FROM THE DEPARTMENT OF NEUROSCIENCE,
KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

DOPAMINE D2 RECEPTOR G PROTEIN COUPLING AND ITS REGULATION

BY

ANTON TERASMAA

STOCKHOLM 2003

Printed in Sweden by Karolinska University Press
ISBN 91-7349-788-6

ABSTRACT

Dopamine (DA) receptors belong to the superfamily of G protein coupled receptors. The D_2 DA receptor is negatively coupled with adenylate cyclase via pertussis toxin sensitive ($G_{i/o}$) G proteins. In the brain the D_2 DA receptor is mainly expressed in the caudate-putamen, the nucleus accumbens and the olfactory tubercle but also in the substantia nigra and the ventral tegmental area. The nigrostriatal dopaminergic system innervates the dorsal striatum. Most of the dorsal striatal D_2 DA receptors are localized on the cell bodies of striatopallidal inhibitory GABAergic neurons that send their projections to the globus pallidus. DA D_2 agonists inhibit striatopallidal GABA-ergic neurons thereby increasing motor activity since in this way the indirect pathway of the basal ganglia mediating motor inhibition will be inhibited. Degeneration of the nigrostriatal DA neurons causes a marked decrease in striatal DA levels and inhibition of motor functions. These changes are associated with a marked supersensitivity development in the striatal D_2 receptors. In a hemiparkinsonian rat model with 6-hydroxydopamine (6-OHDA) induced unilateral lesions of the nigrastriatal DA system D_2 agonists induce a strong contralateral rotational behaviour in very low doses due to preferential activation of the supersensitive D_2 receptors on the DA denervated side. The aim of this study was to characterize in the above model potential changes in the coupling of the D_2 receptor to the $G_{i/o}$ protein that may play a role in the D_2 receptor supersensitivity development.

[^{35}S]-guanosine 5'-O-(gamma-thio) triphosphate ([^{35}S]GTPγS) binding as a method for direct visualization of G protein activation by receptors was adapted to study D_{2short} receptors and their signalling in CHO cells transfected with the D_2 receptor. Pharmacological characterization of 18 dopaminergic ligands revealed a good correlation of potencies of all ligands to modulate [^{35}S]GTPγS binding with their potencies to inhibit [^{3}H]raclopride binding. Also efficacies of dopaminergic ligands at D_2 receptor were characterized. It was found that serotonin and other serotoninergic agonists have partial agonistic activity at the D_2 receptor expressed in CHO cells.

Studies on [^{35}S]GTPγS, [^{3}H]DA and [^{3}H]raclopride binding were used to investigate the cross regulation between G proteins and D_2 DA receptors expressed in CHO cells. The obtained results indicate that not only analogues of GTP but also GDP and GMP turned D_2 DA receptors into a low affinity state for DA. On the other hand, activation of the D_2 receptor by DA caused a decrease in the binding affinity for GDP, but not for analogues of GTP. According to these results, the high-affinity state of agonist binding can be achieved only when no nucleotides are bound in the agonist receptor-G protein complex.

The role of G-proteins in D_2 receptor supersensitivity was studied in striatal membranes from rats with unilateral 6-OHDA induced lesions of the nigrostriatal DA cells. The number of [^{3}H]raclopride binding sites was increased in the DA denervated striatum, but no changes in ligand binding affinities and in proportion of high-affinity agonist binding sites could be detected. The number and the affinity of [^{35}S]GTPγS binding sites was unaltered after the striatal DA denervation, whereas the binding affinity of GDP was decreased in the DA denervated versus the intact striatum. It is proposed that the decrease in GDP binding affinity to D_2 DA receptor-coupled G proteins is an important factor in D_2 receptor supersensitivity appearing after degeneration of the striatal DA terminals.

DSP4 induced lesions of locus coeruleus (LC) noradrenergic neurons influence the ascending mesencephalic DA systems by reducing striatal DA turnover and inducing behavioural supersensitivity to dopaminergic drugs, the latter effect being similar to that observed after the loss of striatal DA terminals. The density of striatal D_2 receptors was increased following DSP4 treatment as also is the case after DA denervation of the striatum. In contrast, such NA lesions had no effect on D_2 receptor G protein coupling as found after DA denervation.

It is known that Ca^{2-}/calmodulin suppresses the D_2 receptor signaling by interacting with the calmodulin binding motif of the D_2 receptor located in the N-terminal part of the third intracellular loop of the D_2 receptor. This motif is also part of the A_{2A}/D_2 heterodimer interface and A_{2A} strongly antagonizes the D_2 signalling within the A_{2A}/D_2 heterodimer. It is demonstrated that in the A_{2A}/D_2 cotransfected, but not in the D_2 alone transfected CHO cells, expressing endogenous calmodulin, Ca^{2-} substantially increases the basal and DA stimulated [^{35}S]GTPγS binding. These results may be explained on the basis of a competition between calmodulin and A_{2A} for their overlapping binding motifs at the D_2 receptor. The results illustrate the dynamic interplay of A_{2A}/D_2 heterodimers and the D_2 interacting protein in control of the D_2 signalling.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AC	adenylate cyclase
cAMP	adenosine 3'-5'-cyclic monophosphate
CHO	Chinese hamster ovary
DOPAC	3,4-dihydroxyphenylacetic acid
DTT	DL-dithiothreitol
EDTA	Edetic acid
G protein	heterotrimeric GTP binding protein
GABA	γ-aminobutyric acid
GDP	guanosine 5'-diphosphate
GDPβS	guanosine 5'-O-(2-thiodiphosphate)
GMP	guanosine 5'-monophosphate
Gpp[NH]p	guanosine 5'-[β,γ-imido]triphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
mGluR	Metabotropic glutamate receptor
NIPS	N-(p-isothiocyanatophenethyl)spiperone
NPA	R(-)-propylnorapomorphine
s.c.	subcutaneous injection
RGS	Regulator of G protein signaling

ORIGINAL PAPERS

The present study is based on the following papers, which will be referred to by their Roman numerals

I. **Terasmaa A.**, Finnman U.-B., Owman C., Ferré, S., Fuxe K. and Rinken A. Modulation of [^{35}S]GTPγS binding to Chinese hamster ovary cell membranes by $D_{2(short)}$ dopamine receptors. *Neuroscience Letters*, 2000, vol. 280(2), pp 135-138.

