and thalamic excitation of the striato-nigral and striato-pallidal pathways, which regulate the tonic activity of substantia nigra, pars reticulate neurons (Gerfen, 2000). Disturbance in the activity of different portions of these two pathways can disrupt this balance, with consequences that might lead either to the production of involuntary movements or to akinesia and bradykinesia as in Parkinson's disease (Baik et al., 1995; Obeso et al., 2000a; Obeso et al., 2000b).

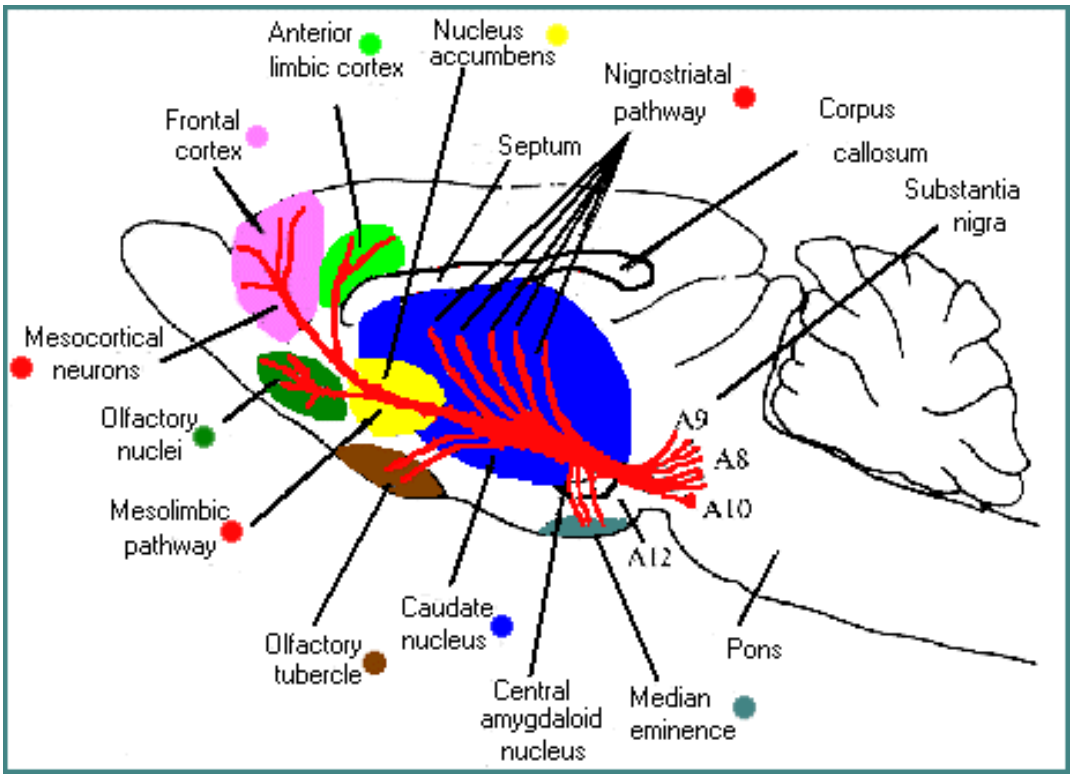

Figure 5: DA neurones are organised into pathways in the brain. DA pathways originate from groups of cells in the rostral areas of the brain. These groups were given the titles A8, A9, and A10. The nigrostriatal system runs from the substantia nigra (A9) forwards to the caudate, putamen, and globus pallidus, these 3 structures are called the corpus striatum. The mesolimbicocortical sytem run from the ventral tegmental area (A10) forwards to the nucleus accumbens, amydala, septum, olfactory nuclei.

8. D2R functions in the CNS

Since the discovery of D2R, a plethora of pharmacological studies had pointed out its prominent role in the CNS. In the past ten years the use of engineered knockout mice has represented a great tool to discriminate the functions of different genes *in vivo*.

At present, two different D2R knockout mice (D2R-KO) have been generated (Baik et al., 1995; Maldonado et al., 1997; Saiardi et al., 1997). These animals have represented a great tool to discriminate the complexity of D2R function *in vivo*.

Locomotor activity requires the coordinated actions of cortical and subcortical structures. The basal ganglia, composed of corpus striatum, globus pallidus, subthalamic nucleus, and substantia nigra, play an important role in modulating the final output of cortical neurons. Despite the differences in signal transduction mechanism, the contribution of D1- and D2-like receptors to locomotion is generally considered synergistic. However, none of the available drugs has complete specificity for any of the five known dopamine receptor subtypes. Furthermore selective functional loss of the dopamine D1 receptor by gene targeting was reported to cause either an increase in baseline activity or no alteration in locomotion (Kobayashi et al., 2004). In contrast D2R-KO mice present a strong reduction in motor performance. In the open field test, that measures the basal locomotor activity, D2R-KO mice show a significant reduction in both locomotion and rearing behavior compared to wild type littermates (Centonze et al., 2003).

Moreover D2R-KO mice spend significantly less time on the rotarod apparatus, which measures their coordination ability, than the WT mice.

All these data strongly indicate that the D2R has a prominent role in the dopaminergic system in the control of locomor activity. None of the other DA receptor deficient mice present such « parkinsonian like phenotype » in motor coordination and activity (Usiello et al., 2000; Kobayashi et al., 2004).

The majority of dopaminergic neurones in the mammalian CNS are located in the midbrain, where they form the substantia nigra (SN), the ventral tegmental area (VTA) and the retrorubral nuclei. Midbrain DA neurones project to the striatum (or caudate-putamen complex), limbic system and frontal cortex, and receive afferents from multiple structures in the diencephalon and telencephalon. The D2Rs show a very particular distribution since they are at post-synaptic level (striatum) where they control the motor out-put and pre-synaptic level (SN and VTA) where they control the DA syntesis and release. Pre-synaptic inhibition of DA release is a physiological phenomenon by which extra-cellular DA release stimulates pre-synaptic autoreceptors to further inhibit DA release. In particular, pharmacological as well as anatomical studies have identified D2R and D3R as potential DA autoreceptors. Studies of in *vivo* voltammetry and microdialysis using WT and D2R-KO mice have shown that the expression of D2R is a necessary condition for the maintenance of the pre-synaptic inhibition (Calabresi et al., 1997). The effect of haloperidol (D2R antagonist) or quinpirole (D2R antagonist) on dopamine release was

completely suppressed in D2R-KO mice. Importantly, in D2R-KO mice the extracellular increase of DA induced by cocaine or morphine is greatly enhanced versus WT, although no significant changes were observed in basal extracellular concentration of DA (Usiello et al., 2000). All these data demonstrate that the D2R is a key element in regulating the increase in DA in the inter-synaptic space and could play a crucial role in the physiology of addiction.

The mesolimbic dopaminergic system consists of neurons originating in the VTA which have axonal projections to the limbic system. In particular, dopaminergic projections to the Acb have been implicated in the control of the reward mechanisms and in the psychomotor effects generated by drugs of abuse, including opiates, cocaine, amphetamine and alcohol. Studies of D2-R null mice have demonstrated the crucial role of this receptor in the rewarding effects of opiates (Rouge-Pont et al., 2002). In the Place Conditioning paradigm the D2-KO mice failed to show a preference for opiates since they spent the same time in the morphine- and in saline associated compartments. In contrast, the wild-type littermates showed a significant increase in the time spent in the drug-associated compartment during the testing phase. Interestingly, it has been demonstrated that the D2-R is not required for the development of physical opiate dependence or for the locomotor response to acute administration of morphine. In addition, D2-R activity seems to be specifically involved in the motivational component

to opioid dependence. Indeed, when these mice were tested in a similar behavioral paradigm in their response to a natural reinforce such as food, no difference were observed between D2-R deficient and wild-type mice (Maldonado et al., 1997; Usiello et al., 2000; Rouge-Pont et al., 2002; Centonze et al., 2003).

Notably, DA is the major catecholamine in the vertebrate retina playing a central role in neural adaptation to light (Witkovsky, 2004). Yet, the physiological contribution of light-dependent dopaminergic signaling to nonvisual functions has remained unexplored. Recent findings show that signaling mediated throught the D2R greatly influences central molecules participating in the regulation of the circadian clock, playing a central role in the control of light masking of circadian locomotor activity (Doi et al., 2006; Yujnovsky et al., 2006). D2R mediating signaling is required in regulating the proper organization of daily locomotor activity in light-dark cycles (Doi et al., 2006).

In conclusion the use of knock-out mice has shown that D2R has a prominent role between the different DA receptor in the dopaminergic neurotransmission. It is able to modulate a large variety of physiological function *in vivo*: from locomotor activity to drug of abuse, ethanol intake and control of DA release.

9. D2R signal transduction pathways

At present, many signal transduction pathways have been shown to be affected by D2R activation (Vallar and Meldolesi, 1989). D2R was first characterised as inhibitor of intracellular cAMP levels (Stoof and Kebabian, 1984). Indeed, an inhibition of adenylyl cyclase (AC) activity mediated by DA has been reported *in vivo* systems such as the anterior and intermediate lobe of the pituitary gland, and in striatal cells (Caccavelli et al., 1992). All these tissues express high levels of D2R (Jackson and Westlind-Danielsson, 1994). The evidence that D2R signalling was blocked by pertussis toxin (PTX) (Cote et al., 1984), indicated that these receptors are associated with members of the Gi/Go-protein family (Senogles et al., 1987; Elazar et al., 1989). These proteins trigger the inhibition of AC, resulting in a decrease in intracellular cAMP concentration (Gilman, 1984). In particular, the activation of Gαi- and Gαo-subunits has been shown to affect the activity of type I, V and VI of the AC (Tang and Gilman, 1992; Taussig and Gilman, 1995). The final product of this intricate interactions leads to variable levels of cAMP and consequently of functional protein kinase A (PKA). PKA, in turn, phosphorylates cytoplasmic and nuclear proteins, regulates cellular metabolism, including ion channel function, and finally desensitises 7TM G-protein coupled receptors (Choi et al., 1993; Hofmann et al., 1994) leading to the cellular response to neurotransmitter release (Fig. 6 and Fig. 7).

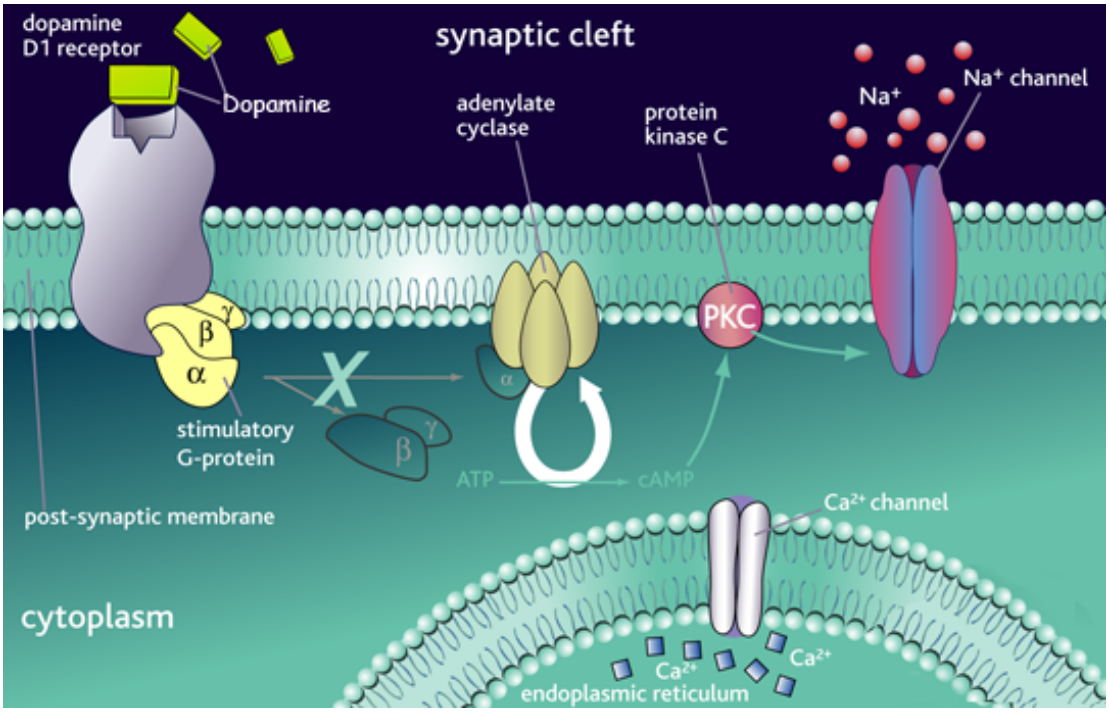

Figure 6. Dopamine D1 receptor. DA binds D1 receptor and leads the activation of the receptor. The D1 receptor is coupled to stimulatory G-proteins, which dissociate from the receptor on agonist binding and initiate secondary messenger signaling mechanisms. This causes cell depolarisation, which is inhibited by antagonist binding.

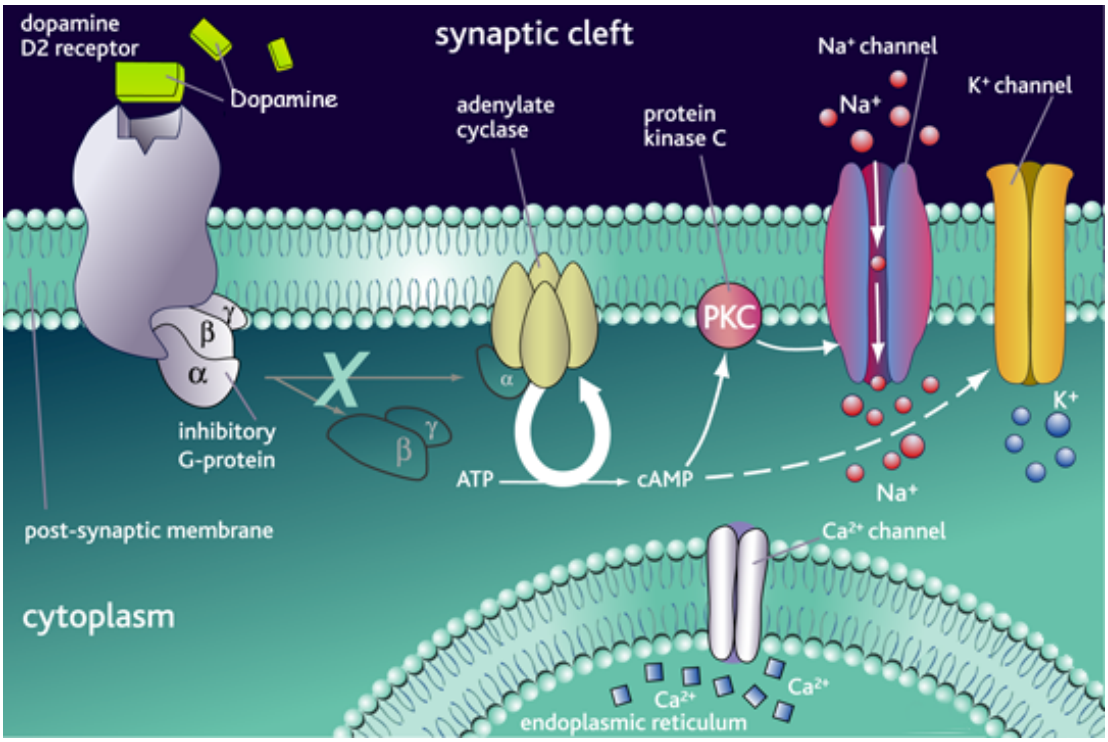

Figure 7. Dopamine D2 receptor. DA binds D2 receptor and leads the activation of the receptor. The D2 receptor is coupled to inhibitory G-proteins, which dissociate from the receptor on agonist binding and inhibit secondary messenger signaling mechanisms. This causes inhibition of down-stream signaling mechanisms. Antagonist binding inhibits this usual process, resulting in cell depolarisation.

A number of reports have linked the D2R stimulation with a decrease in intracellular Ca^{2+}. The effect seems to be sensible to PTX but independent changes in cAMP levels. The negative regulation of PRL and MSH release, respectively from lactotrops and melanotrps, has been related to the inhibition of of voltage-sensitive calcium channels that leads to a reduction of intracellular Ca^{2+}. (Jackson and Westlind-Danielsson, 1994). In the rat pituitary GH4C1 cell line, D2 receptors inhibit Ca^{2+} currents through

coupling to Gαo (Liu et al., 1994). In anterior pituitary cells D2 receptors seem to inhibit two voltage-activated Ca^{2+} currents (Lledo et al., 1992), through coupling to Gαi1 and 3. Interestingly, it has been shown that the ability of D2 receptors to block Ca^{2+} channels varies between D2L and D2S (Liu et al., 1994).

D2R had also been shown to influence the PKC pathway (Vallar and Meldolesi, 1989; Demaurex and Distelhorst, 2003) although a direct modulation of the phospholipase C (PLC) by D2R is still controversial. Interestingly, the activation of D2R in pituitary lactotrophs inhibits AC, but also Ca^{2+} release from intracellular stores through the inhibition of IP3 levels (Vallar and Meldolesi, 1989).

Whether this is due to a direct cAMP-independent inhibition of PLC (Rasolonjanahary et al., 2002)by the D2 receptor, or indirectly to the decrease of Ca^{2+} concentration (Banihashemi and Albert, 2002) via D2 action on Ca^{2+} channels has to be elucidated.

D2R, acting on intracellular Ca^{2+} concentration, is also able to influence the DA synthesis by affecting the function of tyrosine hydroxylase, the rate-limiting enzyme in DA production (Meissner et al., 2003). The increase of intracellular Ca^{2+} activates the calcium/calmodulin dependent protein kinase (CaM kinase or CaM II) by binding to calmodulin (Braun and Schulman, 1995); CaM kinase in turn activates tyrosine hydroxylase.

In addition to the previously described pathways, D2R affects the intracellular concentration of potassium ions. Indeed, D2R mediated activation of K^+ channels is mediated by PTX sensitive Gα. subunits (Rahman and McBride, 2001). The involvement of D2R in the control of voltage-dependent cAMP-independent K^+ channels and of Ca^{2+}-sensitive K^+ channels cAMP dependent has also been postulated (Jackson and Westlind-Danielsson, 1994).

The role of D2R in modelling potassium currents has been extensively studied. It has been shown that D2R increases outward potassium currents leading to cell hyperpolarization. Such effects have been observed in rat striatal and mesencephalic neurons as well as in the anterior pituitary (Davison et al., 2004): the activation of potassium currents appears to be modulated by a G-protein mechanism (Congar et al., 2002). The effect of DA on potassium currents in melanotrops is abolished by pertussis toxin (PTX) treatement. In addition, treatment of cells with G-protein antibodies or antisense oligonucleotides blocks the D2R stimulation of potassium currents. In the pituitary, activation of potassium currents appears to be mediated by Gαi3, whereas, in rat mesencephalon cultures by Gαo. K^+ influx also decreases Ca^{2+} concentration through voltage-dependent Ca^{2+} channels (Koch and Schonbrunn, 1988). The functional significance of cell

hyperpolarization appears to be the inhibition of DA release by autoreceptors in the brain and of prolactin secretion in the pitutary.

D2R can also influence arachidonic acid (AA) synthesis. AA is synthesised from phospholipids by the activity of phospholipase A2 (PLA2): PLA2 needs Ca^{2+} to work. It has been suggested that D2R acting in synergism with D1R might amplify AA synthesis initiated by the increase of intracellular Ca^{2+}, due to the D1R activated pathway (Piomelli et al., 1991; Piomelli and Di Marzo, 1993)**.** Interestingly, PTX-sensitive G-proteins are involved in these effects, strongly suggesting a D2 receptor mediated effect; PKC, PKA and other kinases can also indirectly activate PLA2 {Balsinde, 2005 #121; Piomelli, 1993 #120; Piomelli, 1991 #119}; consequently, it cannot be excluded that the PLA2 regulation by D2 receptors could also be indirect.

The AA elevation upon D2 receptor activation could also explain the stimulation of K^+ channels already described. Released AA can be metabolised by several enzymes, but in the nervous tissue the most important are 12-lipoxygenase (that produces (12s)-hydroperoxyeicosatetraeonic acid or 12-HPETE) and 5-lipoxygenase (that produces 5-HPETE) (Piomelli and Di Marzo, 1993)**.** 12-HPETE could directly activate K^+ channels, thus decreasing intracellular [Ca^{2+}]. Moreover, 12-HPETE or its metabolites act directly on CaM kinase II,

playing a role in synaptic vesicle secretion (Piomelli and Di Marzo, 1993). In addition, AA metabolites derived from the epoxygenase pathway are known to inhibit the Na^+/K^+ ATPase. This ion pump maintains the ion gradient by extruding Na^+ and importing K^+ in neurones (Tseng and O'Donnell, 2004). D2 receptors acting synergistically with D1 receptors inhibit the activity of this enzyme. This inhibition can lead to a transient membrane depolarization, thus affecting cell excitability and consequently neurotransmitter release (Aizman et al., 2000).

A large array of cellular responses follows the activation of DA D2 receptors. In most of the example given it is clear that the response is dependent on the G-proteins available in the cellular system used. Thus, it can be suggested that the presence of two isoforms of D2R, each coupling to different G-proteins in the same cell, is a mechanism to amplify and diversify the response to DA.

Furthermore, recently it has been indicated that D2R stimulation can lead to both the phosphorylation and activation of CREB and MAPK in vitro as well as to phosphorylation of MAPK in vivo (Lee et al., 2006; Pozzi et al., 2003; Cai et al., 2000; Yan et al., 1999)**.** Interestingly, CREB and MAPK seem activated by two different transduction pathways. Indeed, D2R agonists increase intracellular Ca^{2+} and PKC activity leading to the activation of the Ras/Raf/MEK/MAPK cascade, while intracellular Ca^{2+}

and CaMK are required for the activation of CREB (Cai et al., 2000). Importantly, it has been shown that activated MAPK in the CNS are mainly cytoplasmic and localised in the cell bodies and dendrites. Conversely, activated CREB is found in the nucleus. The different subcellular localization of these two activated components indicates that DA induced activation of D2R might result in the contemporary stimulation of multiple targets. It has been proposed that MAPK phosphorylation occurring primarily in dendrites would regulate protein synthesis, cytoskeletal dynamics, and ion channel activities at synapses. On the other hand, phosphorylated CREB would regulate gene expression (Yan et al., 1999) by acting at the nuclear level.

In CHO cell line, stably transfected with D2L or D2S , D2R agonists induce a transient ERK phosphorylation (Choi et al., 1999). This stimulation was markedly attenuated by co-expression of the C-terminus of β-adrenergic receptor, which selectively inhibits Gβγ-mediated signal transduction. Further analysis of D2L- and D2S-mediated MAPK activation demonstrated that D2L-mediated MAPK activation was not significantly affected by PKC depletion or partially by genestein. In contrast, ERK activation mediated by D2S was potentially inhibited by PKC depletion and genestein pre-treatment (Choi et al., 1999).

Further levels of regulation of D2R-mediated signal transduction might be dependent upon intracellular interactions with membrane receptors for other

neurotransmitters and modulators. Indeed, a direct intramembrane interaction between D2R and the somatostatin receptor has been recently shown (Rocheville et al., 2000). However, intra-membrane and intracellular modulation of the D2R-mediated signalling was already evoked for the adenosine A2A receptor as well as for other heterologous receptors (Schindler et al., 2004).

In addition, recent studies have shown that D1R and D2R colocalized in striatal neurons (Emamian et al., 2004). These observations are of interest since the described synergistic and antagonistic actions exerted by activation of D1R and D2R subclasses of DA receptors might well be mediated by intracellular rather than exclusively by intercellular mechanisms.

10. D2R isoforms and G protein-coupling

Two isoforms of DA D2R are present *in vivo*, D2 long isoform (D2L) and D2 short isoform (D2S), generated by alternative splicing of the same RNA transcript. They are co-expressed in all tissue or cell expressing D2Rs, normally in a ratio favouring the D2L with the exception of SN were they are expressed at the same ratio. The long isoform differs from the short isoform in the presence of the sixth exon. This exon codes for 29 amino acids that constitute part of the third intracellular loop (IL3) of the D2R.