II. Rinken A., Ferré S., **Terasmaa A.**, Owman C. and Fuxe K. Serotonergic agonists behave as partial agonists for D_2 dopamine receptor. *NeuroReport*, 1999, vol. 10(3), pp 493-495.

III. Rinken A., **Terasmaa A.**, Raidaru G. and Fuxe K. D_2 dopamine receptor-G protein coupling. Cross-regulation of agonist and guanosine nucleotide binding sites. *Neuroscience Letters*, 2001, vol. 302(1), pp 5-8.

IV. **Terasmaa A.**, Andbjer B., Fuxe K. and Rinken A. Striatal dopamine denervation decreases the GDP binding affinity in rat striatal membranes. *Neuroreport*, 2000, vol. 11(12), pp 2691-2694

V. Harro J., **Terasmaa A.**, Eller M. and Rinken A. Effect of denervation of the locus coeruleus projections by DSP-4 treatment on [^{3}H]-raclopride binding to dopamine D_2 receptors and D_2 receptor-G-protein interaction in the rat striatum. *Brain Research*, 2003, vol. 976, pp209-216.

VI. **Terasmaa A.**, Lindgren N. Canals M. and Fuxe K. Adenosine A_{2A} receptor reverses the effect of calcium on the dopamine D_2 receptor signaling. A [^{35}S]GTP-gamma-S binding study in D_2 and A_{2A}/D_2 transfected CHO cell lines. *Manuscript.*

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INTRODUCTION

BASAL GANGLIA

Basal ganglia are a heterogeneous group of subcortical nuclei and the principal components in rodents are the neostriatum, the globus pallidus, the substantia nigra, the entopeduncular nucleus and the subthalamic nucleus. The functional role of basal ganglia is to integrate sensory-motor, associative and limbic information into motor behaviours.

The ascending dopaminergic neuron system to the basal ganglia is comprised of two main pathways. (1) The nigrostriatal pathway originates in the substantia nigra (A9 region), projects to the dorsal striatum, and is involved in motor function. (2) The mesolimbic pathways originate in the ventral tegmental area (A10) and project mainly to the ventral striatum but also to cortical regions and are considered to be crucial for motivational aspects of motor activity and have been implicated in reward and reinforcement mechanisms. Thus, administration of psychostimulant drugs of abuse elicits an increase in dopamine release in the mesolimbic areas, whereas withdrawal of these drugs results in a reduction of limbic dopaminergic transmission (Ferre *et al.*, 1997).

Striatum is the main input area of the basal ganglia and an important component of the motor system. It receives information through glutamatergic afferent pathways mainly from cortical and thalamic areas (Heimer *et al.*, 1993; Gerfen & Wilson, 1996). Striatum can be divided according to morphological criteria (caudate-putamen, nucleus accumbens and tuberculum olfattorium) and according to functional output (dorsal and ventral striatum) (Heimer *et al.*, 1995; Gerfen & Wilson, 1996). Dorsal striatum consists of the nucleus caudate-putamen. Ventral striatum consists of the nucleus accumbens and the tuberculum olfactorium (Björklund & Lindvall, 1984). Striatal neurons consists mainly of GABAergic medium-spiny neurons (90%) which represent the efferent striatal projections to the globus pallidus (GPe in human), the entopeduncular nucleus (GPi in humans) and the substantia nigra pars reticulata. The striatal interneurons are the large cholinergic aspiny interneurons (5%) (Pasik *et al.*, 1988; Heimer *et al.*, 1995). The striatal GABAergic efferent neurons are divided into two subtypes, giving rise to two

different pathways: the direct and the indirect pathways (Alexander & Crutcher, 1990; Gerfen & Wilson, 1996), see Figure 1. The direct pathway, also called the striato-nigral / striato-entopenducular pathway, is composed of GABAergic neurons, which contain the peptides substance P and dynorphin, and sends its projections to entopeduncular nucleus (EP) and to the substantia nigra pars reticulata (SNr), which are the two major output nuclei from the basal ganglia to the thalamus. The indirect pathway, also called the striato-pallidal pathway, consists of GABAergic neurons that contain peptide enkephalin, and sends its projections to the globus pallidus (GP), which in turn sends its projections to the subthalamic nucleus (STN). From the STN, glutamatergic projections are sent to the EP and SNr, see Figure 1. Both types of neurons form a local striatal network through collaterals before the axons leave the striatum.

Dopamine D_1 receptors are mostly localized in the soma dendritic region of the direct pathway and dopamine D_2 receptors on the soma dendritic region of the indirect one (Alexander & Crutcher, 1990; Yung *et al.*, 1995; Gerfen & Wilson, 1996).

Substantia nigra is anatomically divided in zona compacta (dorsal part, SNc) and zona reticulata (ventral part, SNr). From SNc dopaminergic pathways project to the dorsal striatum (nigrostriatal pathway) (Andén *et al.*, 1964; Dahlström & Fuxe, 1964; Anden *et al.*, 1966; Fuxe *et al.*, 1967). The SNr is composed by inhibitory GABAergic neurons which project to thalamus and it receives inhibitory GABAergic inputs from the striatum and excitatory glutamatergic inputs from the nucleus subthalamicus (Groenewegen & Berendse, 1990).

Globus pallidus is divided in internal (GPi) and external (GPe) segments. GPe receives the GABAergic innervation from the striatum (indirect pathway) and sends its GABAergic output to the nucleus subthalamicus (STN). From the STN excitatory glutamatergic neurons innervate the SNr and GPi.

SIGNALING BY GPCRS

Dopamine receptors, like other members of the G protein coupled receptor family, transduce signaling through GTP binding proteins (G proteins). G proteins consist of the three subunits – the GTP-binding α-subunit (MW~40000 daltons) and a tightly bound complex consisting of a β-subunit (MW~35000 daltons) and a γ-

subunit (MW~8000 daltons). Sixteen genes for α-subunits (leading to 20 gene products), 6 for β-subunits and 12 for γ-subunits are known until now, generating large numbers of theoretically possible different G protein αβγ-heterotrimers (Lohse, 1999). Both the α-subunits and the βγ-dimers are capable of regulating effector systems (reviewed by (Muller & Lohse, 1995; Clapham & Neer, 1997)). G proteins cycle from an inactive, GDP bound, heterotrimeric (αβγ) state to an active GTP bound state in which the GTP bound α-subunit is dissociated from the βγ subunit complex. Activated receptors catalyse this step by promoting the release of GDP from the G protein α subunit, permitting the subsequent rapid binding of GTP. The dissociated α- and βγ-subunits can each regulate the function of appropriate effector molecules. G proteins return to their inactive state by hydrolysis of GTP and subsequent reassociation of the GDP bound α-subunit and the βγ-subunit complex. At least two families of G protein regulatory proteins are well known, namely, the regulators of G protein signaling (RGS-proteins) and the phosducins (Lohse, 1999). The RGS-proteins act on the α-subunit and accelerate the hydrolysis of GTP. The phosducins interact with the βγ-complex, and prevent the βγ subunit to bind to effectors as well as to associate the βγ-complex to α-subunits. In this way a disruption of the G protein cycle and an inhibition of βγ-dependent signaling is produced (Lohse, 1999). Thus, G proteins are transducers and amplifiers of transmitter induced signaling, and are substantially regulated by a number of proteins (Offermanns, 1999).

There are several methods for measuring receptor mediated signaling through G proteins such as measurement of activity of enzymes modulated by G proteins (e.g. adenylate cyclase), of the GTP-ase activity of α-subunits, which is increased upon receptor stimulation, and of the initial rate of GDP/GTP exchange using the non-hydrolysable GTP analogue [^{35}S]GTPγS, which binds covalently to the α-subunit.

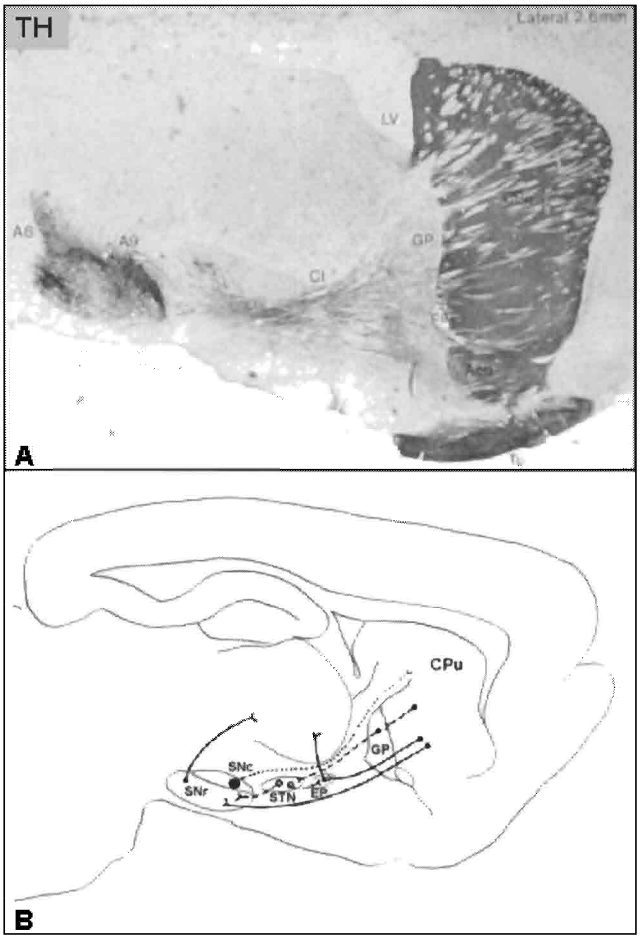

Figure 1 A) Tyrosine hydroxylase (TH) immunoreactivity in the ascending dopaminergic system from the mesechephalic cell groups A9/A8 to the caudate-putamen. B) A schematic represenation of the direct (solid line) and indirect (dashed line) pathways of the basal ganglia, and the nigro-striatal dopaminergic patway (dotted line). CPu-caudate putamen/striatum, GP- globus pallidus, EP- entopeduncular nucleus, STN- subthalamic nucleus, SNr-substantia nigra pars reticulata, SNc-substantia nigra pars compacta, Acb- nucleus accumbens, Tu- olfactory tubercle, LV- lateral ventricle, ca- anterior commissure, Cl- claustrum.

DOPAMINE RECEPTORS

Dopamine receptors belong to the superfamily of seven transmembrane spanning G protein coupled receptors. Initially, dopamine receptors have been divided into two groups on a pharmacological basis, D1 receptors that inter alia activate adenylate cyclase and D2 receptors that inter alia inhibit adenylate cyclase activity. Molecular cloning techniques have revealed five subtypes of dopamine receptors, the D_1 and the D_5 belonging to the D1-like group, and D_2, D_3 and D_4 receptors belonging to the D2-like group (for review see (Missale *et al.*, 1998)).

The D_1 receptor is the most widespread dopamine receptor and is expressed at a higher density than any other dopamine receptor subtype (Missale *et al.*, 1998; Emilien *et al.*, 1999). D_1 mRNA and protein have been found in highest densities in the caudate putamen, the nucleus accumbens, and the olfactory tubercle. In addition, D_1 receptors have been detected in many limbic areas, the hypothalamus and the thalamus. D_1 receptors in the entopeduncular nucleus and in the substantia nigra pars reticulata are preferentially localized on nerve terminals of striatal GABAergic projection neurons co-expressing substance P and dynorphin (Missale *et al.*, 1998; Emilien *et al.*, 1999).

The distribution of D_2 receptors is similar to that of the D_1 receptor, being localized primarily to the striatum (Weiner *et al.*, 1991; Emilien *et al.*, 1999).It is noteworthy that co-localization of the D_2 receptor with the D_1 receptor has been regarded as rare (Missale *et al.*, 1998), but recently Aizman and co-workers (Aizman *et al.*, 2000) have suggested that co-localization of D_1 and D_2 receptors is a more common feature although one or the other of the two dopamine receptors dominates.