D2R belongs to the family of 7TM G protein-coupled receptors. These receptors share similarities in size and structure, with the highest homology in the transmembrane regions (Dohlman et al., 1991): once the binding of a specific ligand activates them, the G-protein/receptor complex dissociates thus leading to the activation of intracellular signaling cascades (Neer, 1995; Hermans, 2003). The third intracellular loop (IL3) is the most variable region among this group of receptors: this region is responsible for the interaction of the receptor with the G-proteins (Fig. 8). In addition to IL3, the second intracellular loop (IL2) be involved in the interaction of the receptors with the G-proteins. Moreover, the generation of several D1R/D2R hybrids has shown that D2R coupling to Gi is due to the IL3 loop in cooperation with regions in the IL2 (Kozell et al., 1997). Mutagenesis studies have shown that the region inside the IL3 loop responsible for the interaction with the G protein is contained in the N-terminal and the C-terminal portions of the loop (Grishina et al., 2000). Interestingly, this is the only variable region between the two isoforms of DA D2 receptors. Indeed, a 29 amino acids insertion is present in a region close to the center of the IL3 of the D2L isoform. All these data suggest that the insertion might play a role in modulating the D2R/G proteins interaction (Montmayeur et al., 1991; Liu et al., 1994; Guiramand et al., 1995).

The expression of D2L and D2S receptor isoforms in JEG3 cells results in the decrease of the intracellular level of cAMP by the inhibition of AC

(Montmayeur et al., 1991) indicating that these receptors are functionally coupled to G-proteins in this cell line. Cotransfection of an antisense Gαi2 vector partially blocked the activity of D2L, but not that of D2S. Taken together, these data clearly indicate that the 29 amino acids insertion present in D2L confers specificity for coupling to Gαi2 and therefore discriminates functionally between the two DA D2R isoforms (Montmayeur et al., 1993).

Although the classical signal transduction cascade operates at the plasma membrane, it has been known that heterotrimeric G-proteins are also found on intracellular membranes such as endosomes, secretory granules, the endoplasmic reticulum and the Golgi complex (Helms, 1995). For example, one subclass of Gα can be detected in several membranes depending on the cell type (Wilson et al., 1994). Recent evidences show that G-proteins are involved in diverse functions such as in vesicular transport (Helms, 1995), in the binding of the coatomer to the membranes of the vesicles involved in the endoplasmic reticulum, Golgi and intra-Golgi transport (Serafini et al., 1991; Wilson et al., 1994) as well as in maintaining the Golgi structure (Jamora et al., 1997; Le-Niculescu et al., 2005).

A novel α subunit, sGi2, has been identified in the laboratory of Dr. Borrelli, which is the product of the alternative splicing of the Gαi2 gene (Montmayeur and Borrelli, 1994). A splicing event replaces the last 24 amino acids of Gαi2 with a new segment of 35 amino acids in the C-terminal region of the protein. This substitution is responsible for the localization of

the protein in the Golgi apparatus (Downes and Gautam, 1999; Picetti and Borrelli, 2000). A similar mechanism has also been reported for Gαs, in which the N-terminal 46 amino acids are substituted with a new 498 amino-acid sequence (Kehlenbach et al., 1994).

Other G proteins localized intracellularly suggest that they perform different functions from the G proteins at the plasma membrane.

It has been reported that activation of G proteins with AlF4- are involved in the intracellular membrane protein trafficking (Bomsel and Mostov, 1992; Helms, 1995). G proteins have been shown to be involved in secretory (Melancon et al., 1987) as well as in endocytic (Mayogara et al., 1989) mechanisms.

It was previously shown in Cos cells that sGi2 localizes in the Golgi apparatus by a specific 14 amino-acids proline-rich sequence contained in the C-terminal sequence of the protein (Picetti and Borrelli, 2000). In 2002, Wedegaertner reported that the disruption of the normal C-terminus of αi2 causes mislocalization and rapid degradation of sGi2 suggesting the cellular instability of sGi2. Recently, Khan and Gutierrez, in 2004, showed that sGi2 protein is widely expressed in rat and monkey brain regions and that depending on the cell subtype is localized in intracellular compartments such as the endoplasmic reticulum, Golgi, mitochondria and nucleus but also in dendrites, axons and spines suggesting: that this protein is stable as it is transported in the cell after synthesis.

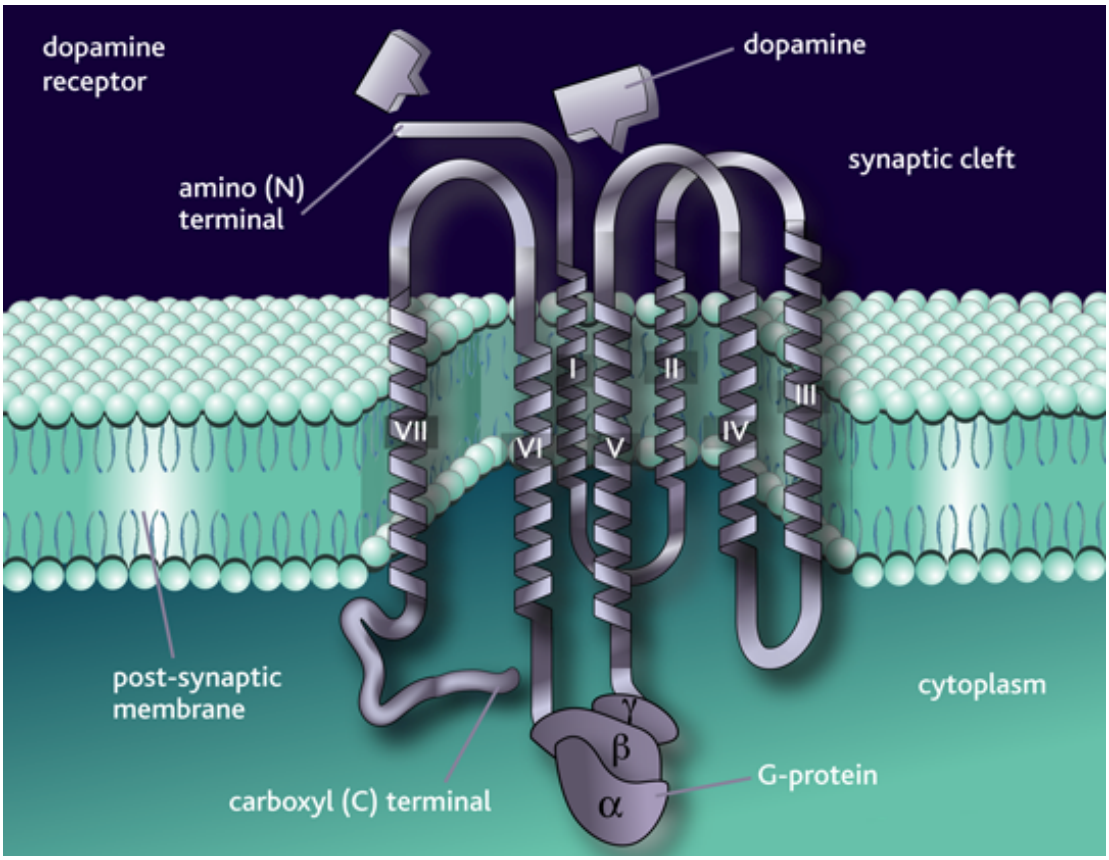

Figure 8: Dopamine D2 Receptor coupled with a G-protein. Dopamine D1-like receptors are coupled to stimulatory G-proteins and they have a stimulatory effect on neurotransmission when bound by an agonist. The D2-like receptors are coupled to inhibitory G-proteins. Dopamine D2-like receptors have an inhibitory effect on neurotransmission when bound by an agonist. Many neuroleptic drugs are antagonists of the D2 receptors. This class of drug is used to treat psychotic disorders, such as schizophrenia.

11. Gene targeting: D2R-deficient mice

In the beginning of the 80's, cells from embryonic origin, the embryonic stem (ES) cells, have been isolated for the first time (Evans and Kaufman, 1981); these are totipotent cells and can be cultured and manipulated in vitro without

altering their potential. By a process called homologous recombination, a DNA fragment, which has been incorporated into these ES cells, can integrate into their genome and thus introduce a mutation in the gene of interest. Once implanted into an embryo, these modified ES cells can give rise to mice mutant for the gene of interest.

Recombination between homologous DNA-sequences is a natural mechanism occurring rarely and very little is still know about the molecular mechanisms of this process in vertebrates (Smithies et al., 1985). However, use of appropriate DNA vectors has taken advantage of this event to introduce a fragment of interest into the genome and selection for the cells that have undergone homologous recombination (Thomas and Capecchi, 1987).

The vector for the homologous recombination is a circular DNA plasmid, which contains sequences homologous to the targeted locus. A selection marker (e.g. the gene neo offering resistance to neomycin) interrupts these sequences. Introducing the vector via electroporation into the ES cells can cause chromosomal rearrangements leading to the insertion of the vector into the genome by replacement or integration (Capecchi, 1989). The selection marker has a double function. Its presence in the coding sequence of the gene of interest disturbs the normal transcription of this gene: the gene is thus inactivated. On the other hand, the gene *neo* enables the modified ES cells to resist to G418, a synthetic analogue of neomycin. Thus, the cells that have integrated by homologous recombination into their genome from the vector

can be isolated (Thomas and Capecchi, 1987). A second selection process may be necessary to eliminate those ES cells that have integrated from the vector randomly into their genome, by non-homologous recombination. The gene coding for the *thymidine kinase (TK)* of the *Herpes simplex* virus makes the ES cells vulnerable when applying the modified nucleotide gancyclovir. Introducing the *TK* gene at one extremity of the vector for homologous recombination allows for a second selection procedure of the electroporated ES cells. Both selection procedures can be combined to augment the frequency of cells that have undergone homologous recombination: integration of the neo gene (resistance to G418) and absence of TK (resistance to Gancyclovir) in the cells that have been harvested after electroporation (Thomas and Capecchi, 1987).

The embryonic stem cells are derived from the inner cell mass of mouse blastocyst (Evans and Kaufman, 1981). The ES cells which have been selected after homologous recombination can be re-injected into mouse host embryos where they can contribute to all different tissues including the germ line (Bradley et al., 1984). The ES cells generally used are derived from the pure genetic mouse strain 129/sv, a mouse strain with agouti coat color. The host embryos are of mixed (heterologous) origin (e.g. C57BL/6J, black mice) and are implanted into a (hormonally-induced) pseudo-pregnant female (Thomas and Capecchi, 1987). The chimera animals that derive from these embryos are made up of a mixture of two coat colors: black for the tissues

derived from wild type blastocysts and agouti for the tissues derived from the injected ES cells. The germline of these chimeras also derives from two genetic backgrounds: C57BL/6J for the wildtype germlayer-cells and 129/sv for the germlayer-cells derived from the modified ES cells. By the end of meiosis, half of the gametes on 129/sv background contain the introduced mutation. The chimeras are then bred into an animal of the host-strain C57BL/6J. The offspring of this crossing with black coat color will then be of wild type phenotype whereas half of the offspring with agouti coat color will be heterozygous for the desired mutation (the agouti color being dominant over the black color). The continuous crossing of the latter ones will allow for the establishment of an animal strain which is homozygous for the modified gene (a "null" mutant) (Thomas and Capecchi, 1987).

Using the gene targeting approach, in the last few years, different DAR mutant mice have been generated (Giros et al., 1989; Drago et al., 1994; Baik et al., 1995; Rubinstein et al., 1997; Xu et al., 1997). These animals have represented a great tool to discriminate the function of each receptor *in vivo*. In addiction, they allow the overcome the bias created by the use of other model system, in which either chemically or mechanically lesions are induced, which although very useful can be non-selective. Similarly, the use of ligands to test the function of receptors *in vivo* presents the bias of drugs acting at multiple receptors with the consequent lack of specificity. Targeted mutagenesis of desired genes *in vivo*, by homologous recombination, is

offering a mean to estimate the physiological effects of genes of interest. Using this technique, null mice for several components of dopaminergic system have been generated, providing animal models to evaluate, in a more selective manner, the role of dopaminergic transmission (Giros et al., 1989; Drago et al., 1994; Baik et al., 1995; Rubinstein et al., 1997; Xu et al., 1997) . Mutant mice have been generated to analyze the physiological involvement of D2R *in vivo* (Baik et al., 1995). To generate D2R null mice, the first coding exon (exon 2) and flanking introns were deleted and replaced with a neomycin resistance gene under the control of the phosphoglycerate kinase I promoter (Adra et al., 1987). One ES clone was identified that contained the mutated gene inserted by homologous recombination. This clone was injected into the blastocysts of recipient mice and five chimeras' male mice were obtained. These mice were able to establish a line of D2R heterozygote null mice. Viable homozygous mice were then obtained. The D2R deficient mice presented a reduction of their body weight as compared with normal littermates. The analysis of food and water intake revealed a slight decrease (10-15%) in homozygous mice; the body temperature was reduced of 0.7°C in D2R-KO with respect to the WT littermates. The pharmacological analysis of homozygote mice showed a complete absence of D2R binding site (Baik et al., 1995). D2R mutant mice have helped to establishment the role of D1R and D2R in locomotion. The behavioral phenotype of the D2R-KO is characterized by a significant motor deficit, when compared to the WT

littermates (Baik et al., 1995). These observations indicate that D2R-KO present a “Parkinson-like phenotype”, intending that lack of D2R signaling affects movements in a similar manner that DA reduction in other models, although to a much lower extent.

D2R-KO mice have been used to elucidate the autoreceptor presynaptic functions of D2R *in vivo*. Interestingly, D2R-KO mice lead to a total abrogation of the inhibitory effects of DA on the firing of dopaminergic neurons of SN (Mercuri et al., 1997). In addition, also the DA release assessed in the striatal synaptosome in D2R-KO mice suggests a pivotal role of D2R in the control of DA release. More recently, these results have been confirmed by *in vivo* microdialysis and by voltammetry (Benoit-Marand et al., 2001; Rougé-Ponte et al., 2002). Taken together these results indicate that D2R is the major DA autoreceptor.

12. D2L-deficient mice

Despite all the pharmacological observations, the lack of specific compounds able to discriminate between the two isoforms, D2L and D2S, has not yet allowed to establish their individual contributions in the D2R mediated functions. Two different D2L-KO have been generated (Usiello et al., 2000; Wang et al., 2000) that express only the short isoform, D2S, without changing the total mRNA and protein D2R level (Usiello et al., 2000; Wang et al.,

2000). The analysis of these mice permitted to definitively clarify that the two isoforms have a distinct function, *in vivo*. The only expression of short isoform in D2L-KO mice at presynaptic level results in an increase of autoreceptor function: the experiments strongly indicate that the D2S can be considered the presynaptic D2R (Usiello et al., 2000; Rougé-Ponte et al., 2002) .

Furthermore at postsynaptic level the over expression of D2S in the striatum of D2L-KO mice completely alters the function of D2R (Centonze et al., 2004). A cooperative/synergism interaction between D1R and D2L can be proposed on the basis of pharmacological and behavioral studies (Centonze et al., 2003; Usiello et al., 2000) while D2S seems to interfere, at postsynaptic sites, with D1R-mediated functions. Moreover, analysis of D2L-KO mice have show that the D2L isoform is the target of the antipsychotic haloperidol, since in D2L-KO mice this compound does not elicit catalepsy any longer as it does in WT mice. Thus, D2L and D2S have a different function *in vivo*: D2S is principally the D2 presynaptic autoreceptor, which at postsynaptic level negatively modulates D1R-dependent responses. In contrast, the D2R-mediated postsynaptic effects and their cooperative/synergistic activity with D1R seem likely to be mediated by D2L.

These two mouse model, D2R-KO and D2L-KO, have been used during my PhD work.

RESULTS AND DISCUSSION

At the beginning of my PhD studies in the laboratory of Dr. Borrelli, I focused my attention on developing a new D2R monoclonal antibody (4H6-7-3) recognizing both isoforms of the D2R.This antibody is directed against a peptide corresponding to the mouse D2R aminoacid (aa) sequence spanning from aa 309 to 322 (D-P-S-H-H-G-L-H-S-N-P-D-S-P). Since after more than ten years Dr Borrelli's lab was unable to obtain an antibody which would recognize D2R, we used a different strategy. We reasoned that lack of immunogenicity might depend from an escape mechanism to avoid self destruction of D2R-mediaed functions. We thus immunized D2R-KO mice, indeed these animals were born without D2R and thereby they could have a better response to the immunization protocol.

Monoclonal D2R antibody was produced by fusing single antibody-forming cells to tumor cells grown in culture, the hybridoma. Each hybridoma was able to produce relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it was possible to produce a population of cells, each of which produced identical D2R antibody molecules (Fig. 9). These cells where then injected in nude mice for ascite production.

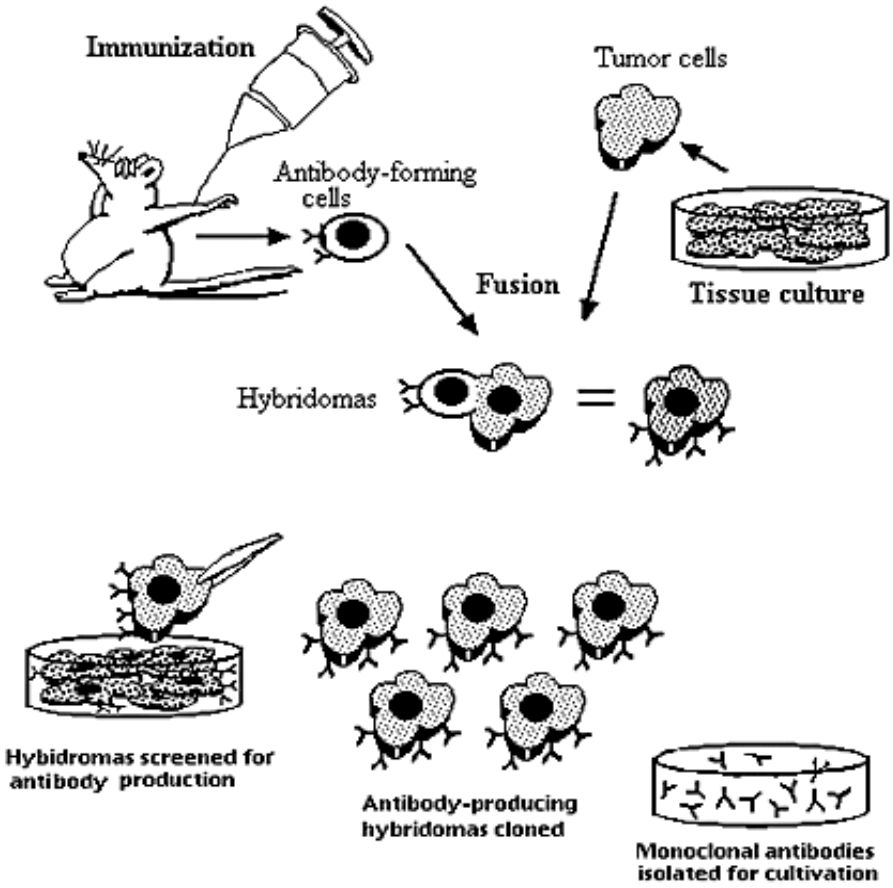

Figure 9: Monoclonal Antibody Production. Process by which the D2R antibody was produced.

Once the antibody was made, I tested its specificity by Western blot using striatal membrane protein extracts, rich in dopaminergic receptors from WT, D2L-/- and D2R-/- mice as well as COS cells transfected with the expression vectors of either D2S, D2L or both receptors. I carried out the solubilization of D2R from striatal membranes, with different detergents and detergent-salt combination. CHAPS, a zwitterionic detergent (3-[(3-deoxycholamidopropyl)-dimethylammonio]-1-propane sulfonate) was found

to be the best solubilizing agent, among all detergents used. D2Rs were detected in the striatum as a wide band corresponding to a group of proteins of 70-120 kDaltons (fig. 10). The presence of multiple bands is due to receptor specific posttranslational modifications as well as to the oligomerization of receptors that belong to the seven transmembrane domain G-protein coupled receptor family. As expected, no signal was detected in D2R-/- striatum.

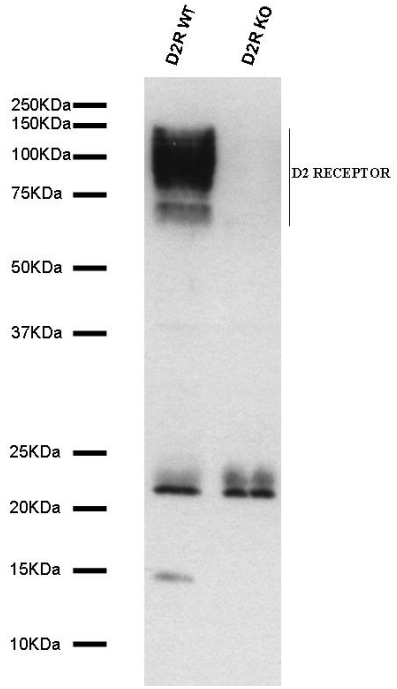

Figure 10: Generation of a specific antibody against D2R. I raised a mouse monoclonal anti-D2R antibody (4H6-7-3) directed against a peptide corresponding to the mouse D2R aminoacid residues 309-322. Western blot analyses using the 4H6 antibody revealed the presence of positive bands only in WT, but not in D2R-KO membrane preparations. The presence of multiple bands is due to receptor specific posttranslational modifications as well as to the oligomerization of receptors that belong to the seven transmembrane domain G-protein coupled receptor family.

Using this antibody, I was able to perform immunoprecipitation and immunoistochemistry assays, as well as Western blot, that will be presented during my discussion.

The 4H6-7-3 antibody was useful to analyze the subcellular localization of D2R. It has been reported that G-proteins are involved in the intracellular membrane protein trafficking (Bomsel and Mostov, 1992; Helms, 1995). G proteins have been shown to be implicated in secretory (Melancon et al., 1987) as well as in endocytic (Mayogara et al., 1989) mechanisms. A novel α subunit, sGi2, has been identified in the laboratory of Dr. Borrelli, which is the product of the alternative splicing of the Gαi2 gene (Montmayeur and Borrelli, 1994). A splicing event replaces the last 24 amino acids of Gαi2 with a new segment of 35 amino acids in the C-terminal region of the protein. This substitution is responsible for the localization of the protein in the Golgi apparatus (Downes and Gautam, 1999; Picetti and Borrelli, 2000).

In the Dr. Borrelli's laboratory, I have analyzed the role of sGi2, *in vivo*. I started by performing *in vitro* analyses on transtected cells. Cell extracts immunoprecipitated with the D2R-antibody showed that each isoform of D2R could interact with sGi2 *in vitro*. However, I then analyzed whether these complexes can also form *in vivo*, by preparing extracts from D2R-KO and D2L-KO mice. Importantly, I was able to show that sGi2 preferentially interacts with D2L *in vivo* and not with D2S (fig. 11). Indeed, in in D2L-KO mice the anti-D2R is not able to immunoprecipitae sGi2. These findings are

important since they show that studies *in vitro* are less specific than those *in vivo*.