The D_3 receptors are enriched in the nucleus accumbens and islands of Calleja, but are poorly expressed in dorsal striatum (for review see (Missale *et al.*, 1998; Emilien *et al.*, 1999)). The D_4 receptor seems to occur at much lower densities than the D_1 and D_2 receptors in the striatum (Emilien *et al.*, 1999). Finally, the D_5 receptor is poorly expressed in rat brain when compared with the D_1 receptor (for review see (Missale *et al.*, 1998)).

DOPAMINE D_2 RECEPTOR

The D_2 receptor exists as two alternatively spliced isoforms (D_{2S} and D_{2L}) that differ by the insertion of a stretch of 29 amino acids in the third intracellular loop (AA242-270 in the human D_{2L} sequence) (Dal Toso *et al.*, 1989; Missale *et al.*, 1998).The rat dopamine D_{2L} receptor protein consist of 444 amino acid residues (Bunzow *et al.*, 1988; Monsma *et al.*, 1989; Chio *et al.*, 1990; Rao *et al.*, 1990) while human D_{2L} receptor consists of 443aa (Dal Toso *et al.*, 1989; Grandy *et al.*, 1989; Selbie *et al.*, 1989; Robakis *et al.*, 1990; Seeman *et al.*, 1993). Both isoforms revealed the same pharmacological profile and both isoforms inhibit AC when expressed in mammalian cells (Dal Toso *et al.*, 1989). However, the D_{2S} receptor isoform displayed a higher affinity than the D_{2L} in the ability to inhibit AC activity (Missale *et al.*, 1998). There is a third alternative splice variant of the D_2 receptor gene, $D_{2longer}$, resulting in insertion of valine and glutamine residues into the third intracellular loop of the D_{2L} receptor between residues 270 and 271 (Seeman *et al.*, 2000).

The D_2 receptor is located both pre- and postjunctionally. It is of interest that the splice variants of the D_2 receptor are differentially distributed and may have different functions. The D_{2S} receptor predominates in the cell bodies and nerve terminals of the dopaminergic cells of the mesencephalon and hypothalamus. The D_{2L} is in fact more strongly expressed by neurons in the striatum and nucleus accumbens, structures highly innervated by the dopaminergic nerve terminals (Khan *et al.*, 1998; Emilien *et al.*, 1999). It appears that the D_{2S} isoform is the likely dopamine autoreceptor, whereas the D_{2L} isoform primarily represents a postjunctional receptor (ibid). Thus, in the striatum, the D_{2L} splice variant is expressed most strongly in medium sized GABA-ergic and large cholinergic neurons, whereas the D_{2S} receptor is found mainly in dopamine terminals.

The striatopallidal GABA-ergic neurons where the D_2 receptors are the major receptor represent the first component of the indirect pathway of the striatum extending into the globus pallidus. Transmission in this pathway is increased in Parkinson's disease, contributing to the parkinsonian symptomology. The D_2 receptor, therefore, may be implicated in Parkinson's disease. In agreement with a role of D_2 receptors in Parkinson's disease, the D_2 receptor deficient mice show signs

of inhibited motor activity (a 74% reduction of locomotion and absence of rearing) although other members of the dopamine receptor family are unaffected (Saiardi *et al.*, 1998; Emilien *et al.*, 1999).

D_2 RECEPTOR INTERACTING PROTEINS

Both, the D_3 and the D_2 receptors were shown to interact with FLN-A (nonmuscle filamin or actin binding protein 280) (Li *et al.*, 2000; Lin *et al.*, 2001) using the yeast two hybrid approach. Interaction of D_{2L} receptor with FLN-A was verified by the coimmunoprcipitation of FLN-A with D_{2L} receptors in membranes from HEK293 cells stably expressing D_{2L} receptor (Lin *et al.*, 2001). Amino acids 211-241 localized to the N-terminal end of the third intracellular loop of the D_{2L} receptor comprise a core domain sufficient for interaction with FLN-A (Lin *et al.*, 2001). Interaction of the D_3 receptor with FLN-A is spread to the N- and C terminus of the third intracellular loop (AA 211-227 and 281-329 of the D_3 receptor) (Lin *et al.*, 2001). FLN-A protein has been shown to regulate D_2 receptor signaling (Li *et al.*, 2000). The FLN-A protein also interacts with other G protein coupled receptors, namely metabotropic glutamate receptor type 7b, and a number of other metabotropic glutamate receptor subtypes (Enz, 2002).

Protein 4.1N interacts with the same locus of the D_2 receptor as the FLN-A and was identified in a yeast two hybrid screen, confirmed in pulldown and coimmunoprecipitation assays (Binda *et al.*, 2002). The protein 4.1N and D_2 receptor are localized to the plasma membrane in the transfected HEK293 cells. However, the presence of a protein 4.1 truncation fragment reduces the level of D_2 receptor expression at the plasma membrane, suggesting a role of protein 4.1N/D_2 receptor interaction for the localization or stability of dopamine receptors at the neuronal plasma membrane (Binda *et al.*, 2002).

A mechanism of cross-talk between intracellular Ca^{2+} and D_2 receptor is suggested by Bofill-Cardona and co-workers (Bofill-Cardona *et al.*, 2000). The D_2 receptor contains a calmodulin binding motif localized to the N-terminal end of the third intracellular loop (AA208-226), and the corresponding peptide binds to calmodulin in Ca^{2+} dependent manner. Furthermore, D_2 receptor promoted [^{35}S]GTPγS binding was suppressed by Ca^{2+}/calmodulin, and the dopamine agonist dependent inhibition of forskolin stimulated cAMP formation was decreased with

elicited Ca^{2+} influx. It is therefore suggested that calmodulin directly targets the D_2 receptor to block the receptor operated G protein activation switch (Bofill-Cardona *et al.*, 2000).

Spinophilin, a protein phosphatase regulatory protein that is enriched in the dendritic spines (Allen *et al.*, 1997) is another protein interacting with the third intracellular loop of the D_2 receptor as shown with a yeast two hybrid assay (Smith et al 1999 JBC). However, the functional significance of this interaction is still unknown.

Stimulation of adenosine A_{2A} receptors reduces the affinity of D_2 agonist binding in the plasma membrane, thus suggesting direct intramembrane interaction between these receptors (Ferre *et al.*, 1991). Indeed, D_2 and A_{2A} receptors co-localize on the plasma membrane as shown with confocal microscopy in primary striatal cultures (Hillion *et al.*, 2002). Long term exposure to A_{2A} or D_2 agonists results in coaggregation, cointernalization and codensitization of A_{2A} and D_2 receptors in the transfected SH-SY5Y neuroblastoma cells (Hillion *et al.*, 2002). Co-immuno precipitation experiments showed that the molecular basis for for these A_{2A}/D_2 interactions was an A_{2A}/D_2 heteromeric complex (Hillion *et al.*, 2002). Furthermore, an A_{2A}/D_2 heterodimer may be formed since a strong BRET (bioluminescence resonance energy transfer) signal is detected in HEK293-T cells cotransfected with A_{2A}R_R.luciferase and D_2R-YFP (Canals *et al.*, 2003). Studies with chimeric D_2/D_1 receptors and computational experiments using docking simulation on theoretical models of D_2 and A_{2A} receptors suggest that the locus of the A_{2A}/D_2 interaction involves the N-terminal part of the D_2 receptor (Canals *et al.*, 2003). Indeed, mutation of the arginine residues of the third intracellular loop of the D_2 receptor prevents A_{2A}/D_2 hetero-dimerization as studied with the BRET technique and also peptides derived from the N-terminal region of the third intracellular loop of the D_2 receptor (AA215-224) interacts with the A_{2A} receptors and more precisely with domains of the C-terminal part of the A_{2A} receptor as shown with mass spectrometry and in a pull-down assay (Ciruela *et al.*, 2003).

6-HYDROXYDOPAMINE LESION AND DOPAMINE SUPERSENSITIVITY

6-hydroxydopamine induced lesions of the nigrostriatal dopamine pathway is one of the animal models of Parkinson's disease. In this model injection of the neurotoxin 6-hydroxydopamine into the substantia nigra induces degeneration of the ascending dopamine pathways (Ungerstedt, 1968) and the striatal levels of dopamine and DOPAC are decreased by the order of 95 and 90%, respectively (Breese *et al.*, 1987; Bjelke *et al.*, 1994). Striatal dopamine denervation leads to dopamine receptor supersensitivity, manifested by enhanced responsiveness to dopamine (Ungerstedt, 1971; Staunton *et al.*, 1981; Neve *et al.*, 1982; LaHoste & Marshall, 1992; Labandeira-Garcia *et al.*, 1996). In fact, behavioural sensitivity to dopamine agonists is enhanced up to 40-fold following close to total destruction of the ascending dopamine system in rats (Marshall & Ungerstedt, 1977; Mandel *et al.*, 1992). Thus, dopamine agonists like apomorphine, a mixed D_1 and D_2 dopamine receptor agonist, activates preferentially the dopamine receptors on the dopamine denervated side due to the dopamine receptor supersensitivity development (Ungerstedt, 1971; Creese *et al.*, 1977; Labandeira-Garcia *et al.*, 1996). In fact, striatal dopamine denervation has been found to increase the density of D_2 receptors by 29-40% in the dopamine denervated striatum, compared to the intact striatum (Creese *et al.*, 1977; Neve *et al.*, 1991). However, the relative proportion (ratio) of D_{2L} and D_{2S} dopamine receptor mRNAs was unchanged after dopamine denervation (Neve *et al.*, 1991). Similarly, chronic (3 weeks) daily treatment with reserpine increases D_2 receptor density in striatal membranes by 25% (Burt *et al.*, 1977). However, the time courses of the development of behavioral supersensitivity and the increase in the D_2 receptor density does not match each other (Staunton *et al.*, 1981; Neve *et al.*, 1982). Rats have been observed to rotate contralaterally in response to apomorphine within a few days after unilateral 6-hydroxydopamine induced nigral lesions (Ungerstedt, 1971; Staunton *et al.*, 1981; Labandeira-Garcia *et al.*, 1996), while an increase in the density of striatal dopamine receptors has been reported to occur only two or more weeks following lesion (Labandeira-Garcia *et al.*, 1996). Indeed, behavioral supersensitivity to dopamine receptor agonists has been observed after unilateral dopamine depleting lesions that do not induce an increase in the D_1 or D_2 receptor

density. Rats with 6-hydroxydopamine induced unilateral lesions of nigral dopamine cells treated chronically with D_2 receptor antagonists have in contrast equivalent densities of D_2 receptors in the striatum of both sides and still develop contralateral rotational behaviour in response to dopamine agonists (Breese *et al.*, 1987; Mileson *et al.*, 1991). Therefore, it is proposed that the increase in the dopamine receptor density is not the reason for the increased sensitivity to dopamine agonists in behavioural and other functional studies found after dopamine denervation (Inoue *et al.*, 1994; Kostrzewa, 1995). Furthermore, the catalytic activity of adenylate cyclase is not altered in association with development of dopamine receptor supersensitivity (as measured by direct activation of AC with forskolin), whereas the response of the enzyme to dopamine agonists is enhanced (Missale *et al.*, 1989; Cowburn *et al.*, 1991). This indicates that the mechanism of the dopamine receptor supersensitivity may involve alterations in G protein function. In fact, after one to five weeks following unilateral 6-hydroxydopamine induced nigral dopamine cell lesions, both basal and dopamine induced [^{35}S]GTPγS binding is significantly increased (Geurts *et al.*, 1999), indicating involvement of alterations in the receptor G protein coupling in the development of D_2 receptor supersensitivity.