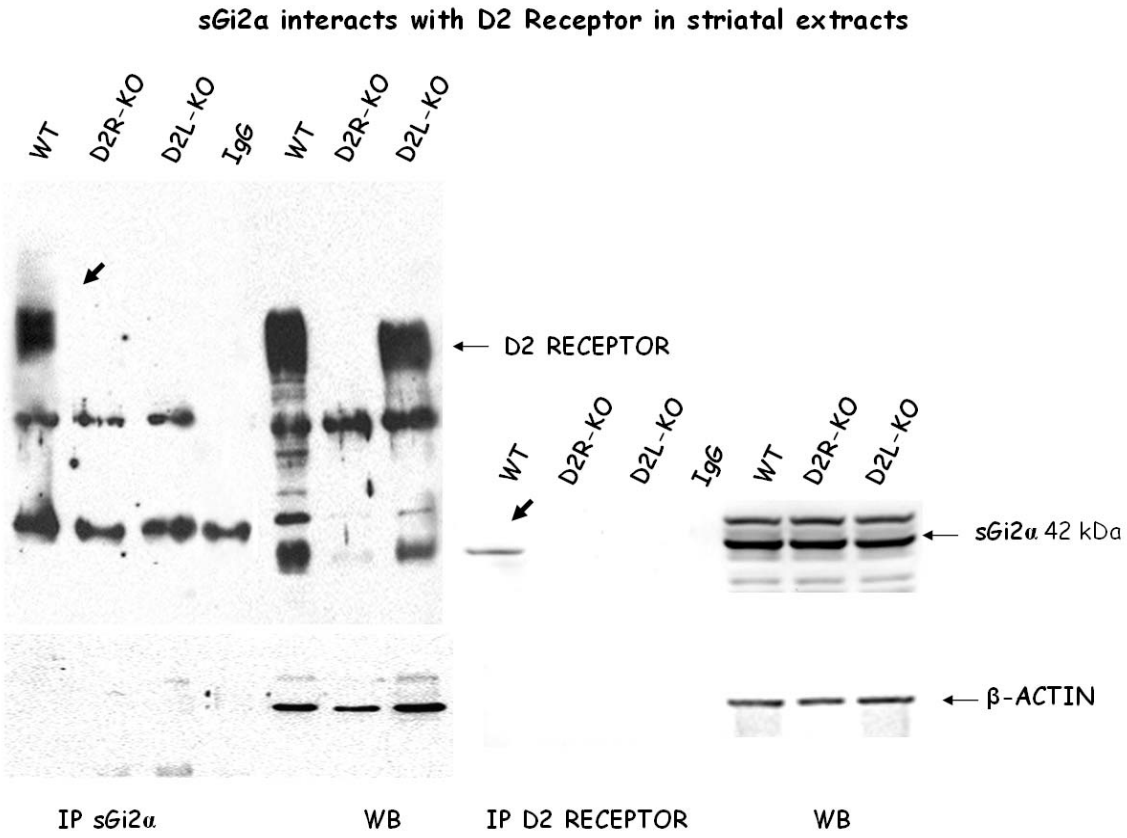

Figure 11. sG_{i2} and D2L are co-expressed in the striatum. Membrane proteins from WT, D2R-KO and D2L-KO mouse striatum were immunoprecipitated with a pab anti-sG_{i2}, processed by Western blot and revealed with a mab anti-D2R, on the left, or samples were immunoprecipitated using a mab anti-D2R and the reveled by Western blot using a pab anti-$sG_{i2\alpha}$, on the right. For control WT proteins were immunoprecipitated with rabbit or mouse IgG respectively. Proteins were loaded without immunoprecipitation. These results showed a co-immunoprecipitation in WT extracts but not in D2R-/- and D2L-/- thus suggesting that in vivo sGi2 interacts specifically with D2L but not with D2S. IP, immunoprecipitation, WB, Western blot.

More recently, I have observed by immunofluorescence analyses on primary culture of dopaminergic striatal neurons, that addition of D2R agonists as well as of antagonists in the medium, induces mobilization of D2R and of sGi2 from the Golgi (fig. 12 and fig. 13). This suggests that sGi2 is very likely implicated in the trafficking of D2R from this subcellular structure to the membrane. The observation that D2R agonists and antagonists have the same properties on D2R trafficking, suggest that these compounds might act as molecular chaperones (i.e. molecules that might help receptor trafficking) in D2R mobilization. These results show the existence of mechanisms regulating dopamine receptors recruitment to the plasma membrane. These results might be relevant to clinical studies, in which it has been shown that the effect of agonists or antagonists is not immediate. We might speculate that the therapeutical effect of these compounds becomes evident also thanks to the traslocation of the D2R at the membrane.

The interaction between D2R and sG_{i2} might have a critical role in the physiology of the central nervous system (CNS), since it appears to be responsible for the intracellular retention of D2Rs. However, sG_{i2} also is widely expressed in the CNS (Montmayeur and Borrelli, 1994) suggesting that it might in addition regulate other 7-TMD receptors in the same way.

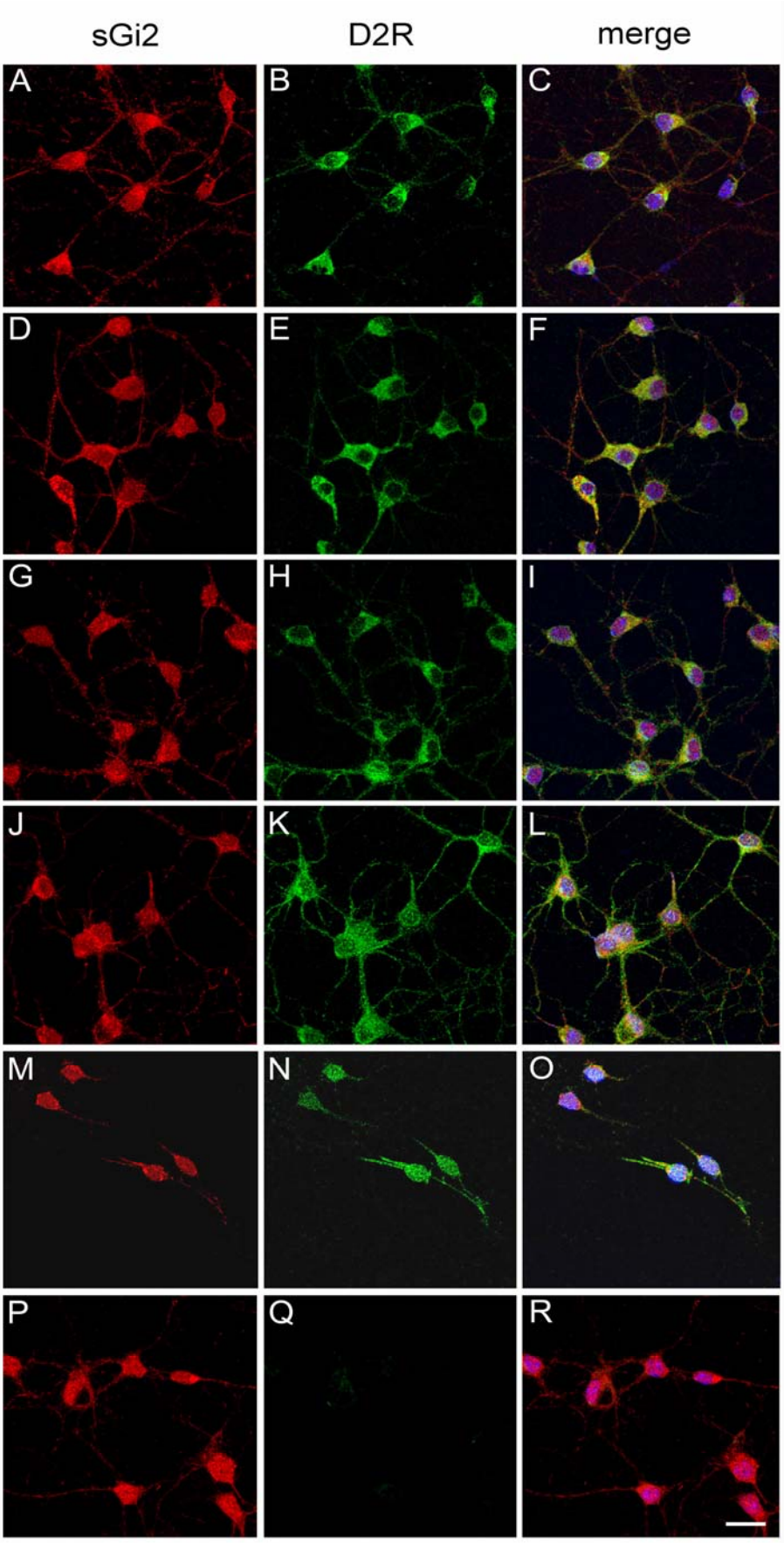
sGi2
D2R
merge
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Figure 12. D2R agonists and antagonist induce the mobilization of D2R from the Golgi to the membranes. Immunostainings using a pab anti-sG_{i2} (A, D, G, J, M, P) and a mab anti-D2R (B, E, H, K, N, Q) were performed on PN1 mouse striatum neuronal cultures treated with different D2R agonists [dopamine (D-F), bromocriptine (G-I), quinpirole (J-L)] and antagonist [haloperidol (M-O)], and 0.6 µM thick planes were visualized with a confocalmicroscope. (A-C): control without treatment.; (P-R): effect of quinpirole on neurons from D2R ko animal. Depending of the treatment, note the D2R overexpression at the neurite membrane level following quinpirole and haloperidol treatments (K, N). Scale bar 23 µl.

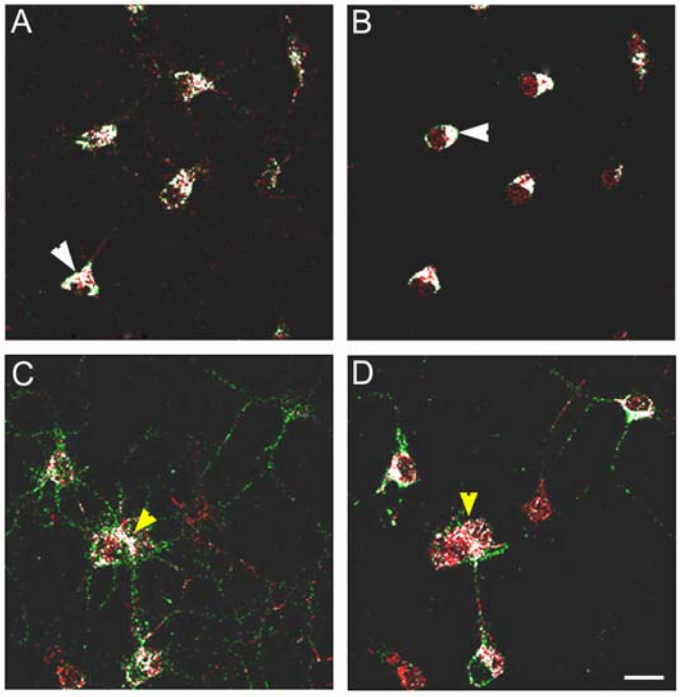


Figure 13. sG_{i2} and D2R are co-localized in the Golgi apparatus of striatal neurons *in vitro*. Colocalization of D2R and sG_{i2} were analysed in control (A, C) and quinpirole (B,D) treated striatal neurons and visualized in white on pictures. (A, C) and (B,D) correspond to two different planes of the same confocal analysis. In the control, the colocalization is strong and concentrated in the Golgi (white arrows), whereas after quinpirole treatment it is less abundant and more diffused in the cytoplasm (yellow arrows). Scale bar 23 µM.

CHAPTER I

Manuscript N°1

Impaired light masking in dopamine D2 receptor–null mice

Masao Doi1, Irene Yujnovsky, Jun Hirayama1, Monica Malerba, Emanuele Tirotta, Paolo Sassone-Corsi, & Emiliana Borrelli

Environmental cues modulate a variety of intracellular pathways whose signaling is integrated by the molecular mechanism that constitutes the circadian clock. Although the essential gears of the circadian machinery have been elucidated, very little is known about the signaling systems regulating it. It has been reported that signaling mediated by the dopamine D2 receptor (D2R) enhances the transcriptional capacity of the CLOCK:BMAL1 complex, *in vitro* (Yujnovsky et al., 2006). This effect involves the mitogen-activated protein kinase transduction cascade and is associated with a D2R-induced increase in the recruiting and phosphorylation of the transcriptional coactivator cAMP-responsive element-binding protein (CREB) binding protein. Importantly, CLOCK:BMAL1-dependent activation and light-inducibility of mPer1 gene transcription is drastically dampened in retinas of D2R-null mice (Fig. 14). Because dopamine is the major catecholamine in the retina, central for the neural adaptation to light, our findings establish a physiological link among photic input, dopamine signaling, and the molecular clock machinery. Notably, DA is the major catecholamine in the vertebrate retina playing a

central role in neural adaptation to light (Witkovsky, 2004). Yet, the physiological contribution of light-dependent dopaminergic signaling to nonvisual functions has remained unexplored. Our results show that signaling mediated throught the D2R greatly influences central molecules participating in the regulation of the circadian clock, playing a central role in the control of light masking of circadian locomotor activity. D2R mediating signaling is required in regulating the proper organization of daily locomotor activity in light-dark cycles.

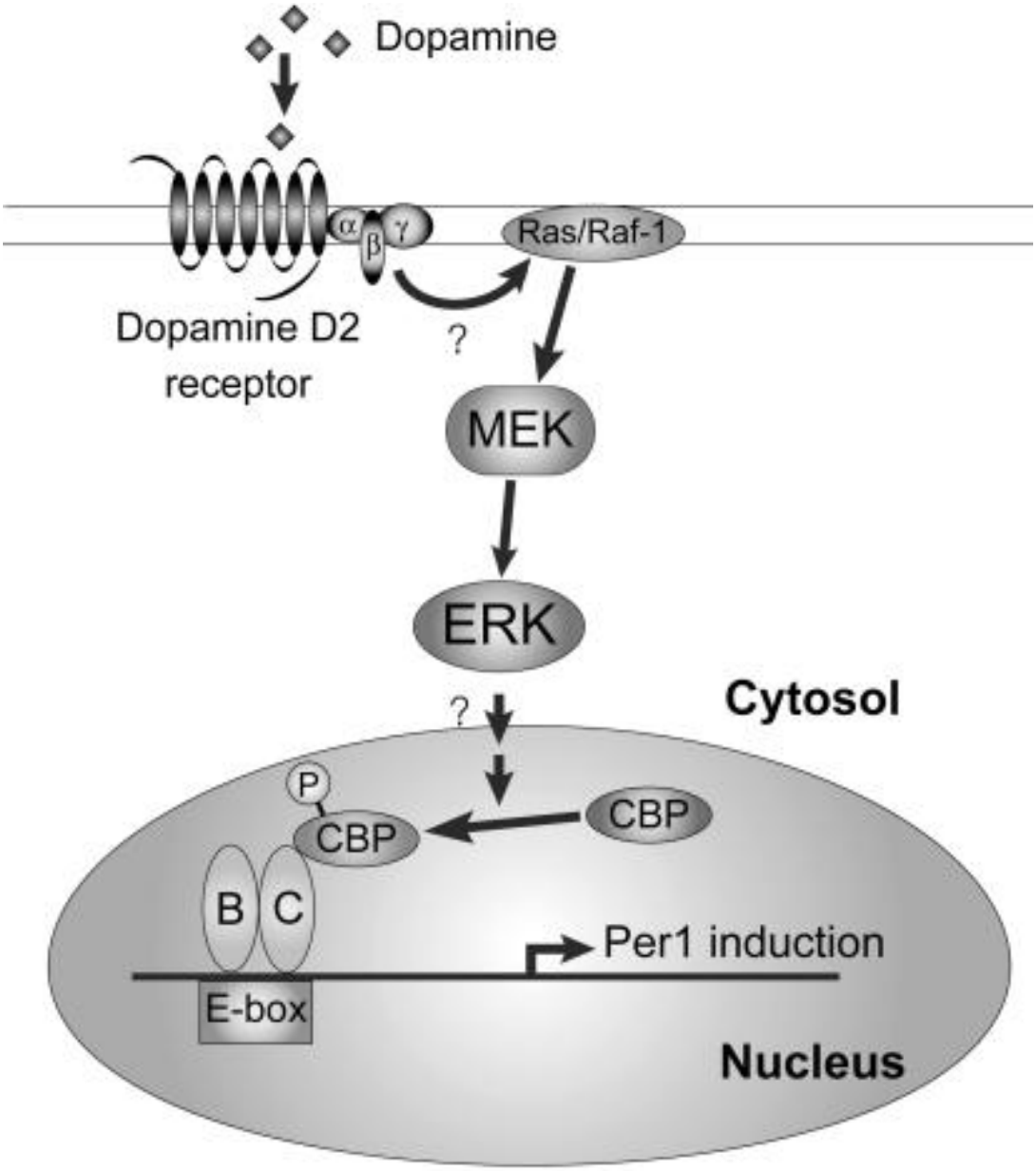


Figure 14: Proposed mechanism of D2R-mediated *mPer1* induction. Activation of the dopamine D2R signaling cascade results in enhancement of CLOCK:BMAL1-driven transcription of clock genes. This signaling system involves the activation of MAPKs and the increased recruitment and phosphorylation of the transcriptional coactivator CBP to the CLOCK:BMAL1 complex. P, phosphate; B, BMAL1; C, CLOCK.

CHAPTER II

Manuscript N°II

Regulation of Akt signaling by D2 and D3 dopamine receptors *in vivo*

Jean-Martin Beaulieu, Emanuele Tirotta, Tatyana D. Sotnikova, Bernard Masri, Ali Salahpour, Raul R. Gainetdinov1, Emiliana Borrelli and Marc G. Caron

Recent *in vivo* studies revealed that striatal D2-class receptors exert their action in a cAMP independent fashion by promoting the formation of a signaling complex composed of Akt, protein phosphatase-2A (PP2A) and β-arrestin 2 (Beaulieu et al., 2004; Beaulieu et al., 2005). Formation of this complex leads to the inactivation of Akt following the dephosphorylation of its regulatory threonine 308 (Thr-308) residue by PP2A (Beaulieu et al., 2005). Inactivation of Akt in response to DA results in the activation of glycogen synthase kinase 3 (GSK-3), as indicated in figure 15 which in turn contributes to the expression of DA-associated behaviors (Beaulieu et al., 2004). Interestingly, reduced Akt functions have been reported in schizophrenic patients, while administration of the antipsychotic haloperidol, a D2-class receptor antagonist, activates Akt and inhibits GSK-3 in the mouse brain (Emamian et al., 2004). Recently, I have been involved in a study aimed at elucidate the role of D2R in Akt phosphorilation. For this, I have collaborated with a postdoctoral fellow, Martin Bealieu, from the laboratory of Prof. M. Caon, at Duke University, North Carolina. The results of this collaboration have now been submitted at the Journal of Neuroscience, and appear in the following article.

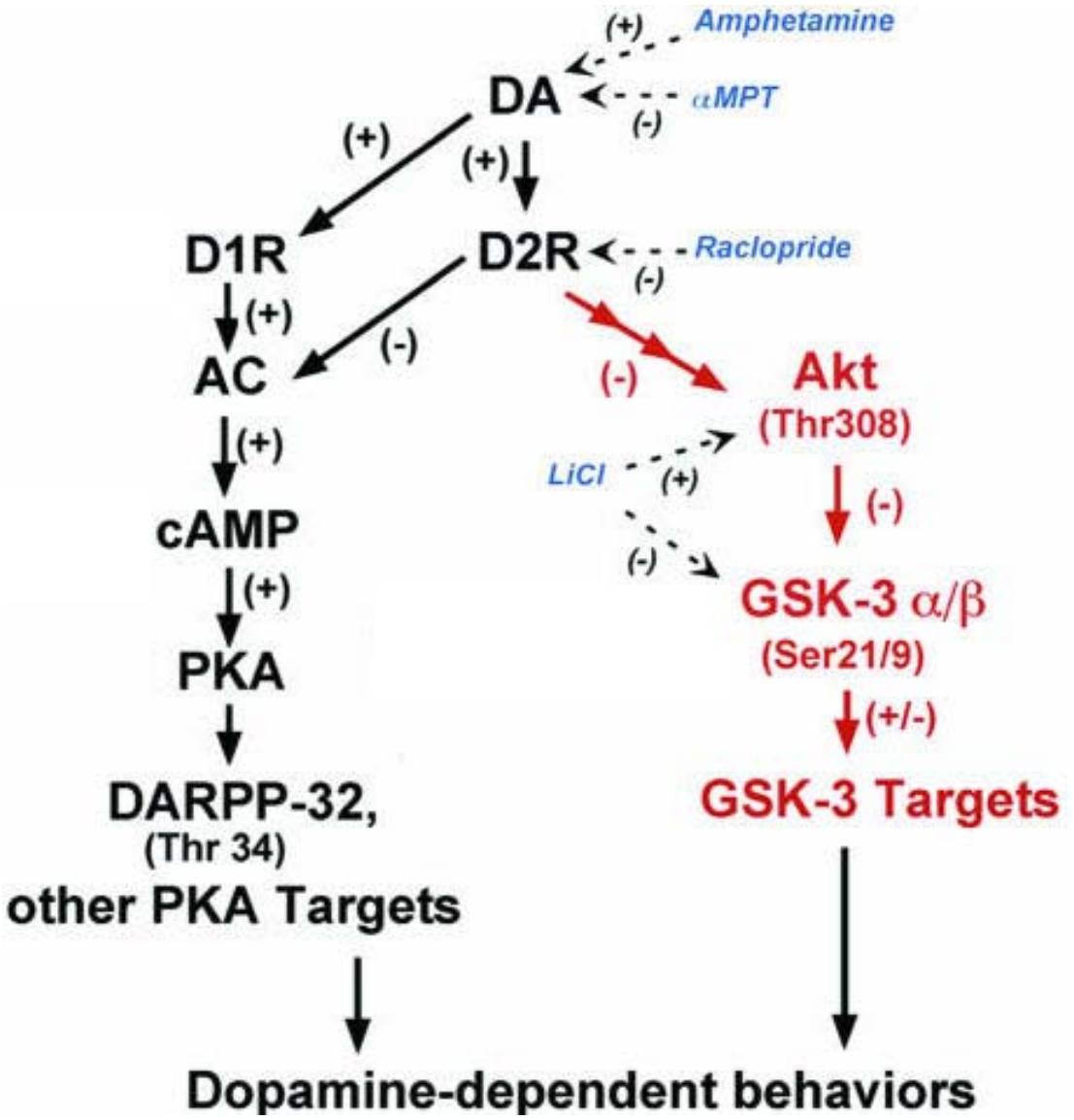


Figure 15. Working model of potential mechanisms of DA receptor signaling. In red, regulation of the Akt/GSK-3 pathway by DA. Elevated DA tones lead to activation of GSK-3 through a signaling cascade involving D2-class receptors and the inactivation of Akt caused by a reduction of its phosphorylation on Thr-308. This reduction in Akt activity results in a reduced phosphorylation/increased activation of GSK-3α and GSK-3β that, in turn, regulate DA-associated behaviors. Note that, in contrast to DARPP-32, the phosphorylation of Akt and GSK-3 was not affected by cAMP. Dashed lines indicate the effect of different drugs on this signaling cascade. D1R, DA D1 receptor; D2R, DA D2 receptor; AC, adenylyl cyclase.

Dopamine D2 receptors-mediated signaling and neuronal aging: implications with Parkinson's disease.

D2R and Parkinson's disease

Parkinson's disease (PD), together with other major age-related neurodegenerative disorders such as dementia with Levy bodies, multiple system atrophy and Alzheimer's disease, imposes a huge economic and emotional burden that will increase greatly as people live longer. Understanding how specific sets of neurons are lost in these disorders has become one of the major imperatives of biomedical research.

PD is characterized by altered motor function comprising: resting tremor, rigidity, difficulty in initiating voluntary movements and poor postural reflexes. These deficits are due to the loss of dopaminergic neurons of the Substantia Nigra (SN), which results in diminished synthesis and release of dopamine at nerve terminals in the Striatum. Post-mortem analyses of the brain of patients with PD shows that the surviving dopamine neurons commonly contain intracytoplasmatic eosinophilic inclusions, named Lewy bodies, which represent a hallmark of the disease.

Although symptoms of some chronic neurodegenerative disorders can be temporally relieved by pharmacological or surgical manipulations, it is not

possible to stop the progression of these diseases. Furthermore, with time, the response to pharmacological treatments declines with the appearance of adverse effects.

The discovery that most neurodegenerative disorders feature excessive protein misfolding and intracellular protein aggregation has given a strong input to the study of these diseases (Sherman and Goldberg, 2001). The mechanism by which protein aggregates impair cell function and survival are slowly being elucidated. Several proteins have been implicated in the composition of the cellular aggregates.

One of the best characterized proteins and major constituent of these aggregates is α-synuclein (α-syn), a protein highly enriched in the synapses. A role of α-synuclein in the pathogenesis of PD was first shown in 1997 when an Ala53Thr substitution was found in a large Italian family as well as in several smaller Greek kindred affected by a familial form of PD (Polymeropoulos et al., 1997).

The pathogenetic nature of the mutation was soon confirmed by the identification of a second mutation, Ala30Pro, in a german kindred (Kruger et al., 1998) and by the demonstration of α-syn as a major component of Lewy bodies in sporadic forms of the disease (Spillantini et al., 1997). Interestingly, intracellular protein aggregates containing α-syn have been found to be hallmarks of several other neurodegenerative disorders including, dementia with Levy bodies and multiple system atrophy.

The discovery that missense mutations in α-syn are associated with a small subset of PD (Lucking and Brice, 2000) suggests a causal role of this protein in neurodegeneration and has triggered a growing research interest on its function. However, at present, neither α-syn function nor its contribution to neurodegeneration is completely understood. One possibility is that α-syn may facilitate vesicle transport from the cell body to the synapse. Consistent with this notion, α-syn is enriched in presynaptic terminals, and its N-terminal structurally resembles an apolipoprotein α-helical region that appears to reversibly bind vesicular membranes (Fig. 16).

Importantly, α-syn knockout mice exhibit a defect in dopamine release and reuptake, supporting a role for α-syn in the regulation of dopamine neurotransmission (Abeliovich et al., 2000). A-syn is not the only factor determining the vulnerability of neurons in PD and Parkinsonism; α-syn expression occurs in neurons throughout the central nervous system, yet the neurodegeneration in PD exhibits predominant selectivity for only one region of the brain, the dopaminergic neurons in the SN.

Xu et al. (Xu et al., 2002) recently identified a second contributing factor to the selective neural vulnerability in PD by showing that α-syn neurotoxicity may stem from its interaction with dopamine in dopaminergic neurons. Their conclusions are consistent with earlier reports (Tabrizi et al., 2000; Jae Kim et al., 2006) demonstrating that α-syn expression is toxic to dopaminergic neuronal cell lines.

Dopamine induces protein damage through oxidation, by generating reactive quinones and oxygen radicals, which in turn can cause apoptosis. Oxidative stress resulting from dopamine metabolites has long been suspected of playing a role in the selective death of dopaminergic neurons that leads to PD (Giasson et al., 2003).

Other neurodegenerative disorders may be generated by variations in the composition of protoaggregates producing toxicity and mature aggregates providing a means of sequestering neuron specific proteins.

The causes of the abnormal folding of proteins are various and still poorly understood. Obvious causes might be genetic defects producing a single amino acid substitution or expansion of a repeated amino acid tract, as it occurs in the familial forms of many neurodegenerative diseases (Moore et al., 2005). However for most neurodegenerative disorders that occurs sporadically or in a non-Mendelian fashion, other causes of abnormal folding might lie at the source of the pathogenic cascade.

An aspect presently ignored in Parkinson's as well as in other related neurodegenerative diseases, is represented by the participation of dopamine receptors to these pathologies. In particular, several evidence (Bozzi et al., 2000; Bozzi and Borrelli, 2002) showing that D2R signalling might exert a neuroprotective effect on dopaminergic and D2R containing neurons previously ignored. Maybe the discovery of the molecular mechanism of such

neuroprotection will offer novel therapeutic targets for the treatment of Parkinson and related disorders.

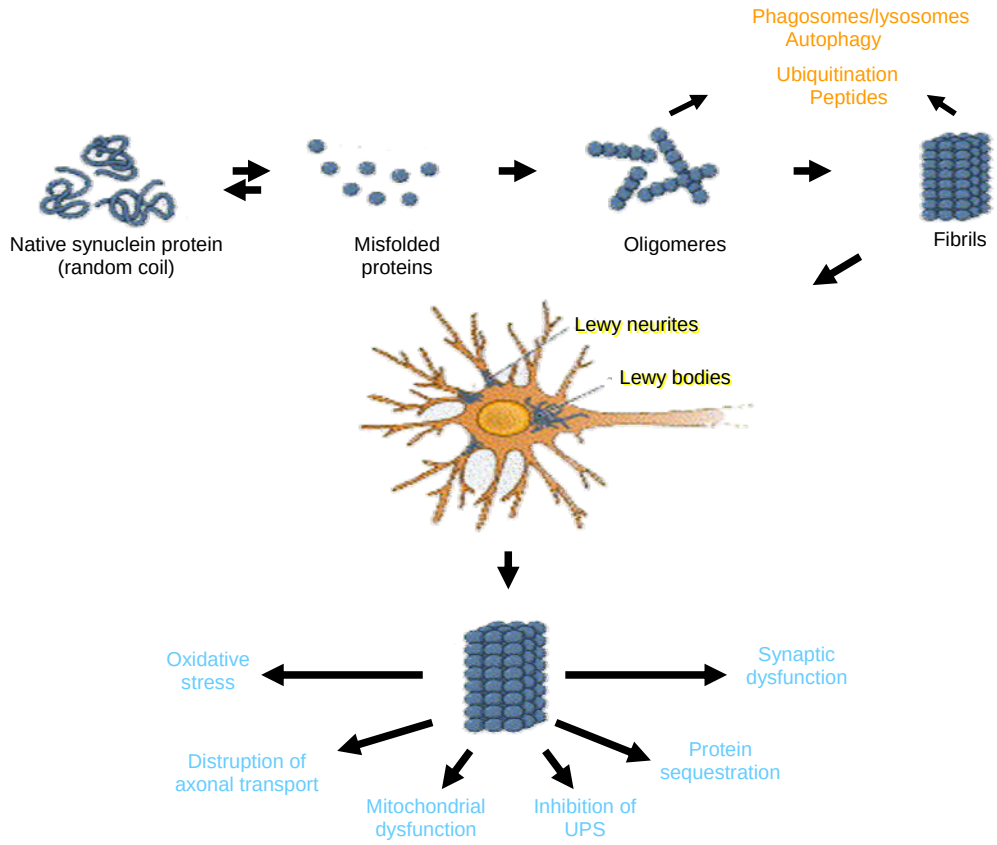


Figure 16: Model of α-syn misfolding and aggregation and the downstream consequences. Schematic illustration of the stepwise process whereby normal, highly soluble α-syn misfolds and is converted into pathological oligomers and higher-order aggregates that fibrillize and deposit into Lewy bodies and Lewy neurites in affected neurons of the PD brain. Genetic abnormalities, as well as poorly understood environmental factors (e.g., pesticides, head trauma), may accelerate this process and overwhelm the ability of normal quality-control systems (molecular chaperones, ubiquitin proteosome system (UPS), phagosome/lysosome system) to prevent or reverse protein misfolding or eliminate proteins that have misfolded or assembled into pathological aggregates and amyloid fibrils. Several toxic consequences of the accumulation of fibrillar deposits of α-syn are illustrated in the lower part of the figure.

Dopamine dependent neurotoxicity of α-syn

Several lines of evidence have shown that the accumulation of α-syn renders endogenous levels of dopamine toxic by potentiating the generation of reactive oxygen species, thus providing a potential mechanism for the selective loss of dopaminergic neurons in PD (Rochet et al., 2004). However, α-syn is not intrinsically a cell-death protein, as it exhibits neuroprotective activity in non dopaminergic human cortical neurons. Several reports suggest that this selective vulnerability is likely due to the toxic potential of endogenous dopamine (Conway et al., 2001). Rather an early stage of aggregate consisting of fewer than 30 α-syn molecules, a "protoaggregate" or "oligomer", is probably the offending species. It may exert toxic action by creating pores in lipid membranes (Volles and Lansbury, 2002). One result is leakage of dopamine from vesicle to the cytoplasm. Free dopamine aside from its direct oxidative toxicity, exacerbate the pathogenetic process by inhibiting the further aggregation of the protoaggregates into the Lewy bodies (Conway et al., 2001; Volles and Lansbury, 2002). By sequestering protoaggregates, the Lewy bodies may provide a protective function. The interest in protein misfolding, aggregation, and proteosomal activity has provided further insight into potential pathogenetic pathways in PD.

More recently, there has been increasing interest in the development of drugs able to modify these biochemical abnormalities, either by delaying the rate of cell death or by restoring function to neurons that are likely to be damaged

but not dead; this way altering the course of PD. In this context, dopamine agonists have shown significant promise. Not only do these drugs provide symptomatic relief of PD but they also appear to be associated with a significant decrease in the rate of motor complications and to be capable of protecting against some of the adverse consequences of levodopa use (Schapira, 2002; Maratos et al., 2003; Weber et al., 2003). Additional neuroprotective properties of dopamine agonists may depend on their antioxidant actions in vitro and in vivo. In particular, some D(2)/D(3) dopamine agonists may have neuroprotective activity (Maratos et al., 2003). Protection in cell and animal models against a variety of toxins, including MPTP and 6-hydroxydopamine, confirms that these agonists have in vitro and in vivo neuroprotective action (Jenner, 2003). If the neuroprotective action of this drug is confirmed in patients with PD, this will have important implications for its early use in patients (Schapira, 2002). However, it is not clear yet how dopamine agonists might exert their protective activity. Some evidence suggests that this may be mediated by modulation of mitochondrial membrane potential and the inhibition of apoptosis. It is therefore important to explore the mechanism of action of these compounds in preclinical model systems.

It was been evaluating the influence of D2R-mediated functions in neuroprotection. Previously studies shown that D2R-mediated signalling is neuroprotective against glutamate and cholinergic outbursts in vivo (Bozzi et

al., 2000; Bozzi and Borrelli, 2002). Thus suggesting that D2R-mediated signaling might have a general protective function in different areas of the CNS. This is of particular relevance with respect to Parkinson's as well as other neurodegenerative diseases arising in brain areas that receive dopaminergic inputs such as Alzheimer disease and MSA (Multiple System Atrophy). The rationale is that D2Rs have a pre- and postsynaptic localization and function. In addition, Rougé-Pont et al. have shown that the D2 receptors are the bona fide dopaminergic receptors regulating dopamine release (Rouge-Pont et al., 2002). These observations led us to investigate the role of D2R on α-syn accumulation in the CNS, *in vivo*. D2R-mediated effect might promote or prevent neurodegeneration and/or might accelerate the physiological aging of dopamine neurons as well as of neurons in the projecting areas.

SPECIFIC AIM

During my thesis, I have attempted to elucidate the role of D2R signaling in aging of dopaminergic and striatal neurons, *in vivo*. My work has been concentrated on the analysis of two animal models previously generated in the laboratory. These mutant are characterized either by the specific ablation of the two isoforms of the D2 receptor (D2R-KO mice) or by the selective ablation of the long isoform of the receptor (D2L-KO mice).

Signaling through dopamine D2 receptors governs physiological functions related to locomotion, hormone production and drug abuse (Montmayeur et al., 1991; Maldonado et al., 1997; Mercuri et al., 1997; Saiardi et al., 1997). By alternative splicing the D2 receptor gene encodes two distinct isoforms: D2L which acts mainly at postsynaptic sites and D2S (Fig. 17) which serves presynaptic autoreceptor functions controlling dopamine (DA) release (Usiello et al., 2000). **A**blation of D2 receptor results in locomotor impairment, altered response to drugs of abuse, pituitary tumors and electrophysiological alterations of D2R-expressing neurons (Montmayeur et al., 1991; Maldonado et al., 1997; Mercuri et al., 1997; Saiardi et al., 1997).

D2R is target of dopamino-mimetic agents which are the first-line treatment in Parkinson's disease (PD). Reportedly, early treatment of PD with dopamine (DA) agonists, selectively stimulating D2 receptors, may modify the disease progression by possibly preventing cell death (Nair and Sealfon, 2003). Moreover DA agonists through D2 receptors may activate

neuroprotective signaling pathways and mediate increased cell survival (Kihara et al., 2002).

DA synthesis and release is regulated by D2R, I thus evaluated the contribution of D2R mediated signaling to the pathogenetic mechanism underlying the formation of α-syn intracellular aggregates. Here I show the occurrence of neuronal sufferance in mice lacking D2 receptors, as indicated by α-synuclein accumulation, chaperon inductions and accelerated aging in different brain areas. Thus, absence or downregulation of D2R expression might promote neurodegeneration and/or might accelerate the physiological aging of dopamine neurons as well as of neurons in the projecting areas. The possibility to analyze mouse models in which either both the D2R isoforms are missing (D2R-KO) or only D2L (D2L-KO), allowed to get insights into the mechanisms by which these aggregates are formed. In particular, using these mice I has been able to explore whether α-syn aggregates are dependent from the lack of D2 pre- and post-synaptic mediated signaling, as in D2R-KO or only by loss of D2-mediated postsynaptic signaling as in D2L-KO.

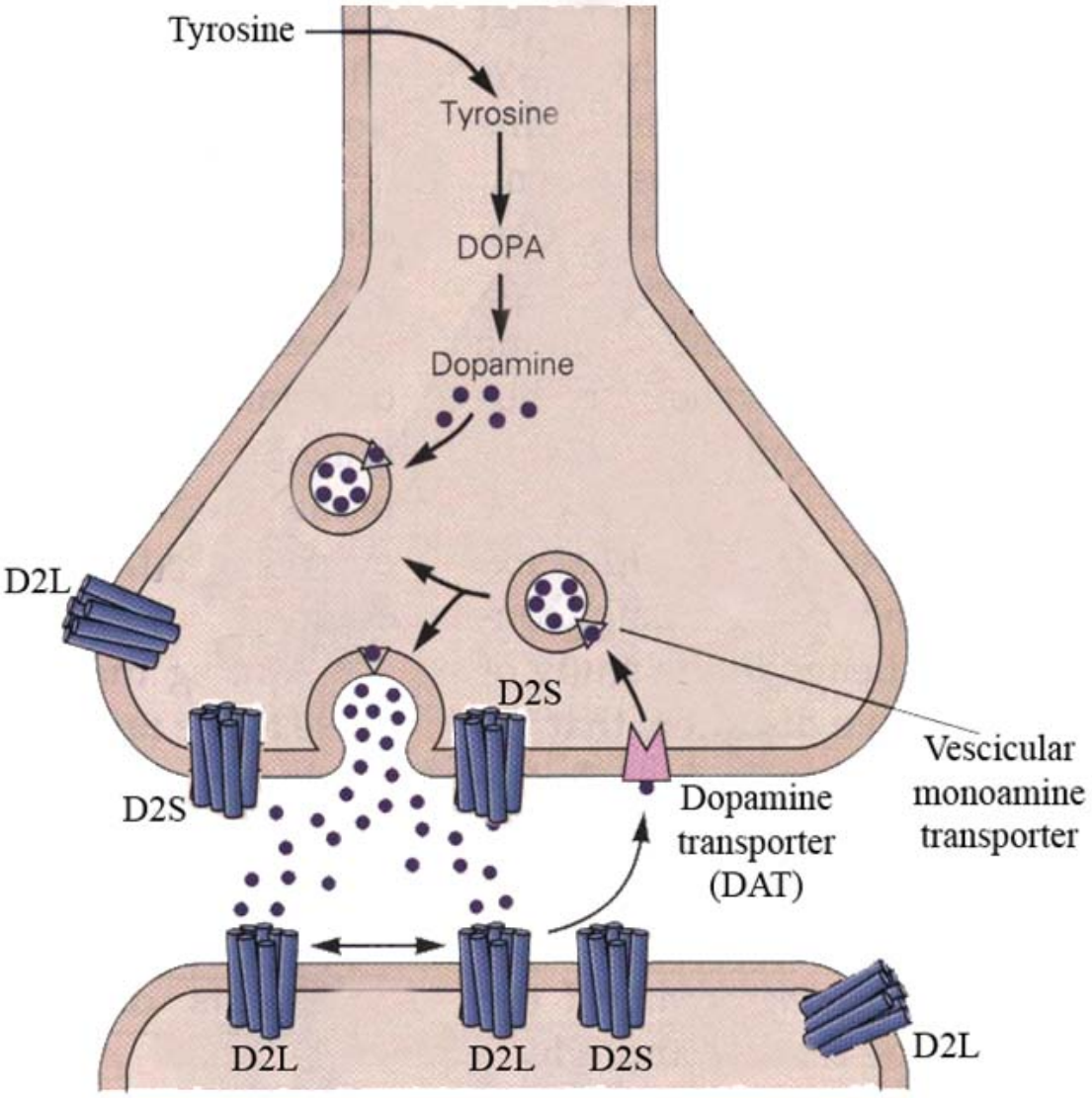


Figure 17: Schematic representation of DA syntesis, release and reuptake. Presynaptic nurons (top) are able to syntetize the DA. Once DA is released, it goes to bind and activate D2R. As indicate in the picture, D2S is mostly presents at presynatpic level, while D2L is more postsynaptic receptor. DAT is responsible for the presynatic DA reuptake.

MATERIALS AND METHODS

Construction of Mutant Forms of the D2R Receptor

The D2R gene was isolated from a mouse 129-ES/EMBL3 genomic library. A 6.5-kb *Kpn*I-*Sal*I genomic fragment corresponding to the *D2* gene was isolated and subcloned into pBlueScript (Stratagene). From this clone, a 0.9-kb *Nco*I fragment, containing exon 2 and flanking intron sequences, was removed and replaced by a 1.4-kb segment of the PGKI-neo cassette, inserted in the opposite transcriptional orientation, relative to the *D2* gene (Baik et al., 1995). This targeting vector was linearized and electroporated in P1-ES cells. ES cells carrying the disrupted *D2* allele were revealed by Southern blot and positive clones were injected into C57BL/6 embryos at the blastocyst stage. Chimeras offspring were mated with C57BL/6. Germline transmission of the mutant allele was assessed by Southern blot analysis of tail DNA isolated from the agouti-coat progeny (Baik et al., 1995).

Construction of Mutant Forms of the D2L Receptor

A 585-base pair *Hinc*II fragment of the mouse D2L cDNA (Montmayeur et al., 1991) containing the 87-base pair D2L-specific sequence was subcloned into pBluescript SK (Stratagene, La Jolla, CA) with deleted endogenous *Sac*I and *Bsp*HI sites to generate pD2L-HII. To generate each mutant, pD2L-HII was digested with two restriction enzymes, and the wild-type fragment was

substituted with the mutagenized fragment. Pairs of complementary oligonucleotides including the mutations were synthesized corresponding to the sequences between the two restriction sites (Guiramand et al., 1995). After annealing, the oligonucleotides were ligated into pD2L-HII, generating the different mutants. The *Hinc*II-mutated fragments were then sequenced and exchanged with that of wild-type D2L. The D2L mutants were verified by restriction digests and sequencing. The full-length inserts containing the entire D2L coding region were finally subcloned into the pSG5 eucaryotic expression vector (Green et al., 1988). To generate mutants K251V and D249V, pD2L-HII was digested with *Bsu*36I and *Bsp*HI at positions 868 and 896 of the full-length mouse D2L cDNA, respectively (Montmayeur et al., 1991). For mutants K3R-V, K5R-V, P264G, S259/262A, and D271V, pD2L-HII was digested with *Bsu*36I and *Sac*I at positions 868 and 953, respectively. Animals were housed four or five to a cage at 23°C on a 12 h light/12 h dark cycle with ad libitum access to food and water. Animal care was approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Membrane preparations

Mouse striatal from D2R- D2L-KO and WT littermas were rapidly dissected . For the isolation of membranes, fresh tissues were homogenized on ice with a

polyethylene pestle, in a Membrane Extraction Buffer,MEB: 50 mM TRIS pH 7.5, 150 mM NaCl, protease and phosphatase inhibitor cocktail (Sigma, St-Louis, MO). The samples were sedimented by ultracentrifugation (40 minutes, 70 000×g, 4°C), and the pellets were resuspended in MEB supplemented with 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and gently rotated at 4°C for 40 minutes. The solubilized fractions were finally centrifuged, to remove the insoluble material (10 minutes, 18000×g, 4°C) collected by ultracentrifugation. The supernatants represented the solubilized protein membrane extracts.

Fractionation of Mice Brain Tissue

Subcellular fractionation of mice brain was performed by dissecting striatal and Substantia Nigra regions of D2R-, D2L-KO and WT littermas, using a rodent brain matrix (Harvard apparatus, USA). Approximately 10-30 mg of tissue was homogenized on ice in 10 volumes of TBS+ (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 1 mM *N*-ethylmaleimide, plus complete proteasome inhibitor mixture). After 5 min of centrifugation at 1,000 x *g*, the supernatant was ultracentrifuged for 30 min at 120,000 x *g* at 4 °C. The resulting supernatant represented the TBS+ soluble fraction. All subsequent centrifugation steps were performed at 120,000 x *g* for 20 min at 4 °C. The pellet was rinsed twice with TBS+ and extracted sequentially with 1

volume of TBS+ containing 1% Triton X-100 and TBS+, 1 M sucrose to remove myelin. The pellet was then extracted with 1 volume of RIPA buffer (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). The extensively washed detergent-insoluble pellet was solubilized in 8 M urea/5% SDS (and termed the urea-soluble fraction) and sonicated. The 1,000 x *g* pellet from the first step was subsequently extracted in the same way.

Western blotting and immunoprecipitation analysis

For Western blotting assay, 30-50 µg of the protein lysates were loaded per lane and separated on 10 or 12% SDS-polyacrylamide gels and electroblotted to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Non specific binding was blocked in 5% non fat dry milk in 0.05% Tween 20 in PBS for 1h, at room temperature. Membranes were then incubated overnight with appropriate primary antibodies.

Immunocomplexes were revealed by using appropriate peroxidase-conjugated secondary antibodies (Jackson Immuno-Research) along with a chemiluminescent reagent (SuperSignal West-Pico, Pierce). Densitometric analysis was carried out within linear range by using Image Analysis Chemigenius2 (Syngene).

For immunoprecipitation assay, 500 ug of membrane protein extracts were incubated for 4 h with protein A/G-agarose beads at 4 °C and centrifuged at 3,000 x *g* for 5 min, to avoid non-specific binding. The supernatants were incubated overnight at 4°C with 10 µg with the appropriate antibody. The day after samples were incubated 2 hours at 4°C with protein-A/G Sepharose (Amersham Pharmacia Bioscience), in 500ul of PBS supplemented by protease and phosphatase inhibitor cocktail. The beads containing bound proteins were washed four times by centrifugation in MEB buffer, then denatured by boiling in Laemmli sample buffer for 5 min, and resolved on SDS 10 or 12% PAGE, to identify the coprecipitating proteins. Immunoprecipitation with mouse or rabbit immunoglobulin (mIgG or rIgG) alone was used as negative control.

Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. The brain was removed and postfixed in 4% PFA for 2 hr at 4°C and cryoprotected in 30% sucrose solution. Seven micrometer sagittal sections were cut on a freezing microtome (Leica, Nussloch, Germany). All sections were mounted on Superfrost slides (Roth, FRG), and analyzed with appropriate protocols.

Statistical Analysis

Data were analyzed by one-way ANOVA. Values in graphs were expressed as mean ± SEM.

RESULTS

Pathological Inclusions of α-syn in Different Brain regions

To assess whether the α-syn aggregates are expressed in the absence of D2R-mediated signaling, α-syn inclusions were studied by immunohistochemical analysis. SN and Striatal neurons (Fig. 18 A and B) of D2R-, D2L-KO aged mice were labeled with the anti- α-syn antibody SYN-1 and compared with WT littermates. We observed that the SN of D2R mutant mice had a significant higher number of neurons containing round shaped α-syn inclusions, with respect to WT littermates (Fig. 18 A). Moreover, in striatal regions, anti- α-syn labeling displayed spatial variations in intensity so that within individual inclusions, some regions were intensely stained, while other regions remained less stained (Fig. 18 B). Quantification of the percentage of neurons that contain α-syn inclusions indicated that the majority of SN neurons in the both KOs mice were α-syn positive, whereas in the striatum only the axonal projections that came from the SN dopaminergic neurons seemed labeled but not the cell body of medium sping neurons.