LOCUS COERULEUS AND DSP-4 LESION

Several studies utilizing anatomical, behavioral, biochemical, and electrophysiological approaches have demonstrated a noradrenergic (NA-ergic) stimulatory input from the locus coeruleus (LC) to the mesencephalic dopaminergic neurons (Anden & Grabowska, 1976; Tassin *et al.*, 1979; Herve *et al.*, 1982; Grenhoff & Svensson, 1989; Lategan *et al.*, 1990; Mavridis *et al.*, 1991; Lategan *et al.*, 1992; Grenhoff *et al.*, 1993), which provide dopaminergic innervation to forebrain regions. Lesions of the LC projections can reduce dopamine release in mesolimbic and mesostriatal projections. Thus, in anesthetized rats, a significant reduction in basal dopamine overflow was observed using in vivo microdialysis in the striatum and nucleus accumbens after either bilateral 6-hydroxydopamine induced lesions of the LC (Lategan *et al.*, 1990) or pretreatment with DSP-4, a selective NA-ergic neurotoxin (Lategan *et al.*, 1992). Treatment with DSP-4 (50 mg/kg), which causes a reduction of NA levels by 70–80% in the LC projection areas, results in an upregulation of dopamine D_2 receptors in the striatum two weeks

after its administration (Harro *et al.*, 2000). Thus, DSP-4 treated animals have a lower locomotor activity, but the effect of amphetamine on horizontal activity is significantly larger in the DSP-4 pretreated rats, indicating supersensitivity development of the D_2 receptors after the LC denervation. Also dopamine β-hydroxylase knockout mice, which lack noradrenaline, show supersensitivity to amphetamine and quinpirole (a D_2 agonist) (Weinshenker *et al.*, 2002).

AIMS OF THE STUDY

To characterize the interaction between dopamine D_2 receptors and G proteins in cotransfected CHO cells as a model system.

To characterize changes in the D_2 receptor G protein coupling associated with development of receptor supersensitivity in an animal model of Parkinson's disease.

To characterize the possible interplay of A_{2A} receptor and Ca^{2+}/calmodulin in the D_2 receptor G protein coupling in CHO cells.

MATERIALS AND METHODS

ANALYSIS OF CHEMICAL COMPOSITION OF GUANOSINE PHOSPHATES

Purity and composition of guanyl nucleotides has been analysed by HPLC on the GILSON HPLC equipment using Mono Q HR 5/5 anion exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden). Compounds were eluted using solvent A, containing 10 % methanol and 3 % of solvent B that contained 1.2 N NH4HCO3. The compounds were eluted at a flow rate 1.0 ml/min, with a linear gradient of increasing concentrations of the solvent B; between 0–2 min the increase was from 0 to 10 % B and between 2–25 min the increase were from 10 to 60% B. The eluted compounds were monitored at 260 nm. All compounds corresponded to the purity level reported by the supplier (85-99%) and none of the preparations contained detectable amounts (more than 0.1%) of nucleotides with higher phosphorylated state than the main compound.

CHO CELLS

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, MD, USA) were stably transfected with the rat dopamine D_{2S} receptor cDNA (2.47 kb cDNA fragment cloned into the EcoR1 sites of pZem 228R, gift from M.G.Caron) with the calcium phosphate precipitation method as described earlier (Chen & Okayama, 1987). Cells transfected with rat D_2 receptor cDNA were grown in α-minimum essential medium without nucleosides, containing 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), geneticin (Gibco, 500 µg/ml) and hygromycin (Calbiochem, 25µg/ml) at 37°C in 5%CO2/95% air. Cells at approximately 80% confluence and at passages 8-10 were used for preparation of membranes.

PREPARATION OF MEMBRANES FROM CHO CELLS

For radioligand binding experiments the cells were collected from Petri dishes, washed twice with ice-cold phosphate buffered saline and centrifuged at 2000 rpm for 5 min at 4°C. The cells were homogenised by sonication in preparation buffer

(20mM K-HEPES, 7mM $MgCl_2$, 100mM NaCl, 1mM EDTA, 1mM DTT, pH 7.6) and the nuclear fraction was precipitated by centrifugation at 3000 rpm for 10 min at 4°C and discarded. The membranes in the supernatant fraction were washed twice in PB by sonication and centrifugation at 20,000 rpm for 40 min at 4°C. The final pellet was homogenized in incubation buffer (20mM K-HEPES, 7mM $MgCl_2$, 100mM NaCl, 1mM EDTA, 1mM DTT, pH 7.6, final protein concentration ~50 µg/ml) and the obtained suspension was used directly for binding experiments. The protein content was measured by the modified method of Lowry (Peterson, 1983) using bovine serum albumin as standard.

6-HYDROXYDOPAMINE INDUCED LESION IN RATS

Pathogen free male Sprague-Dawley rats (BK Universal, Stockholm, Sweden) were housed under standardized conditions, lights on at 07.00 and off at 19.00 h, with free access to water and food pellets. The experimental protocols performed in the present study were approved by the Swedish local committee for ethical experiments on laboratory animals. The rats (b.wt. 150 g) were unilaterally injected with 6-hydroxydopamine (8 µg/4 µl dissolved in 0.9% NaCl, containing ascorbic acid 2 mg/10 ml) into the left substantia nigra, using the following coordinates: bregma level –4.4 mm, lateral 1.2 mm, ventral 7.8 mm (Ungerstedt, 1968; Paxinos & Watson, 1986). To evaluate the efficacy of the lesion the rats were tested for apomorphine induced contralateral rotational behaviour 6-8 weeks and 12 months after the 6-hydroxydopamine induced lesion, since rotational behaviour only develops after a striatal dopamine depletion by 95% or more (Bjelke *et al.*, 1994). Spontaneous rotational behaviour was recorded during 1 h followed by recording for 1 h of apomorphine induced (0.05 mg/kg s.c.) contralateral rotational behaviour.

DSP-4 INDUCED LESION OF LOCUS COERULEUS IN RATS

Male Wistar rats (300-400 g, National Laboratory Animal Center, Kuopio, Finland) were housed in groups of four under 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Lactamin 35, Sweden) and water available ad libitum. DSP-4 [N(2-chloroethyl)-N-ethyl-2-bromobenzylamine] (Astra, Sweden) was administered as a single dose of 10 or 50 mg/kg (expressed as for hydrochloride) intraperitoneally. Each dose was weighed separately, dissolved in distilled water and immediately

injected. Control animals received an injection of distilled water. Rats were killed by decapitation either three days or one month after administration of the neurotoxin. The brains were quickly dissected on ice and the brain tissue was stored at –80°C until biochemical assays.