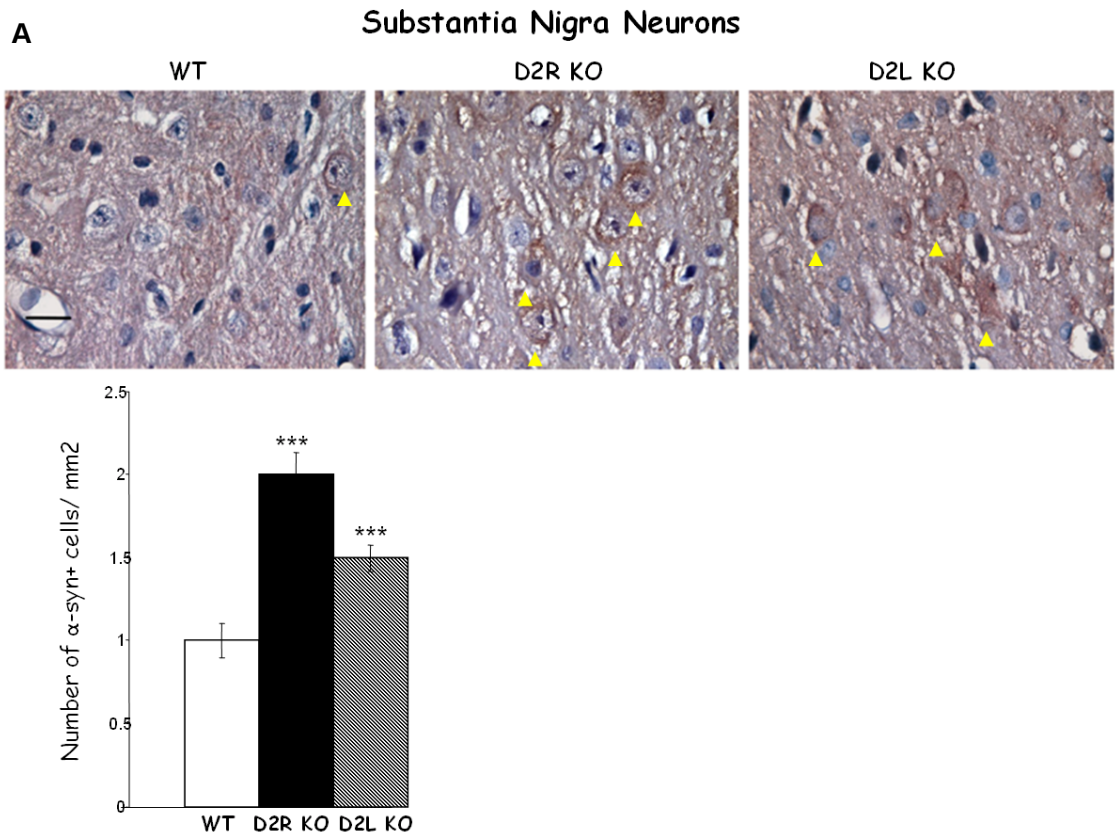


Figure 18 A. Immunohistochemical analyses using a α-synuclein specific antibody, demonstrate that in D2 KO animals α-synuclein accumulates in dopaminergic neurons of Substantia Nigra as well as in the striatum

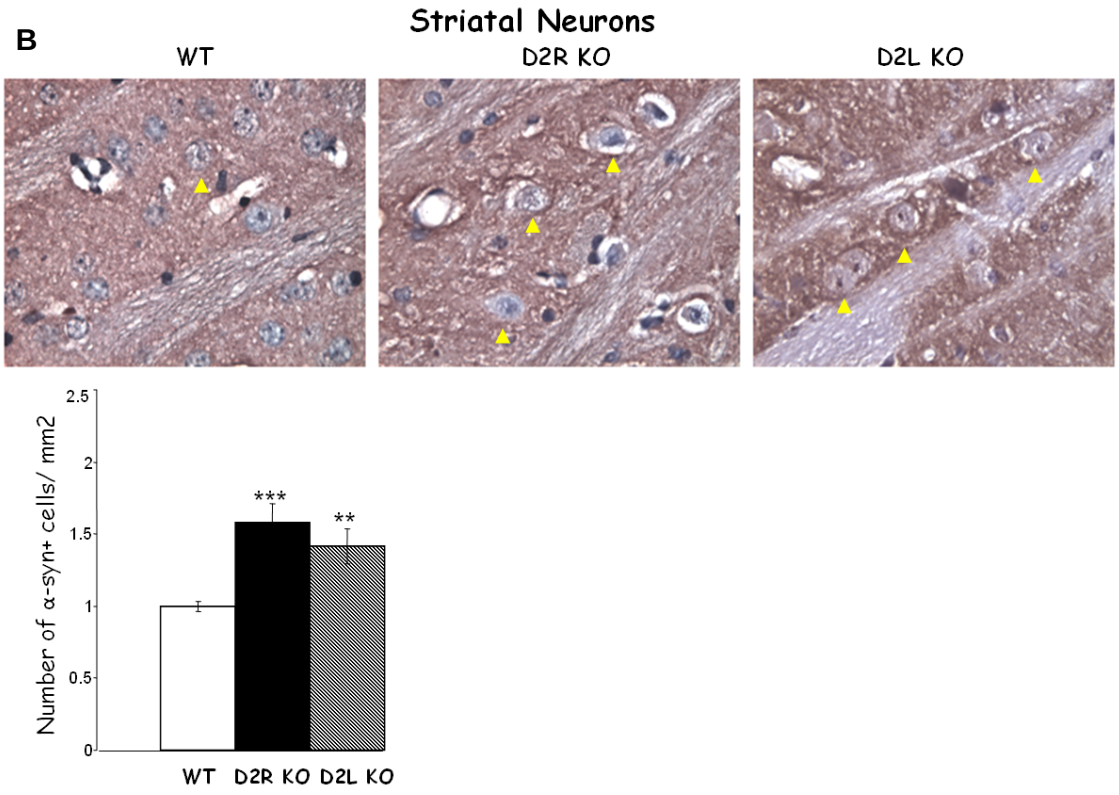


Figure 18 B: Immunostainings of brain slices show specific accumulation of α-syn in D2R mutant mice. Animals lacking either D2R or D2S showed a strong cytosolic labeling of neuronal cells with the specific α-syn antibody (**A** and **B**). A section from a 18-month-old D2R-KO demonstrates the accumulation of α-syn protein in the SN neurons (**A**) as well as in the striatal region (**B**). In contrast, in WT animals immunostainings with α-syn antibody des not reveal any accumulation of endogenous murine α-syn antibody (**A** and **B**) in neuronal cell bodies. Scale bar in A, 200 µm

Detergent Insoluble α-syn in D2R-KO Brains

Neuronal aging is age depended related (Chu and Kordower, 2006): the normal aging contributes to loss dopaminergic nigral neurons in PD expressing alpha-synuclein. I analyzed the temporal evolution of α-syn aggregates in older mice. Strikingly, and in sharp contrast with WT mice in which no α-syn aggregates were detected, 18 month old D2R-KO showed the presence of bigger aggregates in Substantia Nigra (SN) and Striatal extracts with respect with those found in 2 month old mice. To investigate the extent to which α-syn becomes insoluble and forms aggregates, I used a method that allows of soluble versus aggregated forms of α-syn (Tofaris et al., 2003). This method is based upon differential biochemical fractionation under conditions that slowly solubilize aggregates from the bulk of cytoplasmic and membrane proteins. This method is based on the use of different types of detergents starting from the milder to finish with the stronger. Homogenates are primarily made in a buffer without detergent and then trough a series of centrifugations the pellets are solubilized with detergents of increasing strength. The main purpose of this approach was to identify conditions which maximize the recovery of all associated forms of α-syn. The different fractions were analyzed by conventional immunoblotting. A-syn reactivity was detected in cytoplasmic and membrane fractions from SN and Striatal extracts from D2R-KO, D2L-KO, and control Wild type littermates tissues, as shown in figure 19 and figure 20. In these fractions, α-syn was detected by

using monoclonal antibodies Syn-1 (1:1000; Transduction Laboratories, Lexington, KY) as a single band of 19 kDa in accordance with the apparent molecular mass of full-length protein. Higher molecular mass α-syn bands of ~ 40 and ~75 kDa were detected. In contrast to control animals, in which urea-soluble extracts were virtually devoid of α-syn, strong immunoreactivity was found in urea-soluble extracts from D2R-KO. Oligomeric and higher molecular mass aggregates were the predominant species in this fraction. These results suggest the occurrence of a time dependent evolution of α-syn aggregates in the CNS of D2R-KO mice. The presence of these aggregates might be indicative of an accellerated neuronal aging (Feany and Bender, 2000; Ostrerova-Golts et al., 2000) in the absence of D2R-mediated signaling. The immunoistochemichal data together with the fractionation data indicate that the α-syn aggregates occur above all in the dopaminergic neurons of SN. The huge α-syn aggregation detected in the striatal samples represents obviously the α-syn aggregated in the axonal projection of the SN neurons.

Results are presented in arbitrary units normalized to synaptophysin levels observed in WT littermates. Monoclonal antibody directed against synaptophysin (1:5000, Sigma) was used as loading controls.

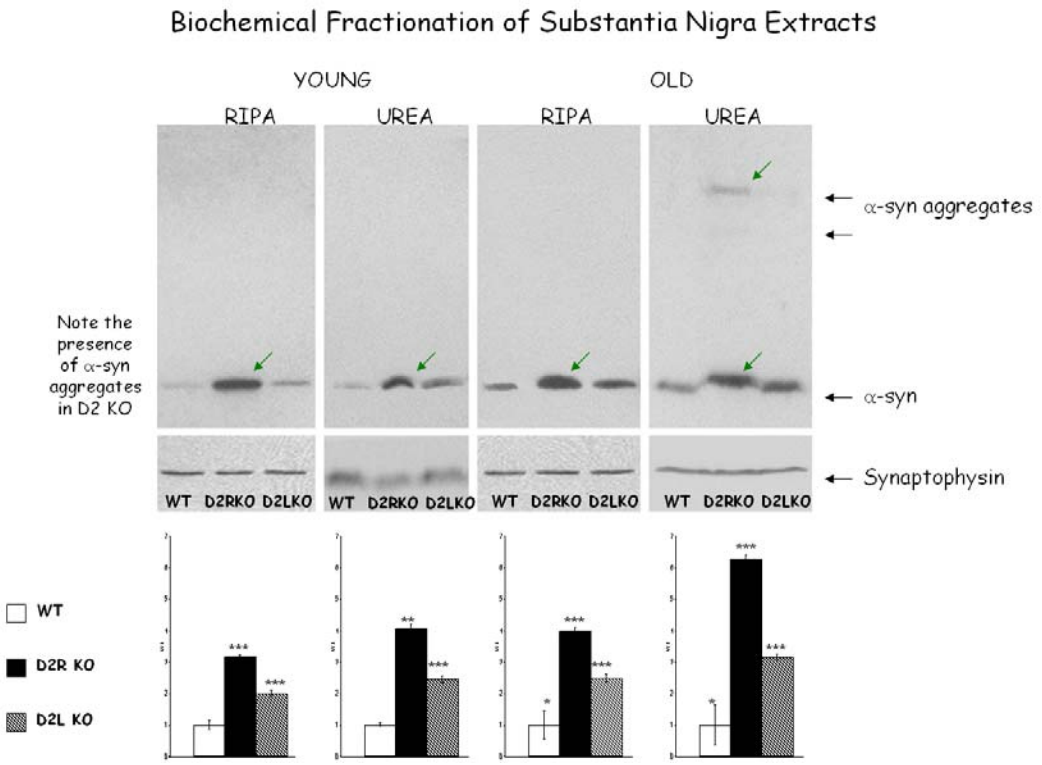


Figure 19: Immunoblotting of α-syn extracted from Substantia Nigra of D2R-, D2L-KO and WT. Immunoblots with Syn-1 antibody from ripa and urea fractions are shown from SN of D2R-KO, D2L-KO, and WT controls. The samples are age-matched. Note the presence of high and low molecular mass aggregates of α-syn in D2R- and D2L-KO but not in control mice. Full-length α-syn was associated with high molecular mass aggregates (*arrows*). N=5 to 10 mice per group, data are average±SEM. *p≤0.05, **p≤0.005, ***p≤0.0005.

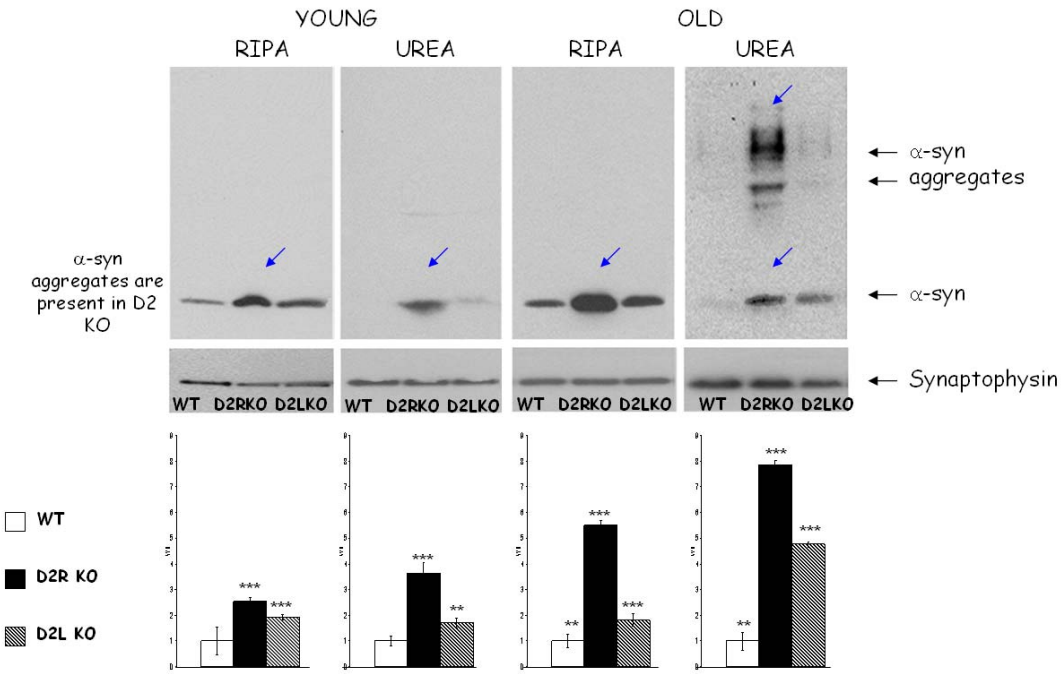


Figure 20: Subcellular fractionation of striatal mice tissues. Sequential detergent extraction methods have been successfully used to detect α-syn in striatal extract of WT, D2R- and D2L-KO mice. Biochemically fractionated striatal extracts were analized by Western blot. Immunoblots were developed with anti-α-syn antibody (SYN-1) as well as anti-synaptophysin antibody mAb. 20 µl of Ripa fraction and 20µl of Urea fraction were loaded in separate lanes of 12% SDS-polyacrylamide gels. Note that the Urea-soluble fraction is eight times more expressed in D2R-KO *versus* WT littermates. Arrowhead indicates α-syn monomer and aggregates. N=5 to 10 mice per group, data are average±SEM. *p≤0.05, **p≤0.005, ***p≤0.0005.

Identification of an interaction between the D2R, DAT and α-syn

While these results demonstrated the presence of α-syn aggregates in D2R deficient mice, it did not clarify whether the absence of D2R both isoforms or only of D2L is responsible of the α-syn aggregates. It was demonstrated that these mutant mice are characterized by a deficit in dopamine release (Usiello et al., 2000), showing increased level of DA in response to different stimuli (Rouge-Pont et al., 2002): thus underlying the prominent role of D2R in the modulation of DA functions. These data suggest that the activity of DAT might also be regulated by D2R and that in the D2R-KO mice the transporter activity might also be alteretad. Data in literature demonstrate that, where the dopamine transporter activity is dysregulated, there might be an increased level of dopamine in the synapse and consequently there might be dopamine-induced neurotoxicity. From co-immunoprecipitation studies, α-syn was found to interact directly with DAT, forming a protein-protein complex in transfected cells, primary cultures of mesencephalic neurons and rat Substantia Nigra (Wersinger et al., 2003; Wersinger and Sidhu, 2003). This suggests that disruption of the ability of α-syn to regulate DAT function may be one of the most important determinants in the genesis of dopaminergic neurodegeneration.

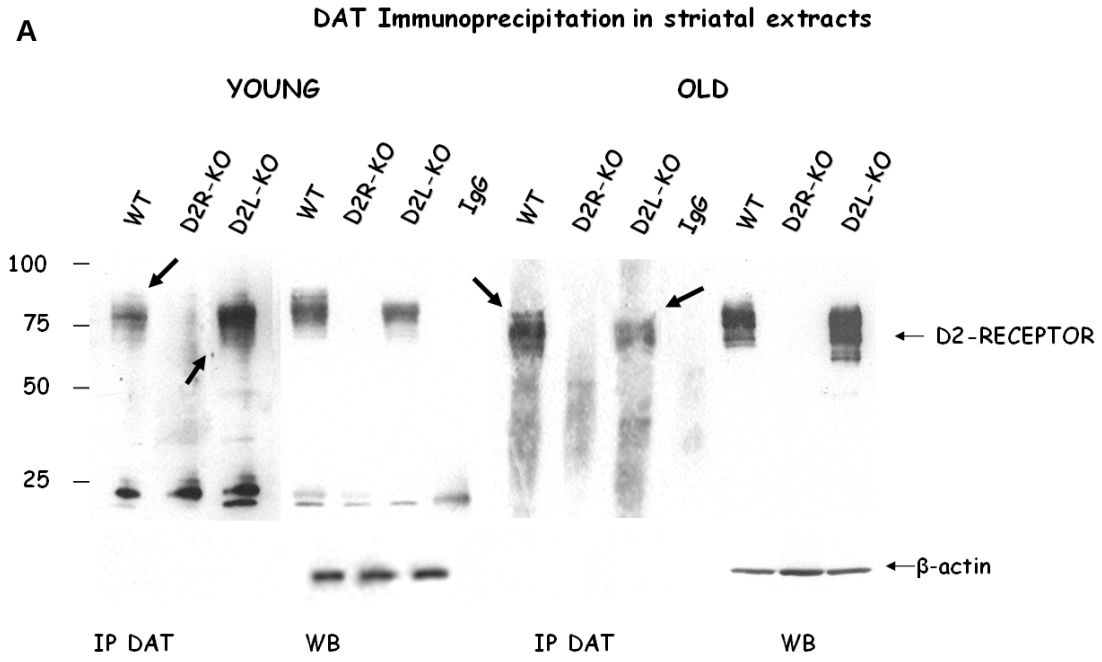
Recently, it has been demonstrated that α-syn enhances the DA signaling pathways by D2R, *in vitro*, thus providing a possible mechanism in the

presynaptic regulation of the synaptic homeostasis in the dopaminergic neurotransmission (Jae Kim et al., 2006).

The potential contribution of the DAT in neurological disease and its role in dopaminergic neurotransmission underline the importance of the DAT regulation, *in vivo*. One possible hypothesis would be that not only DAT interacts with α-syn, but also that D2R might interact with both. Thus in the absence of D2R this complex will not form leading to DAT dysfunctions and α-syn aggregation. In order to support and demonstrate this I performed immunoprecipitation (IP) assays to detect a possible interaction between D2R, DAT and α-syn. I first examined if DAT could form a complex with D2R and thus an anti-DAT antibody could immunoprecipitate D2R. As shown in figure 21 A and B, in the IP using anti-DAT antibody from striatal tissues of mice D2R-, D2L- and WT, D2R appears associate with DAT, providing a very strong *in vivo* evidence for an interaction between these two important proteins. In the striatal tissues are present the axonal projections of the dopaminergic neurons that originate in the SN, expressing DAT and D2R. The interaction seems to involve the D2S isoform, confirming the pre-synaptic function of D2S (Usiello et al., 2000).

An interaction was also detected by co-IP between DAT and α-syn, in striatal tissues of WT and mutant mice (Fig.22 A) and D2R and α-syn (Fig. 22 B), thus indicating that α-syn can participate to the complex, modulating the function of D2R and DAT (Wersinger et al., 2003; Jae Kim et al., 2006). As

shown in figures 21 and 22 the interaction between these proteins is age related, giving emphasis to the neuronal aging effect depending by D2R-mediated DA-signaling in dopaminergic nigro-striatal neurons.



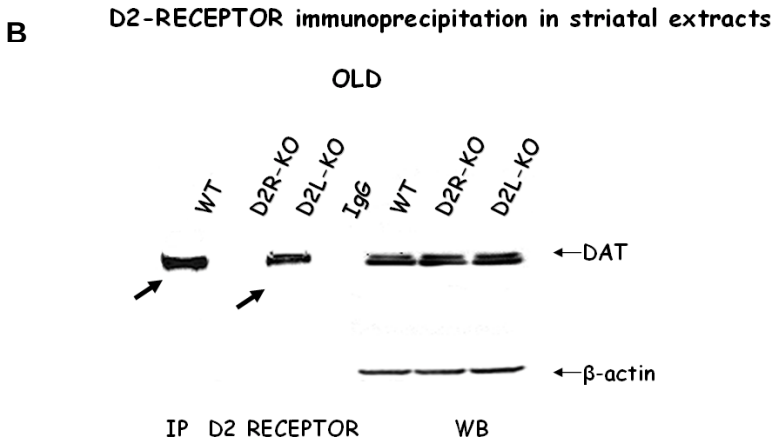
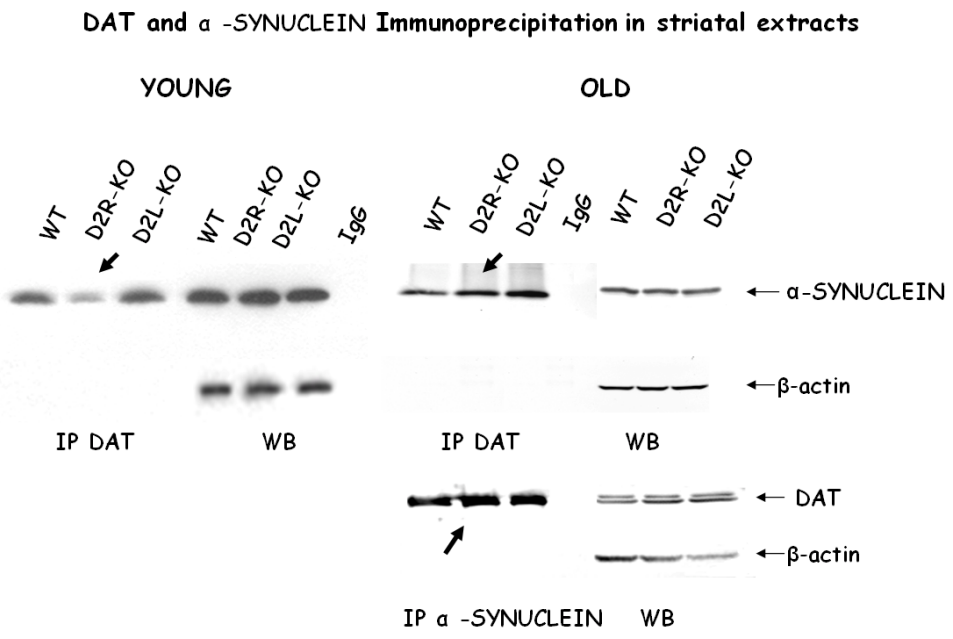


Figure 21: Immunoprecipitations followed by Western blot analysis. Dopamine transporter (DAT) in the membrane fraction of the striatal tissues was isolated by immunoprecipitation (IP) with anti-DAT antibody (Santa Cruz Biotechnology). The samples were analyzed by Western blot using anti-D2R mab (4H6-7-3) raised in the laboratory. 4H6-7-3 mab was directed against a peptide corresponding to the mouse D2R aminoacid (aa) sequence from aa 309 to 322 (**A**). IP of D2R leads to the co-precipitation of DAT from solubilized membrane striatal extracts (**B**). Young and Old mice were compared. In aged mice, the interaction between D2R and DAT is changed with respect to that which is observed in the striatal extracts of young mice. 500 μg of striatal extracts were used for IP, 50 μg of the same extracts were loaded as control. Anti-Immunoglobulin and β-actin (Sigma) were used as negative control for IP.

A


DAT and α -SYNUCLEIN Immunoprecipitation in striatal extracts
YOUNG
OLD
WT
D2R-KO
D2L-KO
IgG
α-SYNUCLEIN
β-actin
IP DAT
WB
DAT
IP α -SYNUCLEIN

B

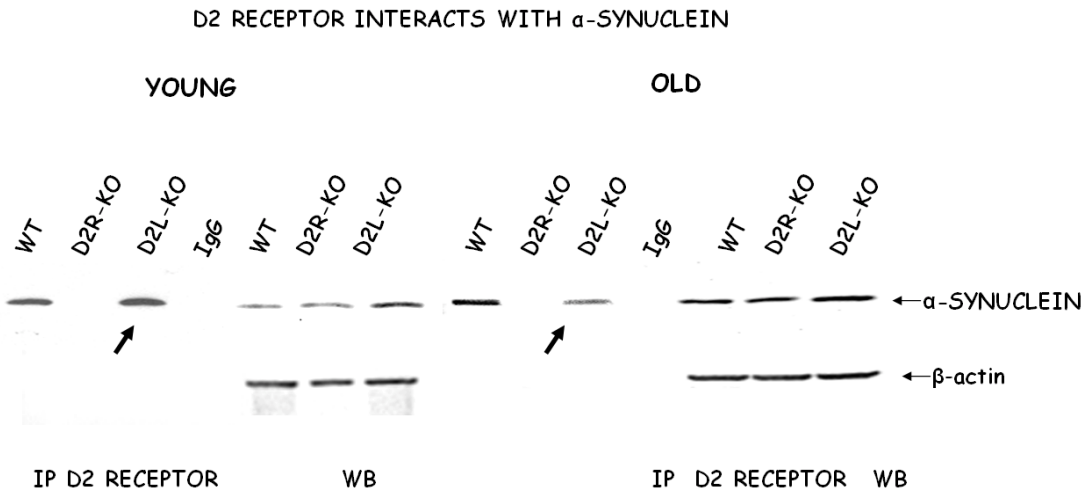

D2 RECEPTOR INTERACTS WITH α-SYNUCLEIN
YOUNG
OLD
WT
D2R-KO
D2L-KO
IgG
α-SYNUCLEIN
β-actin
IP D2 RECEPTOR
WB

Figure 22: Immunoprecipitations followed by Western blot analysis. IP using an anti-DAT leads to the co-precipitation of α-syn from solubilized membrane striatal extracts. Coimmunoprecipitation of α-syn revealed a complex with DAT (**A**).Using an antibody directed against D2R it has been possible to immunoprecipitate the striatal solubilized membrane protein and to detect a complex using the antibody SYN-1 (**B**).

Young and old mice were matched. In the D2R-KO young mice the α-syn is less complex with the DAT than in the WT mice of the same age. In the striatal extracts of 18th Months old D2R-KO mice, more α-syn bound to the DAT was detected with respect to the WT. α-synuclein interacts less with the D2R in the striatal neurons of old D2L-KO mice than in the young D2L-KOs. 500 μg of striatal extracts were used for IP, 50 μg of the same extracts were loaded as control. Anti-Immunoglobulin and β-actin (Sigma) were used as negative control for IP.

CONCLUSION

DA binds specific membrane receptors, which belong to the family of seven transmembrane domain G-protein coupled receptors. Dopamine receptors are subdivided into two subfamilies, the D1- and D2-like. The D1-like subfamily comprises D1- and D5-R, while the D2-like includes D2-, D3- and D4-R.

D2R are highly expressed by dopaminergic neurons as well as by postsynaptic neurons. The D2R exists in vivo in two molecularly distinct isoforms, D2L and D2S, generated by a mechanism of alternative splicing of the same gene (Vallone et al., 2000). Signaling through dopamine D2R governs physiological functions related to locomotion, hormone production and drug abuse. D2R plays a pivotal role both at the postsynaptic and at the presynaptic levels. Presynaptically, by acting as autoreceptors, they regulate dopamine synthesis and release from dopaminergic neurons. Knockout studies have shown that this function is principally performed by D2S while the D2R-mediated postsynaptic effects are D2L-dependent (Picetti et al., 1997; Usiello et al., 2000). Ablation of D2R results in locomotor impairment, altered response to drugs of abuse, pituitary tumors and electrophysiological alterations of D2R-expressing neurons.

Dopaminergic neurons express the DA transporter (DAT), involved in the reuptake of DA from the synaptic cleft. This protein together with DA autoreceptors plays a key role in the control of DA signaling by regulating extracellular DA levels.

Dopaminergic dysfunctions lead to neurological and neuropsychiatric diseases such as Parkinson's and schizophrenia as well as to pituitary tumors. In all these pathologies reductions or elevation of dopamine levels have been implicated demonstrating the key role of this molecule in human physiology.

Parkinson's disease (PD) is characterized by altered motor function comprising: resting tremor, rigidity, difficulty in initiating voluntary movements and poor postural reflexes. These deficits are due to the loss of dopaminergic neurons of the SN, which results in diminished synthesis and release of DA at nerve terminals in the Striatum. D2R is target of dopamino-mimetic agents which are the first-line treatment in Parkinson's disease (PD). Reportedly, early treatment of PD with dopamine (DA) agonists selectively stimulating D2R may modify the disease progression by possibly preventing cell death. Moreover DA agonists through D2R may activate neuroprotective signaling pathways and mediate increased cell survival.

A typical hallmark of Parkinson disease in humans is the formation in dopaminergic neurons of intracytoplasmatic eosinophilic inclusions, termed Lewy bodies. Lewy bodies are composed by aggregates of several proteins.

One of the best characterized proteins and major constituent of these aggregates is α-synuclein (α-syn), a protein of 140 amino acids.

Mutations in α-syn have been isolated from familial forms of early onset parkinsonisms. Two pathogenic mutations have been identified: Ala53T and

Ala30Pro. These mutations have been shown to favor the formation of α-syn aggregates in neurons (Polymeropoulos et al., 1997).

At present, neither α-syn function nor its contribution to neurodegeneration is completely understood. α-syn is enriched in presynaptic terminals, and its N-terminal structurally resembles an apolipoprotein α-helical region that appears to reversibly bind vesicular membranes. This might suggest that this protein plays a role in the function of synapses or synaptic turnover, facilitating vesicle transport from the cell body to the synapse. In addition, α-syn regulates the major factor that controls amounts of free dopamine in the dopaminergic neurons, DAT, thereby conferring the selectivity for degeneration of dopamine-producing neurons seen in PD (Sidhu et al., 2004).

Normal, highly soluble α-syn misfolds and is converted into pathological oligomers and higher-order aggregates that fibrillize and deposit into Lewy bodies and Lewy neurites in affected neurons of the PD brain (Spillantini et al., 1997).

Other than missense mutations in α-syn, many other environmental factors and events can influence the formation of α-syn fibrils, influencing the ability of normal quality-control systems (molecular chaperones, ubiquitin proteosome system (UPS), phagosome/lysosome system) to prevent or reverse protein misfolding or eliminate proteins that have misfolded or assembled into pathological aggregates and amyloid fibrils (Lee and Trojanowski, 2006).

The toxic consequences of the accumulation of fibrillar deposits of α-syn are manyfold. It may exert toxic action by creating pores in lipid membranes. These pore-like structures contribute to cytotoxicity in neurodegenerative diseases by disrupting organelle membranes and altering permeability that could alter mitochondrial or other organelle functions.

The link between α-syn aggregation in PD and dopaminergic signaling is therefore important at the scientific and clinical levels (Chandra et al., 2005).

DA synthesis and release is regulated by D2R, I thus evaluated the contribution of D2R mediated signaling to the pathogenic mechanism underlying the formation of α-syn intracellular aggregates. This was achieved by comparative analyses of α-syn distribution in the CNS of mice in which the expression of D2R has been modified versus WT littermates. I showed the occurrence of neuronal sufferance in mice lacking D2R, as indicated by α-synuclein accumulation and accelerated aging in different brain areas.

The presence of α-syn aggregates was assessed using biochemical and immunohistochemical approaches (Figures 18, 19 and 20). Striatum and Substantia Nigra extracts from both young and old WT, D2R-KO and D2L-KO mice were biochemically fractionated and levels of insoluble α-syn compared. Western blot analyses detected a strong increase of α-syn in the detergent insoluble fractions of old D2R-KO mice in comparison to WT and D2L-KO extracts (Fig.19 and Fig.20). In addition, I observed that while in WT brain sections α-syn immunostaining was undetectable, D2R KO brain

tissues presented cloudy-like and small dotted α-syn-positive cytoplasmic aggregates (Fig.18). Thus suggesting the occurrence of age dependent qualitative changes in biochemical properties of α-syn in the absence of D2R-mediated signaling.

Interestingly, the analysis of young and old D2L-KO mice revealed that also in these mice, despite preserved autoreceptor functions, α-syn insoluble aggregates are present although to a lesser extent with respect to D2R-KO extracts (Fig.19 and Fig.20). This indicates that both pre- and postsynaptic D2-mediated functions are required to preserve the integrity of dopaminergic as well as of dopaminoceptive neurons. In conclusion these data strongly suggest that altered D2-mediated functions through the control of DA release and/or signaling might promote over time an accelerated α-syn misfolding and neuronal aging (fig. 23). The present observation that the D2R can contribute to the aggregation of insoluble α-syn, *in vivo*, may thus be important for the development of more specific pharmaceutical interventions for the management of mental disorders associated with dopaminergic deregulation, like the PD.

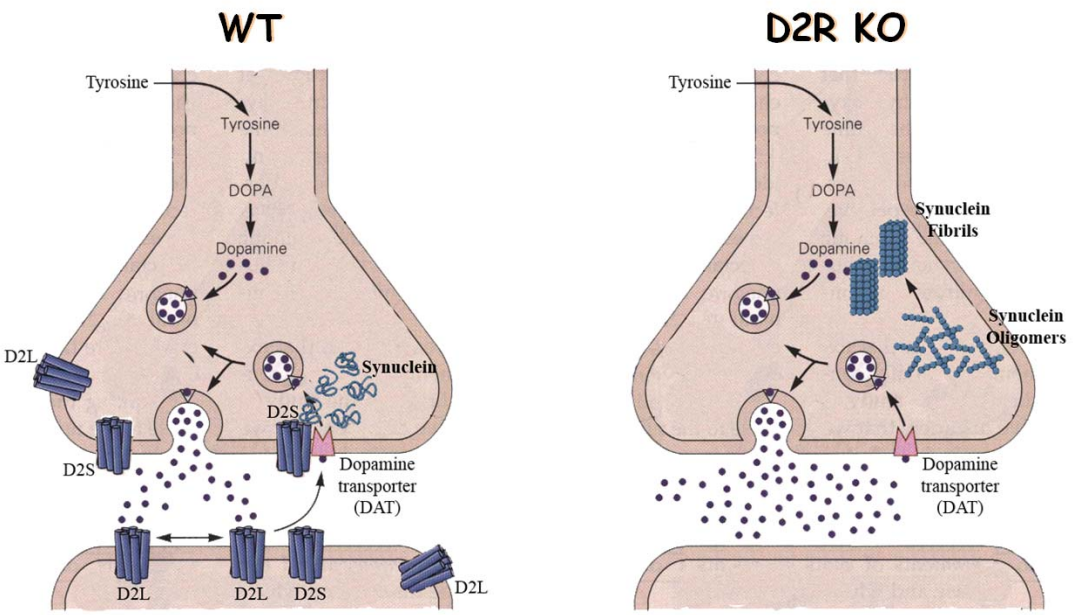


Figure 23: D2R influences on dopaminergic neurons aging and degeneration. The absent of D2R might altered the DA functions with consequent aggregation of α-synuclein in neurons.

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SCIENTIFIC RESEARCH PERFORMED DURING THE PHD PROGRAM

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Impaired light masking in dopamine D2 receptor–null mice

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Disruption of overt circadian rhythms can occur without influencing the endogenous pacemaker, the so-called 'masking' effect classically elicited by light. As the physiological pathways involved in light masking remain elusive, we analyzed mice lacking the dopamine D2 receptor. Although circadian rhythmicity was normal, D2R-null mice showed a markedly deficient light masking response, indicating that D2R-mediated signaling is an essential component of the neuronal pathways leading to light masking of circadian rhythms.

The eye is the principal mediator of light input to the central nervous system in mammals. In addition to photoentrainment of the circadian clock[1,2], the eye mediates other nonvisual responses to light, such as the acute suppression of pineal melatonin and the suppression of locomotor activity—so-called light masking[3]—in nocturnal animals. Despite the considerable progress made toward elucidating the molecular components of the light input pathway[1,2], the identification of the physiological mechanism governing light masking remains elusive. Notably, dopamine is the major catecholamine in the vertebrate retina playing a central role in neural adaptation to light[4]. In vertebrates, dopaminergic activity is higher during the day than at night, and light stimulates the synthesis, turnover and release of retinal dopamine[4–6]. Dopamine is a possible mediator of nonvisual photic responses. For example, light and dopamine are both able to reset the phase of circadian rhythms in the *Xenopus laevis* eye[7] and to induce *per2* expression[8]. A subclass of dopamine receptors, the seven–transmembrane domain, G protein–coupled D2 receptor (D2R)[9,10], seems to have a central role. Indeed, quinpirole, a selective D2R agonist, mimics light in its acute effects on a number of rhythmic retinal phenomena[7]. Yet, the physiological contribution of light-dependent dopaminergic signaling to nonvisual functions has remained unexplored. Here we reveal that D2R-mediated signaling is central in the control of light masking of circadian locomotor activity.

We monitored daily changes in wheel-running activity of D2R-deficient mice[11] (**Supplementary Methods** online). Littermate wild-type and $D2R^{-/-}$ mice were maintained in 12:12 light-dark cycles and

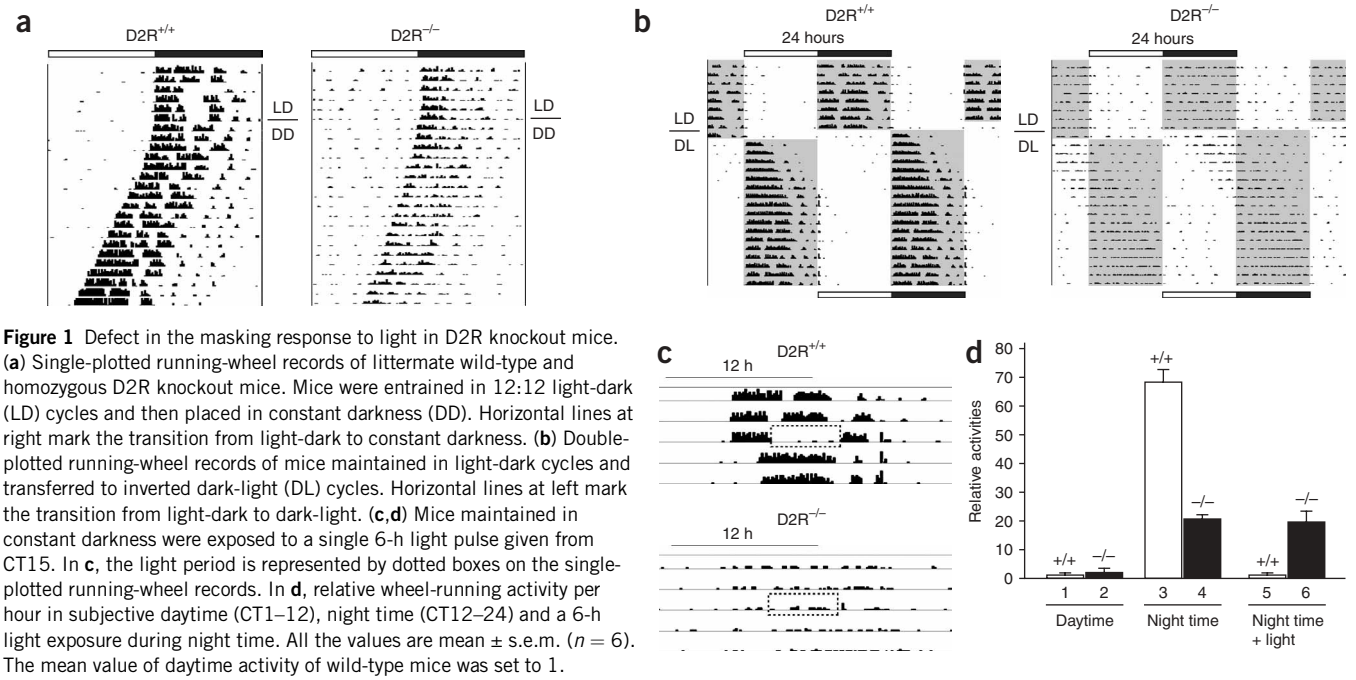

Figure 1 Defect in the masking response to light in D2R knockout mice. (**a**) Single-plotted running-wheel records of littermate wild-type and homozygous D2R knockout mice. Mice were entrained in 12:12 light-dark (LD) cycles and then placed in constant darkness (DD). Horizontal lines at right mark the transition from light-dark to constant darkness. (**b**) Double-plotted running-wheel records of mice maintained in light-dark cycles and transferred to inverted dark-light (DL) cycles. Horizontal lines at left mark the transition from light-dark to dark-light. (**c**,**d**) Mice maintained in constant darkness were exposed to a single 6-h light pulse given from CT15. In **c**, the light period is represented by dotted boxes on the single-plotted running-wheel records. In **d**, relative wheel-running activity per hour in subjective daytime (CT1–12), night time (CT12–24) and a 6-h light exposure during night time. All the values are mean ± s.e.m. ($n = 6$). The mean value of daytime activity of wild-type mice was set to 1.

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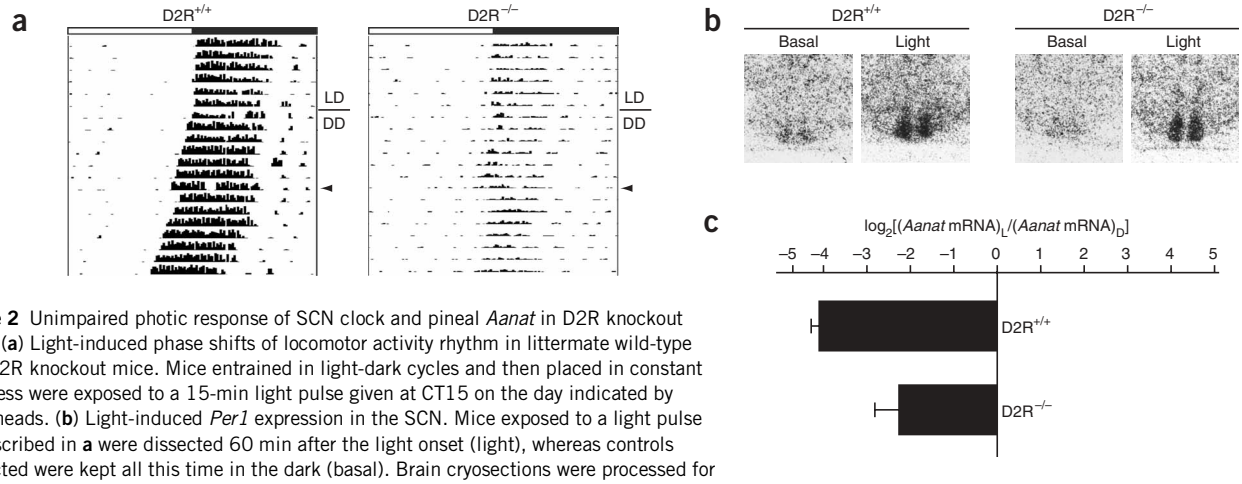

Figure 2 Unimpaired photic response of SCN clock and pineal *Aanat* in D2R knockout mice. (**a**) Light-induced phase shifts of locomotor activity rhythm in littermate wild-type and D2R knockout mice. Mice entrained in light-dark cycles and then placed in constant darkness were exposed to a 15-min light pulse given at CT15 on the day indicated by arrowheads. (**b**) Light-induced *Per1* expression in the SCN. Mice exposed to a light pulse as described in **a** were dissected 60 min after the light onset (light), whereas controls dissected were kept all this time in the dark (basal). Brain cryosections were processed for *in situ* hybridization with *Per1* probe. The region containing the SCN is shown. (**c**) Photoinhibition of nocturnal *Aanat* mRNA levels by extension of light in the pineal gland. Values represent the mean ± s.d. ($n = 3$) for the log-transformed ratio of *Aanat* mRNA in mice exposed to light compared to genotype-paired, dark-exposed controls.

then placed in constant darkness. D2R$^{-/-}$ mice showed normal entrainment to light-dark cycles (**Fig. 1a**) and showed clock-controlled locomotor activity in constant darkness (**Fig. 1a**). Analyses of the circadian free-running period of mice in constant darkness revealed only a very slight difference between wild-type and D2R$^{-/-}$ mice (circadian period, τ (mean ± s.e.m.) = 23.47 ± 0.09 h and 23.72 ± 0.15 h, respectively; $P < 0.005$, Student's *t*-test; an analysis of variance (ANOVA) provided equivalent statistical results). Thus, the fundamental properties of the master clock in D2R$^{-/-}$ mice seemed unimpaired (see also **Supplementary Table 1** online).

D2R$^{-/-}$ mice are known to show reduced locomotor activity[11]. We found that this phenotype was specific for the period of darkness (**Fig. 1a**), as no reduction in locomotor activity was found during the light period (**Fig. 1a** and **Supplementary Table 1**). This suggested a previously unappreciated role of D2Rs in the behavioral response to external light and dark conditions. To test this possibility, mice were transferred to an inverted light-dark cycle so as to be exposed to light in a period when they normally showed higher nocturnal activity (**Fig. 1b**). As expected, running-wheel activity of wild-type mice was strongly inhibited by exposure to light during the inverted light-dark cycle (**Fig. 1b**, left). Hence, clock-controlled changes in running-wheel activity were completely masked by the inverted light-dark cycle. In marked contrast, D2R$^{-/-}$ mice showed a lower inhibition of running-wheel activity during the light period (**Fig. 1b**, right). Indeed, several days after cycle inversion, D2R$^{-/-}$ mice continued to demonstrate wheel-running activity during the light phase, revealing a deficiency in their masking response. These results demonstrate that D2R-mediated signaling is indispensable in order for mice to confine their locomotor activity to a nocturnal niche.

To confirm the deficient masking response of D2R$^{-/-}$ mice, we then monitored changes in running-wheel behavior in response to a 6-h light pulse during the subjective night (**Fig. 1c**). D2R$^{-/-}$ mice maintained in constant darkness showed reduced night-time activity as compared to wild-type littermates (**Fig. 1d**, compare lanes 3 and 4). Upon light exposure, wild-type mice showed the expected light masking effect, illustrated by a marked inhibition of running-wheel activity (**Fig. 1d**, compare lanes 3 and 5). Notably, light masking was totally abolished in D2R$^{-/-}$ mice (**Fig. 1d**, compare lanes 4 and 6; inhibition = 96 ± 2.1% and 2 ± 9.1% for wild-type and D2R$^{-/-}$ mice, respectively; $P < 0.001$, Student's *t*-test; ANOVA provided equivalent statistical results). This substantial difference in response to a light pulse was confirmed by analyzing the light-induced expression of *Per1* in the retina, which was substantially impaired in D2R-null mice (**Supplementary Fig. 1** online). In addition, using a D2R-specific antibody (**Supplementary Fig. 2** online), we found that retinal D2R was localized in the outermost laminae of the inner plexiform layer (**Supplementary Fig. 3** online). D2R is able to modulate light responses of different types of cells, including outer nuclear photoreceptor cells[12] and melanopsin-positive ganglion cells[13], both of which are known to regulate the light masking system[14]. Taken together, these findings indicate that photic signaling through the D2R is required for normal masking responses and, consequently, for the proper organization of daily locomotor activity in light-dark cycles.