MEASUREMENT OF MONOAMINES AND THEIR METABOLITES

The content of monoamines and their metabolites in the frontal cortex was measured by HPLC with electrochemical detection essentially as previously described . The brain tissues were homogenized with a Bandelin Sonoplus ultrasonic homogenizer (Bandelin Electronic, Germany) in ice cold solution (10-20 µl/mg tissue) of 0.09 M perchloric acid containing 5 mM sodium bisulfite and 0.04 mM EDTA to avoid oxidation. The homogenate was then centrifuged at 17 000 x g for 20 min at 4°C. Aliquots (10-20 µl) of the supernatant obtained were chromatographed on a LiChrospher 100 RP-18 column (250 x 3 mm; 5 µm) protected by a Supersphere RP18 (10 x 2 mm; 4µm) guard column. The separation was done in an isocratic elution mode at a column temperature of 30°C using a mobile phase containing 0.05 M citric acid buffer at pH 3.9, 0.9 mM sodium octylsulfonate, 0.3 mM triethylamine, 0.02 mM EDTA, 1 mM KCl and 8% acetonitrile. The chromatography system consisted of Hewlett Packard HP 1100 series isocratic pump, thermostatted autosampler, thermostatted column compartment and HP 1049 electrochemical detector (Hewlett Packard, Germany) with glassy carbon electrode. The measurements were done at electrode potential +0.6V versus an Ag/AgCl reference electrode.

PREPARATIONS OF MEMBRANES FROM RAT STRIATUM

Thirteen months after the 6-hydroxydopamine induced lesion the animals were sacrificed by decapitation, brains removed and the striatum from the left (dopamine denervated) and right (intact) side dissected out and prepared separately. Tissue pieces were homogenized by sonication in 20 volumes of ice-cold preparation buffer (20 mM K-Hepes, 7 mM $MgCl_2$, 1 mM EDTA, pH 7.5). The membranes were collected by centrifugation at 46 000 g for 20 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in PB, centrifuged as above and this procedure was repeated three times. The final pellet was homogenized in PB at a

tissue concentration of 20 mg/ml and stored at -80°C until further studies. Protein content was measured by the modified method of Lowry (Peterson, 1983) using bovine serum albumin as a standard.

BINDING EXPERIMENTS WITH [^{3}H]RACLOPRIDE

Binding of [^{3}H]raclopride to striatal membranes was performed in incubation buffer (IB, 20 mM K-Hepes, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, pH 7.5) as described earlier (Lepiku *et al.*, 1996). Shortly, striatal membranes (0.14 mg tissue/tube) or CHO cell membranes (~12 µg protein/tube) were incubated with different concentrations of [^{3}H]raclopride (0.2-7.5 nM) for 90 minutes at 25°C in a total volume of 400 µl. The reaction was terminated by rapid filtration through glass-fiber filters (GF/B, Whatman Int. Ltd., Maidstone, UK) and the filters were washed three times with 5 ml of ice-cold buffer, containing 20 mM K-HEPES and 100 mM NaCl (pH 7.5). Nonspecific binding was defined as the binding in presence of 100 µM (+)butaclamol. In the competition experiments membranes were incubated with 1-1.5 nM [^{3}H]raclopride and different concentrations of other ligands and/or GDP/GTPγS.

BINDING EXPERIMENTS WITH [^{3}H]DOPAMINE

[^{3}H]dopamine binding was assayed in a reaction mixture containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT (pH7.4). The crude membrane homogenates (~12 µg protein/tube) were incubated with different concentrations of [^{3}H]dopamine (0.1–12 nM) or in the case of competition experiments with [^{3}H]dopamine (3–6 nM) and other ligands for 30 min at 25°C and free ligands were removed by fast filtration through glass-fiber filters (GF/B, Whatman International Ltd., Madistone, UK). The filters were washed with 15 ml (3 times 5 ml) ice-cold washing buffer (WB, 10 mM Tris-HCl, 100 mM NaCl, pH 7.5). Washing with 10-20 ml has been found to be optimal to remove maximal nonspecific binding without affecting specific binding. More extensive washing led already to a loss of specific binding without a significant influence on the nonspecific binding (data not shown). The radioactivity content of the filters was counted in 5 ml of scintillation cocktail Flo-ScintTM V (Packard) by the scintillation counter (Beckman LS 1800, ^{3}H efficiency 41%). The specific binding was defined as the difference between total and nonspecific binding, which

was measured in the absence and presence of 1 μM raclopride or 1 mM dopamine, respectively.

In the studies on the [^{3}H]dopamine association kinetics the crude membrane homogenates were prewarmed for 5 min at 25°C and the reaction started by the rapid addition of [^{3}H]dopamine. At specified time moments aliquots of the reaction mixture (200 μl) were filtered through glass-fiber filters and washed immediately three times with 5 ml WB and specifically bound radioactivity was measured as described above.

Kinetics of [^{3}H]dopamine dissociation was measured after preincubation of the membrane homogenates with 6 nM [^{3}H]dopamine for 30 min at 25°C and dissociation was initiated by the rapid addition of raclopride, dopamine or incubation buffer (dilution). At the specified time moments aliquots of the reaction mixture were taken, filtered through GF/B and the bound radioactivity was determined as described above.

BINDING EXPERIMENTS WITH [^{35}S]GTP-GAMMA-S

Binding of [^{35}S]GTPγS to striatal membranes in IB was carried out as described earlier (Rinken *et al.*, 1999). Shortly, the striatal membranes (0.14 mg tissue/tube) or CHO cell membranes (~12 μg protein tube) were incubated for 90 minutes at 30°C with 0.05-0.2 nM [^{35}S]GTPγS and different concentrations of GDP or GTPγS and/or other ligands. The reaction was terminated by rapid filtration as described above. In the saturation experiments, [^{35}S]GTPγS was diluted with GTPγS to a specific activity of 1.5 Ci/mmol. The membranes were incubated with different concentrations of [^{35}S]GTPγS (0.1-300 nM) for 90 min at 30°C. The non-specific binding was measured in the presence of 0.1 mM Gpp[NH]p.

DATA ANALYSIS

All binding and kinetic data were analyzed by means of the non-linear least squares regression method, using a commercial program GraphPad PRISM™ (GraphPad, San Diego, CA, USA).