Restriction of locomotor activity to a nocturnal niche is achieved by multiple complementary photic responses including light masking[3], photoentrainment of the suprachiasmatic nucleus (SCN) master clock[1,2] and photic suppression of melatonin production[15]. We wondered if D2R was a general mediator of retinal light inputs or whether it played a more specific role by expressly regulating the effect of light on masking. To address this question, we analyzed other light-responsive regions, such as the SCN and the pineal gland. We evaluated the photic response of the SCN master clock in terms of the extent of the phase shift induced by a 15-min light pulse given at circadian time (CT) 15 (**Fig. 2a**). We found no significant differences between wild-type and D2R$^{-/-}$ mice (phase delay = 1.41 ± 0.32 h and 1.39 ± 0.41 h, respectively; $P = 0.47$, Student's *t*-test; ANOVA provided equivalent statistical results). The normal *Per1* photoinduction in the SCN (**Fig. 2b** and **Supplementary Fig. 4** online) confirmed the integrity of the photoresponse in the central clock structure.

We also found an intact photic response in the pineal gland of D2R$^{-/-}$ mice (**Fig. 2c**). Indeed, light is able to acutely suppress the nocturnal expression of arylalkylamine N-acetyltransferase (AA-NAT), the rate-limiting enzyme of the melatonin biosynthetic pathway[15]. Wild-type and D2R$^{-/-}$ mice were subjected to a 2-h light extension into the anticipated dark phase and were then compared with their genotype-paired, dark-exposed controls. Pineal *Aanat* mRNA levels showed photic inhibition of *Aanat* expression in both wild-type and D2R-null mice (**Fig. 2c**). There was a reduced response in

the D2R$^{-/-}$ mice that was probably due to a deficient light input in the retina. However, because these laboratory mice are melatonin deficient[15], the effect of light on *Aanat* expression cannot account for the observed behavioral consequences. Finally, we analyzed visually driven behaviors of D2R$^{-/-}$ mice. Tests of optomotor response (**Supplementary Methods**) demonstrated that D2R$^{-/-}$ mice behaved exactly as wild-type littermates did under both scotopic and photopic conditions (**Supplementary Fig. 5** online). In conclusion, our results indicate that the presence of D2R is critical for proper light input to the retina. In terms of the behavioral consequences of retinal function, D2R seems to be absolutely required for the light-induced suppression of locomotor activity (masking), whereas other visual or nonvisual photic responses seem to be D2R independent. Our findings reveal a yet unappreciated function of D2R-mediated signaling in regulating the proper organization of daily locomotor activity in light-dark cycles.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.D., I.Y., J.H., M.M. and E.T. performed the experiments. P.S.-C. and E.B. designed the project.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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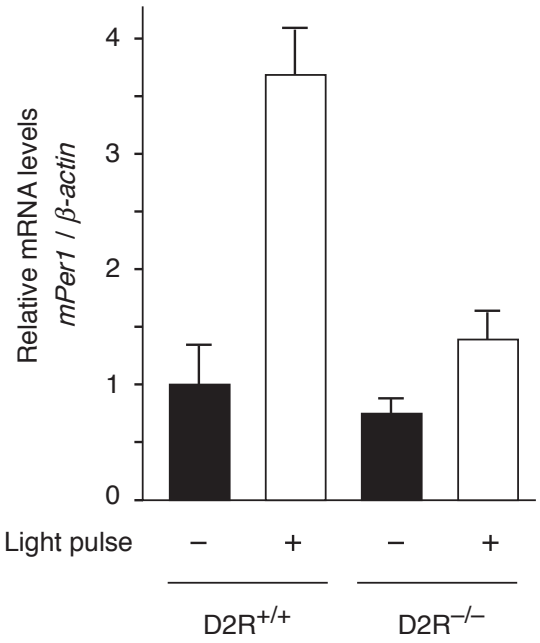
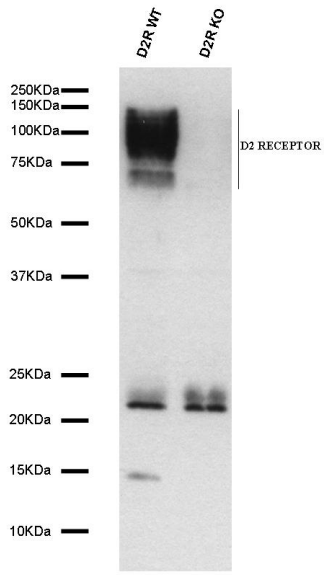
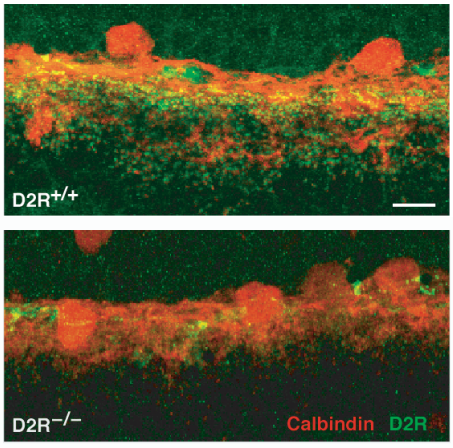


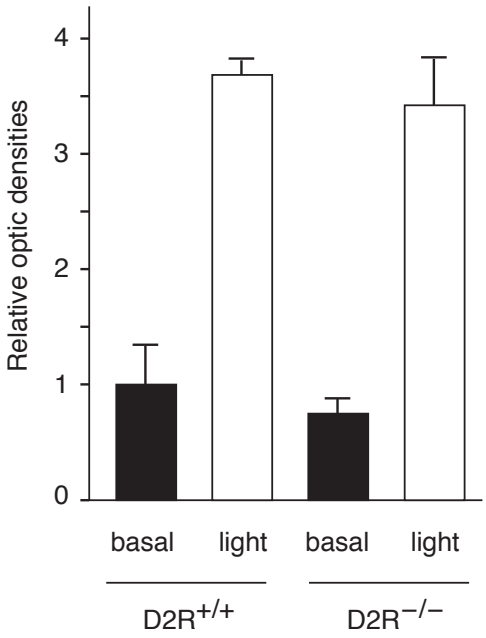
Supplementary Fig. 1 Light induction of *mPer1* is reduced in the retina of D2R knock-out mice Littermate wild-type and D2R knock-out mice were exposed to a 30 min light pulse at CT18 in DD and dissected 60 min after the light onset (light pulse +), whereas controls dissected were kept all this time in the dark (light pulse –). Total RNA was extracted from the isolated retina. Expression levels of *mPer1* were estimated by quantitative real-time PCR and normalized to those of β-*actin*. All the values are the mean ± s.d. (n = 3). Equivalent results on the *mPer1* expression were also obtained by RNase protection assays (not shown).



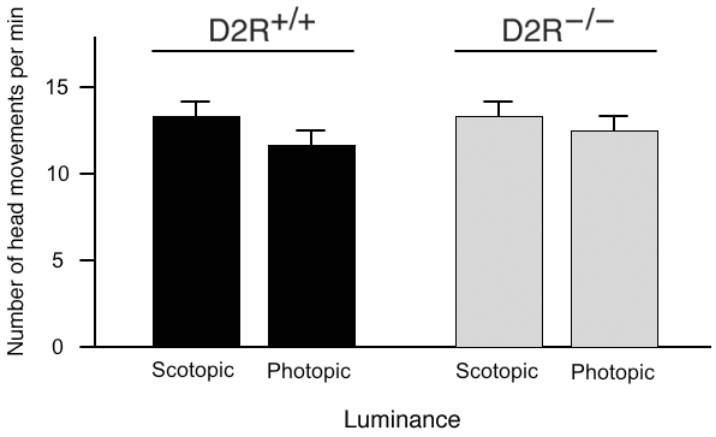
Supplementary Fig. 2 Generation of a specific antibody against D2R. We raised a mouse monoclonal anti-D2R antibody (4H6) directed against a peptide corresponding to the mouse D2R aminoacid residues 309-322. Western blot analyses using the 4H6 antibody (see also **Supplementary Methods**) revealed the presence of positive bands only in WT, but not in D2R$^{-/-}$ membrane preparations. The presence of multiple bands is due to receptor specific posttranslational modifications as well as to the oligomerization of receptors that belong to the seven transmembrane domain G-protein coupled receptor family (Bulenger, S. *et al.*, Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation.*Trends Pharmacol Sci.* 2005, 26:131-7; Urizar, E. *et al*., Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J*. 2005, 24:1954-64; O'Dowd, BF. *et al*., Dopamine receptor oligomerization visualized in living cells.*J Biol Chem*. 2005, 280:37225-35).



Supplementary Fig. 3 Immunofluorescence showing the localisation of the D2 Receptor in the mouse retina. D2R protein (green dots) is visible at the level of the synapses, between horizontal cells labeled with anti-calbindin antibody (red), bipolar cells and photoreceptors (see also **Supplementary Methods**). D2R labelling is absent in the retina from $D2R^{-/-}$ mice. Scale bar 10 μm.



Supplementary Fig. 4 Photic induction of *mPer1* in the SCN. The *in situ* hybridization data shown in Fig. 2b were quantified. Quantification was done by evaluating the optical density of the SCN region on autoradiograms. For each brain, five evenly spaced 10 μm cuts hybridized with *mPer1* probe were quantified and their values summed. Relative values are shown as the mean ± s.d. (n = 3).



Supplementary Fig. 5 Visually-driven behaviors of D2R$^{-/-}$ mice. Optomotor responses of D2R$^{-/-}$ mice were compared to wild type littermates at 0.026 c.p.d. in scotopic and photopic conditions (see **Supplementary Methods**). Numbers of head movements per min are shown as the mean ± s.d. (n = 5).

Supplementary Table I. Phenotypic Characteristics of D2R Knock-Out Mice

Parameter	+/+ (n = 5)	−/− (n = 6)	Student's t-test
Free running period (hr)	23.47 ± 0.09	23.72 ± 0.15	$P < 0.005$
Phase-shift (hr) induced by a light pulse given at CT15	1.41 ± 0.32	1.39 ± 0.41	$P = 0.471$
Amount of activities in a whole day	69.74 ± 14.29	20.00 ± 10.11	$P < 0.001$
Amount of activities in the light time	1.02 ± 0.44	2.07 ± 1.93	$P = 0.124$
Amount of activities in the dark time	68.72 ± 13.99	17.93 ± 8.78	$P < 0.001$
Ratio (%) of the light-time activities to the overall activities in a whole day	1.43 ± 0.57	9.65 ± 4.96	$P < 0.005$

Impaired Light-Masking in Dopamine D2 Receptor-null mice

Doi et al.

Supplementary Methods

Animals

Generation of D2R deficient mice was described[S1]. The D2R deficient mice and wild type mice used were all littermates having equivalent genetic backgrounds (genetic background: 75% C57BL/6J, 25% 129Sv/J). Mice housed in individual cages were entrained on a L12:D12 (12 h light–12 h dark) cycle for two weeks before analyses. All experiments were performed in accordance with institutional guidelines and regulations.

Locomotor activity measurement in running wheels

Locomotor activity was measured as described[S2]. Briefly, wheel-running activity data were collected using the VitalView Data Acquisition System (Minimitter, Sunriver, OR) with a sampling interval of 10 min. Actograms were designed and data were analyzed with the Actiview Biological Rhythm Analysis software (Minimitter).

In situ hybridization

Dissection of mice was done under dim red light conditions. Tissues were placed in OCT (Shandon Cryomatrix TM, Thermo) and frozen on dry ice and 10-□m thick coronal cryosections were prepared. *In situ* hybridization on frozen sections was done as described [S2]. The riboprobe used covers nucleotides 1-336 of the mouse *Per1* reading frame cloned in pBS.

Immunoblotting

We raised a mouse monoclonal anti-D2 antibody (4H6) directed against a peptide corresponding to the mouse D2R aminoacid (aa) sequence from aa 309 to 322. The anti-D2 antibody (4H6) was used for immunoblotting analysis of the mouse striatal membrane. For membrane preparation, striata were rapidly dissected from WT and $D2R^{-/-}$ mice, homogenized on ice with a polyethylene pestle in membrane extraction buffer (MEB)(50 mM TRIS pH 7.5, 150 mM NaCl containing a cocktail of protease inhibitors). Extracts were sedimented by centrifugation (40 min, 70 000 x g, 4°C), pellets were resuspended in MEB containing 10 mM CHAPS (3-[(3-

Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and gently rotated at 4°C for 40 min. The solubilized fractions were finally centrifuged to remove the insoluble material (10 min, 18 000 x g, 4°C). Proteins in supernatants were quantified. 20 µg of membrane preparations in Laemmli buffer were loaded and separated on 12% SDS-polyacrylamide gels and electroblotted to polyvinylidene fluoride (PVDF) membrane. Non specific binding was blocked in 5% non fat dry milk, 0.05% Tween 20 in PBS for 1h, at room temperature. Membranes were then incubated overnight with the 4H6 antibody (1:4000) and revealed using a goat anti-mouse kappa-peroxidase-conjugated secondary antibody (1: 2000, Southern Biotechnology Associates, Inc.) along with chemiluminescent reagent (SuperSignal West-Pico, Pierce).

Immunofluorescence

After deep anesthesia, mice were sacrified and retinas were fixed in 4% paraformaldehyde in PBS and 50-µm vibratome sections were made. Sections were blocked in 5% normal goat serum in PBS containing 0.3% Triton X-100. Antibodies and dilutions used are the following: rabbit anti-calbindin D-28K (Swant), 1:1000; mouse anti-D2R (4H6), 1:2000. Fluorescent secondary antibodies used were: goat anti-mouse, goat anti-rabbit IgG conjugated with Alexa Fluor 488 and 594 respectively (1:800, Molecular Probes).

qRT-PCR and RPA

Whole-tissue RNA was extracted by using RNA-Solv (Omega Bio-tek, Doraville, GA) according to the manufacturer's instructions. Total RNA was then reverse-transcribed into cDNA by using M-MLV Reverse Transcriptase (Gibco BRL) with oligo random hexamers. Prepared cDNA was purified and subjected to quantitative PCR analysis by using Light Cycler (Roche Diagnostics) with SYBR Green PCR Kit (Qiagen). A miniaturized RNase protection assay (RPA) was performed as described[S2]. RNA was equilibrated on agarose gels by ethidium bromide staining. [α^{32}P]UTP-labeled riboprobes were generated by using an in vitro transcription kit (Promega). The probe used cover nucleotides 1-336 of the mouse *Per1* reading frame[S3]. A mouse *β-actin* riboprobe was used as an internal control to monitor the loading of equal amounts of RNA (fragment from +193 to +331 of the mouse coding sequence). The primer sequences used for the qRT-PCR are available upon request.

Optomotor response test

The optomotor response was measured as previously described [S4]. Brefly, animals were placed on a platform in the form of a grid (11.5 cm diameter, 19.0 cm above the bottom of the drum) surrounded by a motorized drum (29.0 cm diameter) that could be revolved clockwise or anticlockwise at two revolutions per minute. After 10 min of adaptation in the dark, vertical black and white stripes of a 0,026 c.p.d. (cycles per degree) spatial frequency were presented to the animal. These stripes were rotated alternately clockwise and anticlockwise, for 2 min in each direction with an interval of 30 s between the two rotations. Animals were videotaped with a digital video camera (Sony, DCR-TRV24E) for subsequent scoring of head tracking movements. Tests were initially performed in scotopic conditions, using the night shot position of the camera. For photopic measurements, animals were then subjected to 400 lux during 5 min to allow them to adapt to the light. Head movements were scored only if the angular speed of the head corresponded to that of the drum rotation.

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Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo

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Regulation of Akt signaling by D2 and D3 dopamine receptors *in vivo*

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Abstract

The serine/threonine kinase Akt is a downstream target of dopamine receptor signaling that is inhibited/dephosphorylated in response to direct and indirect dopamine receptor agonists. While pharmacological studies uncovered the involvement of D2-class dopamine receptors in Akt regulation they did not identify the role of individual receptor subtypes in this process. Here we used knockout mice lacking the D1, D2, D2 long or D3 dopamine receptors as well as a D4 receptor specific antagonist to address the function of each of these receptors in the regulation of Akt *in vivo*. Under basal conditions D2, D2 long and D3 knockout mice display enhanced striatal Akt activation while D1 knockout mice and mice treated with the D4 receptor antagonist L745870 have phospho-Akt levels comparable to those of normal control animals. Furthermore both amphetamine and apomorphine loose their ability to inhibit Akt in D2 knockout mice while retaining their normal effect on this signaling molecule in D1 knockout animals. Finally, D3 knockout mice show a reduced sensitivity of Akt-mediated signaling to dopaminergic drugs but retain the action of these drugs on Akt at high dose regimens. These results indicate that D2 receptors are essential for the inhibition of Akt by dopamine and that D3 receptors also participate to this signaling potentially by enhancing D2 receptor response. Identification of the functions of individual dopamine receptor subtypes in Akt regulation may help the development of new pharmaceutical approaches for mental disorders related to abnormal dopamine transmission signaling such as bipolar disorder and schizophrenia.

Introduction

The monoaminergic neurotransmitter dopamine (DA) has been implicated in multiple brain disorders including schizophrenia, affective disorders, addiction and Parkinson's disease (Snyder, 1976; Carlsson, 1987; Gainetdinov and Caron, 2003). In the brain, the main dopaminergic neuron population arises from the *substantia nigra pars compacta* and projects to striatal neurons. Two classes of G protein-coupled receptors (GPCR) mediate the various physiological functions of DA (Kebabian and Calne, 1979). D1-class receptors (D1 and D5 subtypes) are mostly coupled to Gs_{α} and enhance the production of cAMP while D2-class receptors (D2, D3, and D4 subtypes) are coupled to Gi/o_{α} and inhibit this same process (Kebabian and Greengard, 1971; Enjalbert and Bockaert, 1983; Missale et al., 1998). Moreover, an alternate splicing of the D2 receptor messenger RNA leads to the expression of two D2 receptors isoforms the D2 short (D2S) and D2 long (D2L) which have been associated with pre-synaptic and post-synaptic D2 receptor functions, respectively (Giros et al., 1989; Monsma et al., 1989; Usiello et al., 2000; Lindgren et al., 2003).

Recent *in vivo* studies revealed that striatal D2-class receptors also exert their action in a cAMP independent fashion by promoting the formation of a signaling complex composed of Akt, protein phosphatase-2A (PP2A) and β-arrestin 2 (Beaulieu et al., 2004; Beaulieu et al., 2005). Formation of this complex leads to the inactivation of Akt following the dephosphorylation of its regulatory threonine 308 (Thr-308) residue by PP2A (Beaulieu et al., 2005). Inactivation of Akt in

response to DA results in the activation of glycogen synthase kinase 3 (GSK3) which in turn contributes to the expression of DA-associated behaviors (Beaulieu et al., 2004). Interestingly, reduced Akt functions have been reported in schizophrenic patients, while administration of the antipsychotic haloperidol, a D2-class receptor antagonist, activates Akt and inhibits GSK3 in the mouse brain (Emamian et al., 2004). However, the characterization of Akt and GSK3 regulation by DA receptors has remained limited to the use of pharmacological agents that do not allow the delineation of individual roles played by specific subtypes of DA receptors in this process (Beaulieu et al., 2004; Emamian et al., 2004). Here we used mice lacking D1, D2, D2L or D3 dopamine receptors to elucidate the functions of each of these GPCRs in the regulation of Akt *in vivo*. Our results indicate that the negative regulation of Akt by DA and dopaminergic drugs is dependent on D2 receptors and, to a lesser extent, on D3 receptor activation.

Methods

Experimental Animals

D1 receptor knockout (D1-KO) (Drago et al., 1994), D2 receptor knockout (D2-KO) (Baik et al., 1995), D2L receptor knockout (D2L-KO) (Usiello et al., 2000), D3 receptor knockout (D3-KO) (Joseph et al., 2002), DA transporter knockout (DAT-KO) (Giros et al., 1996; Cyr et al., 2003) and their respective wild-type (WT) C57BL/129SvJ littermates have been described previously. All mice used were from 3 to 4 months of age. For all experiments test and control groups were composed of age and sex matched animal with approximately 50% mice from each

sex. Animals were housed four or five to a cage at 23°C on a 12 h light/12 h dark cycle with ad libitum access to food and water. Animal care was approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Drug Administration

Amphetamine (Sigma-Aldrich, St Louis, MO) and L745870 (Tocris Cookson Inc., Ellisville, MO) were dissolved in saline and injected i.p.. Apomorphine (Sigma-Aldrich) was dissolved in distilled water containing 0.1% ascorbate and injected s.c.. Corresponding vehicle solutions were administered to control animals.

Western Blot Analysis

Western blot were performed as described (Beaulieu et al., 2004). Briefly, mice were killed by decapitation, after which the heads of the animals were immediately cooled by immersion in liquid nitrogen for 6 sec. The right hemistriatum was rapidly dissected out (within 30 sec) on an ice-cold surface and frozen in liquid nitrogen before protein extraction. Tissue samples were homogenized in boiling 1% SDS solution supplemented with 2 µM okadaic acid and boiled for 10 min. Protein concentration was measured by using a DC-protein assay (Bio-Rad, Hercules, CA). Protein extracts (25 or 50 µg) were separated on 10% SDS/PAGE and transferred to nitrocellulose membranes. Blots were immunostained overnight at 4°C with the following primary antibodies: anti-phospho-GSK3/ Ser-21/9 (1:200 dilution); anti-phospho-Akt Thr-308 (1:100); anti-phospho-Akt Ser-473 (1:500); anti-GSK3/ clone 0011-A (1:5,000); anti-Akt

(1:1,000). Immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies along with a chemiluminescent reagent (SuperSignal West-Pico, Pierce). Densitometric analysis was carried out within linear range by using IMAGEQUANT V1.1 (GE healthcare life science). Total protein signal were used as loading controls for phospho-proteins. Results are normalize to respective control conditions and presented has means±SEM Data were analyzed by two-tailed t test. Anti-GSK3/ clone 0011-A were purchased from Santa Cruz Biotechnology. All other primary antibodies were from Cell Signaling Technology (Beverly, MA). Secondary antibodies were obtained from Jackson Immuno-Research (West Grove, PA).

Results

Basal regulation of Akt and GSK3β in dopamine receptor knockout mice

Western blot analysis of the relative levels of striatal phospho-Thr-308 Akt showed no variation in Akt phosphorylation between D1-KO mice and WT littermates (Fig. *1A*) thus confirming previous pharmacological evidence (Beaulieu et al., 2004) that D1 receptors play little role in inhibiting Akt activity under basal conditions. In contrast, elimination of either D2, D2L or D3 receptors in genetically engineered animals led to increased striatal Akt phosphorylation (Fig. *1B-D*). Furthermore, phosphorylation of the Akt substrate GSK3β was also enhanced in these mice (Fig. 1*E*) indicating the both D3 and the long post-synaptic D2 isoform D2L can both contribute to the regulation of Akt/GSK3 signaling by DA.

Overall Akt activity is the result of an equilibrium between its phosphorylation/activation on Thr-308 and Ser-473 in response to phosphatidylinositol kinase (PI3K) -mediated signaling and its dephosphorylation by protein phosphatases. DA receptor signaling through the Akt:β-Arrestin 2:PP2A complex results in a dephosphorylation of Thr-308 Akt by PP2A without affecting its phosphorylation on Ser-473 (Beaulieu et al., 2004; Beaulieu et al., 2005). In contrast, changes in phosphatidylinositol kinase (PI3K) signaling affect the phosphorylation of both Thr-308 and Ser-473 in a similar fashion (Beaulieu et al., 2005). To further establish that changes of Akt phosphorylation observed in DA receptor-KO mice result from impaired Akt deactivation by the Akt:β-Arrestin 2:PP2A complex and not from enhanced PI3K mediated signaling we evaluated relative levels of striatal phospho-Ser-473 Akt in the different DA receptor-KO mice and respective littermates. As shown, (Fig. 1F) phospho-Ser-473 levels were not increased in the different DA receptor-KO mice consistent with the enhanced Thr-308 phosphorylation in D2, D2L and D3-KO mice resulting from a reduction in β-Arrestin 2 mediated DA receptor signaling.

D4 receptor blockade does not affect striatal Akt regulation

To examine the possible contribution of D4 receptors, we then administered an effective dose of the selective D4 receptor antagonists L745870 (Ukai and Mitsunaga, 2005) to WT animals. A similar experiment was also carried out using DAT-KO mice that display a basal reduction of striatal phospho-Thr-308 Akt as a

results of exacerbated dopaminergic neurotransmission (Beaulieu et al., 2004; Beaulieu et al., 2005). DAT-KO mice have persistently increased extracellular dopamine and display high responsiveness to D2-class receptor antagonists (Gainetdinov and Caron, 2003) and may thus represent a more sensitive experimental system to evaluate the impact of such drugs *in vivo*. As shown in Figure 2, D4 receptor blockade did not affect striatal Akt phosphorylation in either WT or DAT-KO mice.

Regulation of Akt by dopaminergic drugs in DA receptor knockout mice.

The D1/D2-class DA receptor the direct agonist apomorphine and the psychostimulant amphetamine, which acts by promoting DA efflux from dopaminergic terminals, both trigger Akt dephosphorylation in the WT mouse striatum (Beaulieu et al., 2004; Beaulieu et al., 2005). Administration of apomorphine (3 mg/kg, s.c.) or amphetamine (3 mg/kg, i.p.) to WT or D1-KO mice resulted in a similar reduction of phospho-Thr-308 Akt levels (Figs. *3 A-D*) thus further confirming that D1 receptors are dispensable for Akt inhibition in response to dopaminergic drugs.

In contrast, administration of apomorphine (3mg/kg, s.c.) or amphetamine (3mg/kg, i.p.) to mice lacking D2 receptors failed to reduce striatal Akt phosphorylation (Figs. *3 A-D*). Instead, apomorphine at a dose of 3mg/kg significantly enhanced the phosphorylation of Akt in the absence of D2 receptors (Fig. *3B*) while amphetamine had no significant effect on Akt in D2-KO mice (Fig. *3D*). Activation of Akt by apomorphine in the absence of D2 probably resulted from

the unmasking of a secondary action of this drug on another receptor(s) that may positively regulate Akt in the striatum (Roth et al., 2004). These observations reveal a central role for D2 receptors in the inhibition of Akt since the remaining striatal DA receptors are not able to mediate the inhibitory action of dopaminergic drugs on this signaling molecule in D2-KO mice.

Injection of 3mg/kg of amphetamine to D3-KO mice resulted in reductions of striatal Akt phosphorylation similar to those observed in WT animals (Fig. *4A,B*). However, elimination of D3 receptors prevented Akt dephosphorylation in response to a dose of 3mg/kg of apomorphine (Fig. *4E,F*). This discrepancy between the effects of the two drugs led us to explore the action of apomorphine and amphetamine on Akt in D3-KO mice over a broader range of doses. As shown in Figure 4 (*C,D*), injection of amphetamine at a dose of 1 mg/kg reduced Akt phosphorylation in the striatum of WT mice while having no effect on Akt activity in D3-KO animals. Furthermore, administration of apomorphine to D3-KO mice at a dose of 6 mg/kg resulted in a reduction of striatal Akt phosphorylation comparable that observed in WT animals in response to a lower dose (3 mg/kg) of this drug (Fig. *4E,F*). Taken together these observations indicate that D3 receptors may play a role in regulating the sensitivity of Akt mediated signaling to dopaminergic drugs but are dispensable for the action of these drugs on Akt at higher dose regimens.

Discussion

Multiple recent lines of evidence identified an involvement of the Akt/GSK3 pathway in DA receptor signaling and functions (Beaulieu et al., 2004; Emamian et

al., 2004; Beaulieu et al., 2005; Gould and Manji, 2005; Beaulieu, 2006). Genetic inactivation of Akt1 or GSK3β, administration of GSK3 inhibitors or uncoupling of Akt from dopamine receptors in β-arrestin 2 knockout mice have been shown to affect DA-related changes in locomotor activity or sensory motor gating (Beaulieu et al., 2004; Emamian et al., 2004; Beaulieu et al., 2005). Characterization of Akt mediated signaling in WT and DAT-KO mice treated with the D2-class receptor antagonists haloperidol and raclopride have pointed toward a role of this DA receptor class in the regulation of Akt mediated signaling (Beaulieu et al., 2004; Emamian et al., 2004). However the role of individual subtypes of DA receptors in triggering Akt dephosphorylation in dopaminoceptive neurons had not been fully examined. The results presented here reveal that two subtypes of D2-class receptors, D2 and D3, are involved in the inhibition of Akt by DA and dopaminergic drugs in the mouse striatum while D4 dopamine receptors do not appear to play a role in this phenomena. Furthermore, these data clearly establish that D1 dopamine receptors are not engaged in this type of signaling.

Genetic inactivation of total D2 or of the post-synaptic D2L receptors led to enhanced Akt phosphorylation in the striatum of knockout mice. Furthermore, absence of D2 receptors also resulted in a loss of normal striatal Akt regulation by dopaminergic drugs. D2 receptors exert both pre and post-synaptic functions. In their pre-synaptic auto-receptor function, mediated by the D2S isoform, these receptors are responsible for the regulation of DA synthesis and, consequently, impulse-dependent dopamine release (Baik et al., 1995; Missale et al., 1998;

Usiello et al., 2000; Benoit-Marand et al., 2001). Post-synaptic D2 receptors have been associated with, among other effects, stimulation of locomotion and the development of haloperidol-induced catalepsy (Baik et al., 1995; Usiello et al., 2000). A loss of D2 auto-receptor function thus should result in an increase of DA release (Benoit-Marand et al., 2001), which would in turn lead to a reduction in striatal Akt phosphorylation as demonstrated in DAT-KO and amphetamine treated WT mice (Beaulieu et al., 2004; Beaulieu et al., 2005). However, the increased basal Akt phosphorylation in D2-KO as well as in D2L-KO mice, which do not display major changes in D2 auto-receptor function (Usiello et al., 2000), indicate that the negative regulation of Akt by DA is mostly a post-synaptic phenomena regulated by D2 receptors.

Like D2, D3 receptors are believed to exert their actions both pre and post-synaptically (Missale et al., 1998; Schwartz et al., 2000; Joseph et al., 2002). However, despite multiple associations between D3 receptor and different neuro-psychiatric disorders (Schwartz et al., 2000), knockout studies have yet to produce a clear picture of the behavioral functions associated with these receptors (Ralph et al., 1999; Joseph et al., 2002; Waddington et al., 2005). Our results suggest that D3 receptors act as modulators that affect the threshold at which Akt is regulated by D2 receptors. At relatively high drug doses (eg: 3 mg/kg amphetamine or 6 mg/kg apomorphine) D3 receptors are dispensable and D2 receptors can regulate Akt dephosphorylation in their absence. However at lower drug doses or under basal conditions, D3 receptors may also contribute to the negative regulation of Akt activity by D2 receptors.

Studies conducted in heterologous systems have shown that D3 receptors possess an affinity for DA that is 100 fold higher than that of D2 receptors (Sokoloff et al., 1992). This difference in affinity may explain why D3 receptors can respond to lower doses of dopaminergic drugs and regulate Akt activity. However, this explanation does not account for the inability of D3 receptors to modulate Akt activity in the absence of D2. Several possible scenarios can explain the observation that DA inhibition of Akt is dependent of D2 receptors and only modulated by D3 receptors. It is known that GPCRs can function as dimers (Angers et al., 2002), one potential mechanism to explain this conundrum is the possibility of dimerization of D2 and D3 dopamine receptors expressed in the same medium spiny neurons. Our results are consistent with the idea that Akt may be regulated by D2/D2 homodimers as well as by D2/D3 heterodimers in which D3 would provide a higher affinity for DA receptor agonists allowing D2 to inhibit Akt in response to lower drug doses. However this explanation remains hypothetical and other possibilities such as an integration of D2 and D3 receptors signal at the level of Akt or other signaling intermediates cannot be excluded. Another alternative is that since our results are derived from a biochemical approach on the whole striatum, the readout coming from a higher number of cells expressing D2 receptors may also mask a possible D2-independent action of D3 receptors in some individuals cells. Characterization of the detailed mechanism by which D2 and D3 DA receptors collaborate to inhibit Akt will certainly require protracted studies *in vivo*, in isolated neurons as well as in heterologous systems and should provide an exciting avenue for future research.

The Akt/GSK3 signaling pathway has recently emerged as a potential culprit and therapeutic target for psychiatric disorders (Beaulieu et al., 2004; Emamian et al., 2004; Beaulieu et al., 2005; Gould and Manji, 2005; Beaulieu, 2006; Li et al., 2006). Brain Akt/GSK3 signaling has been shown to be responsive to DA (Beaulieu et al., 2004; Beaulieu et al., 2005), typical/atypical antipsychotics (Emamian et al., 2004; Beaulieu, 2006; Li et al., 2006), antidepressants (Li et al., 2006) and mood stabilizers (Beaulieu et al., 2004; Gould and Manji, 2005; Beaulieu, 2006). Moreover deregulation of Akt functions have been reported in schizophrenia (Emamian et al., 2004). Interestingly D2 and D3 receptors have previously been linked to schizophrenia (Snyder, 1976; Schwartz et al., 2000) while D3 has also been associated with some cases of bipolar disorder (Schwartz et al., 2000). Our present observations that both D2 and D3 receptors can contribute to the regulation of Akt *in vivo* may thus be important for the development of more selective pharmaceutical interventions for the management of mental disorders associated with dopaminergic deregulation.

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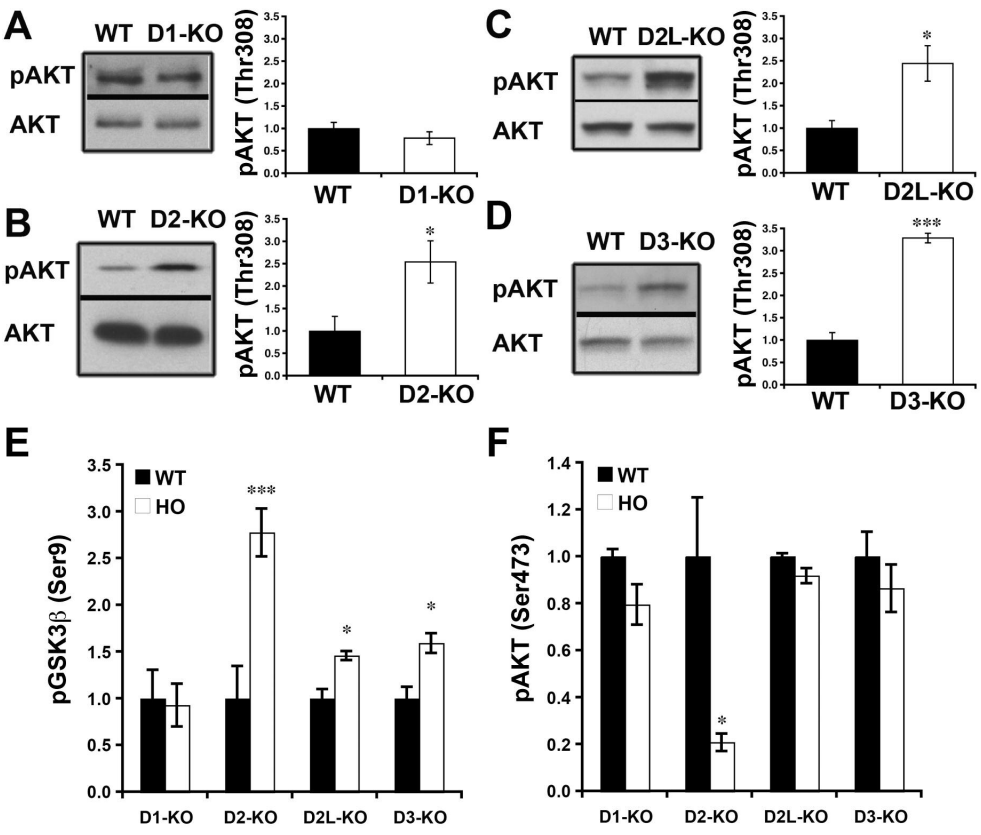
Figure legends

Figure 1. D2 and D3 dopamine receptors regulate Akt phosphorylation under basal conditions. A-D) Western blots-densitometric analysis of phospho-Thr308 Akt levels in extracts prepared from the striatum of different drug naïve DA receptor knockout mice (A: D1, B: D2, C: D2L, D: D3) and WT littermates. E-F) Phospho-Ser9 GSK3β (E) or Phospho-Ser473 Akt (F) levels in extracts prepared from the striatum of drug naïve DA receptor knockout mice and WT littermates. Results are presented in arbitrary units normalized to phospho-protein levels observed in WT littermates. Phospho-independent antibodies directed against respective kinases were used as loading controls. n=5 to 10 mice per group, data are average±SEM. *p≤0.05, ***p≤0.005.

Figure 2. D4 receptor blockade does not affect striatal Akt phosphorylation. Western blots (A) and densitometric analysis (B) of phospho-Akt (Thr308) levels in striatal extracts from WT or DAT-KO mice 30 min after injection of 5mg/kg of the D4 receptor blocker L745870. Results are presented in arbitrary units normalized to phospho-Akt levels observed in vehicle treated mice of the same genotype. Phospho-independent antibodies directed against Akt were used as loading controls. n=5 mice per group, data are average±SEM..

Figure 3. Regulation of Akt by DA drugs in D1 and D2 receptor knockout mice. Phospho-Thr308 Akt levels in extracts prepared from the striatum of WT, D1 and D2 DA-receptor knockout mice injected with apomorphine (3 mg/kg) or amphetamine (3 mg/kg). Representative Western blots (A, C) show results obtained from two separate striatal extracts prepared from different mice. Analyses were conducted at 60 min post-injection. Results in densitometric analysis (B, C) are presented in arbitrary units normalized to vehicle treated mice of the same genotype. n=5 to 10 mice per group, data are average±SEM. *p≤0.05, **p≤0.01

Figure 4. Regulation of Akt by DA drugs in D3 receptor knockout mice. A-B) Relative phospho-Akt (Thr308) levels in extracts prepared from the striatum of WT or D3 DA-receptor knockout mice 60 minutes after injection of amphetamine, 3 mg/kg (A, B) or 1 mg/kg (C, D) or of apomorphine (3 mg/kg or 6 mg/kg) (E, F). Representative Western blots (A, C, E) show results obtained from two separate striatal extracts prepared from different mice. Results in densitometric analysis (B, D, F) are presented in arbitrary units normalized to vehicle treated mice of the same genotype. n=5 to 10 mice per group, data are average±SEM. *p≤0.05, **p≤0.01.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WT D1-KO
pAKT
AKT
pAKT (Thr308)
WT D1-KO
B
WT D2-KO
pAKT
AKT
pAKT (Thr308)
WT D2-KO
C
WT D2L-KO
pAKT
AKT
pAKT (Thr308)
WT D2L-KO
D
WT D3-KO
pAKT
AKT
pAKT (Thr308)
WT D3-KO
E
WT
HO
pGSK3β (Ser9)
D1-KO D2-KO D2L-KO D3-KO
F
WT
HO
pAKT (Ser473)
D1-KO D2-KO D2L-KO D3-KO

Figure 1

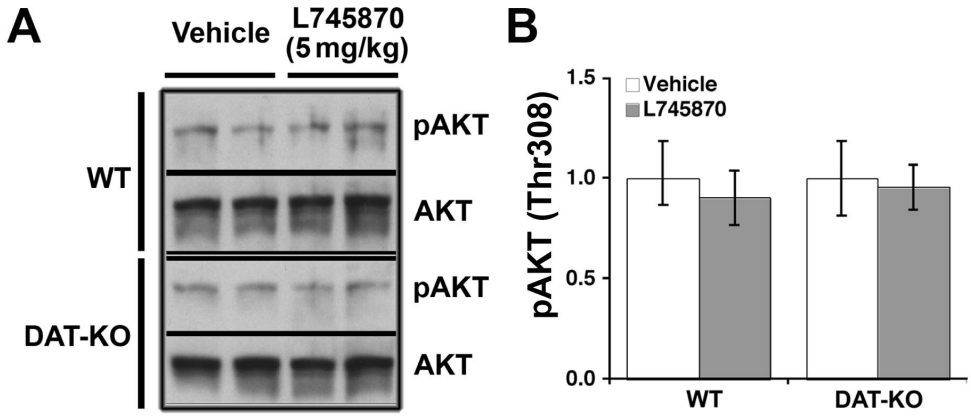
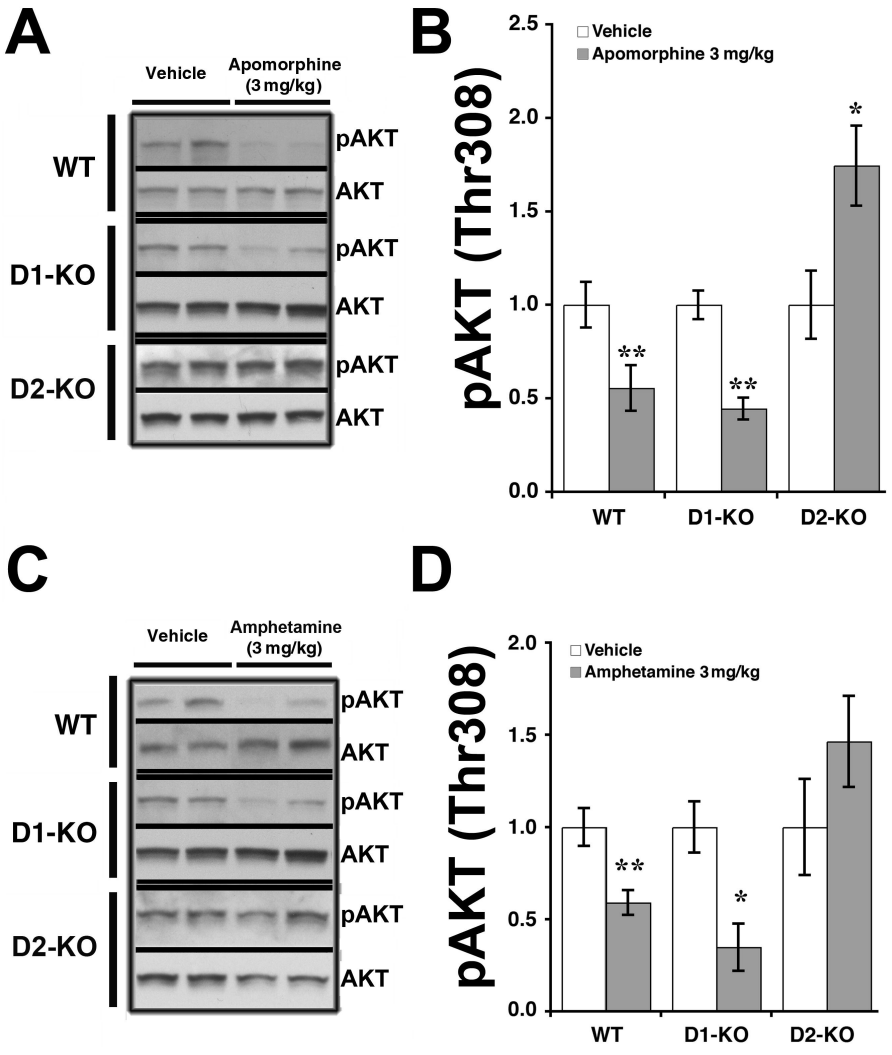

A
Vehicle
L745870
(5 mg/kg)
WT
DAT-KO
pAKT
AKT
pAKT
AKT
B
Vehicle
L745870
pAKT (Thr308)
1.5
1.0
0.5
0.0
WT
DAT-KO

Figure 2


A
Vehicle
Apomorphine (3 mg/kg)
WT
D1-KO
D2-KO
pAKT
AKT
pAKT
AKT
pAKT
AKT
B
Vehicle
Apomorphine 3 mg/kg
pAKT (Thr308)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
WT
D1-KO
D2-KO
C
Vehicle
Amphetamine (3 mg/kg)
WT
D1-KO
D2-KO
pAKT
AKT
pAKT
AKT
pAKT
AKT
D
Vehicle
Amphetamine 3 mg/kg
pAKT (Thr308)
2.0
1.5
1.0
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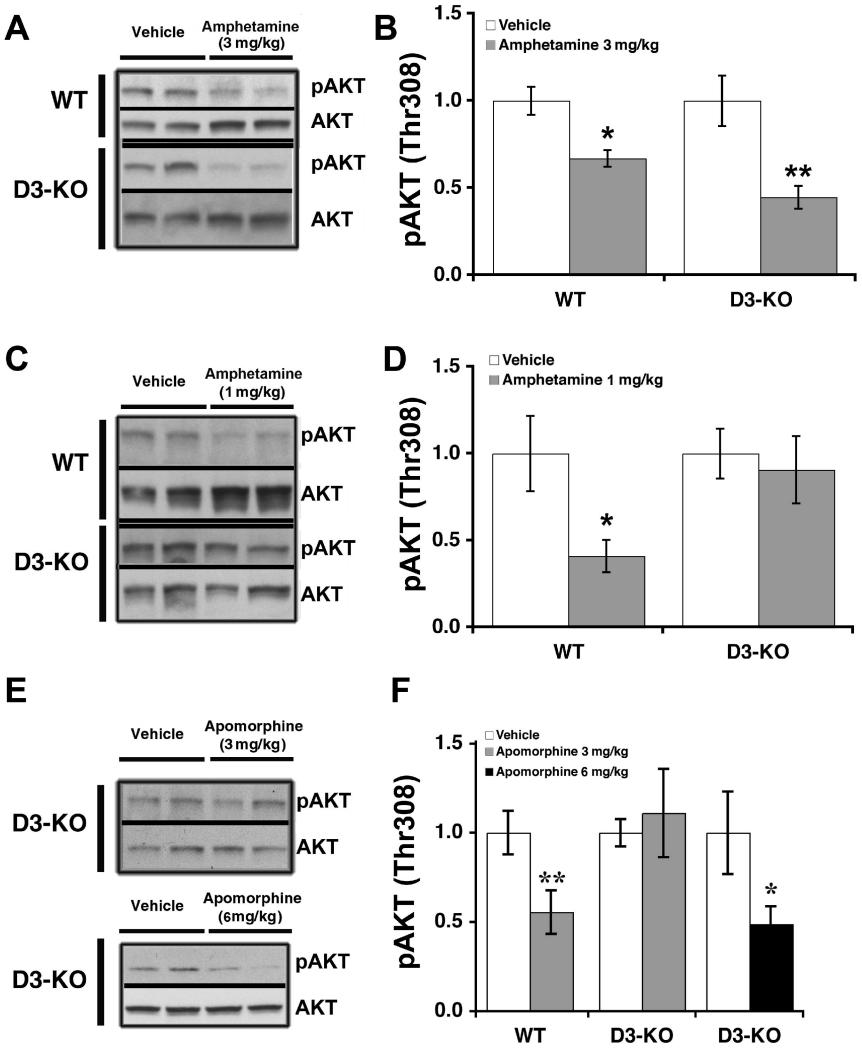
*
WT
D1-KO
D2-KO

Figure 3


A
Vehicle
Amphetamine (3 mg/kg)
WT
D3-KO
pAKT
AKT
pAKT
AKT
B
Vehicle
Amphetamine 3 mg/kg
pAKT (Thr308)
1.5
1.0
0.5
0.0
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**
WT
D3-KO
C
Vehicle
Amphetamine (1 mg/kg)
WT
D3-KO
pAKT
AKT
pAKT
AKT
D
Vehicle
Amphetamine 1 mg/kg
pAKT (Thr308)
1.5
1.0
0.5
0.0
*
WT
D3-KO
E
Vehicle
Apomorphine (3 mg/kg)
D3-KO
pAKT
AKT
Vehicle
Apomorphine (6mg/kg)
D3-KO
pAKT
AKT
F
Vehicle
Apomorphine 3 mg/kg
Apomorphine 6 mg/kg
pAKT (Thr308)
1.5
1.0
0.5
0.0
**
*
WT
D3-KO
D3-KO

Figure 4