RESULTS

PAPER 1

The method for determination of dopamine receptor mediated G protein activation (Weiland & Jakobs, 1994) was adapted to study D_2 receptors expressed in CHO cells. Treatment of cells with pertussis toxin abolished [^{35}S]GTPγS binding, indicating that the dopaminergic effects determined are coupled with Gαi or Gαo, which are the substrates for pertussis toxin (Nurnberg *et al.*, 1995; Cussac *et al.*, 1996). Studies with an irreversible dopaminergic antagonist NIPS indicated that less than 10% of the D_2 dopamine receptors were able to activate all D_2 coupled G proteins, which indicates a substantial amount of spare receptors in the CHO cell membranes. However, the potency of dopamine was decreased 50-fold following such a treatment.

Among 18 ligands studied there were seven ligands that showed only a partial activation of [^{35}S]GTPγS binding in comparison with dopamine. The comparison of the potencies of the dopaminergic agonists to stimulate [^{35}S]GTPγS binding to CHO cell membranes (pEC_{50}) with their ability to inhibit [^{3}H]raclopride binding (pIC_{50}) revealed a good correlation ($r>0.95$) with a slope value not significantly different from unity (1.15±0.13). It indicates a direct connection between receptor occupancy and activation of G proteins by the receptor. Also the pIC50 values of antagonists to inhibit dopamine-activated [^{35}S]GTPγS binding was in good agreement with their ability to inhibit [^{3}H]raclopride binding. Thus, there were no differences between agonists, partial agonists and antagonists when their potencies to modulate [^{35}S]GTPγS binding were compared with their potencies to inhibit [^{3}H]raclopride binding.

PAPER 2

Despite the fact that there were no detectable levels of 5-HT receptors in the CHO cells used, serotonin was able to activate G proteins in D_2 receptor expressing CHO cells. Maximal activation of [^{35}S]GTPγS binding in D_2 expressing CHO cells by serotonin, 8OH-DPAT and 5-methoxytryptamine were 47±7%, 43±5% and 70±7% of the maximal effect of dopamine, respectively. G protein activation by

serotoninergic and dopaminergic agonists were inhibited by D_2 antagonist butaclamol with the same potency (pA2 =8.9±0.1), indicating that only one type of receptor is involved. In competition with [^{3}H]raclopride binding dopaminergic agonists showed that 53±2% of the D_2 binding sites were in the high-affinity state, whereas corresponding value for serotonin was only 20±3%. The results indicate that serotonergic agonists behave as typical partial agonists for D_2 receptors.

PAPER 3

Binding of [^{3}H]dopamine to CHO cell membranes was clearly saturable with a Bmax value being 44% of the Bmax value of the [^{3}H]raclopride. This is in a good agreement with the proportion of D_2 receptors in the high affinity state (R_H=0.48±0.04) as indicated by [^{3}H]raclopride / dopamine competition experiments. The binding of [^{3}H]dopamine was considerably inhibited by sodium ions in the incubation buffer as found earlier (Neve, 1991) which was true also for agonist binding to other G protein-coupled receptors (Tian & Deth, 1993; Tian *et al.*, 1994). In addition to dopaminergic ligands all guanyl nucleotides studied inhibited the binding of [^{3}H]dopamine in a concentration-dependent manner. However, none of the guanyl nucleotides used had an influence on the [^{3}H]raclopride binding properties. Comparison of potencies of different nucleotides to inhibit [^{3}H]dopamine binding to dopamine receptors and to inhibit [^{35}S]GTPγS binding to G proteins revealed no significant differences. As the inhibition of [^{3}H]dopamine binding by nucleotides was complete, it can be proposed that all detectable dopamine radioligand binding corresponds only to the high affinity agonist binding sites of D_2 dopamine receptors. Thus, GTP, GDP as well as GMP turn D_2 dopamine receptors into a low affinity state by binding to the G protein α-subunit. It can therefore be concluded that, the high-affinity agonist state of the D_2 receptors can be achieved only in the case when no nucleotides are bound to G protein α-subunits.

The binding of [^{35}S]GTPγS to the membranes was specific and with high affinity and the maximal number of binding sites was 56 times higher than the number of [^{3}H]raclopride binding sites. All studied nucleotides displaced [^{35}S]GTPγS binding in a concentration dependent manner. Addition of dopaminergic ligands to the membranes had no influence on the GTPγS binding affinity but dopaminergic agonists decreased significantly the affinity of GDP (ΔpKi=0.43-0.68).

In the case of GDPβS and GMP the reductions in affinity caused by dopamine were smaller (ΔpKi~0.4), but still significant. For G proteins only GTP (or its analogue GTPγS) activates the second messenger system, whereas GDP remains bound after deactivation of the G protein by intrinsic GTP-ase activity of Gα subunit. Thus, activation of the D_2 receptors decreases affinity of the nucleotides that do not activate G proteins.

PAPER 4

6-hydroxydopamine induced unilateral lesions of the nigrostriatal dopamine system leads to development of D_2 receptor supersensitivity on the lesioned side. The density of [^{3}H]raclopride binding sites was increased by 30% in the dopamine denervated as compared to the intact striatum, but the lesion had no effect on the affinity of [^{3}H]raclopride. Displacement of [^{3}H]raclopride binding by dopamine was best described according to the two site binding model with no differences in dopamine affinities between dopamine denervated and intact striatum. The proportion of high-affinity binding sites was 69±4% on the intact and 62±5% on the dopamine denervated side. The addition of 100 μM GTPγS decreased the fraction of high affinity binding sites to 36±2% and 37±3% in the intact and dopamine denervated striatum, respectively.

GDP was also able to shift dopamine receptors into a low affinity state, but the potency of GDP to switch dopamine receptors into the low-affinity agonist binding state as shown in dopamine vs. [^{3}H]raclopride competition experiments was decreased in the dopamine denervated striatum (pEC50=5.00±0.19 for intact and pEC50=4.30±0.17 for dopamine denervated striatum ($p<0.001$)). The potency of GTPγS in similar experiments was not altered, having values of pEC50=6.87±0.16 and of pEC50=6.84±0.18 in the intact and dopamine denervated striatum, respectively (Figure 2).

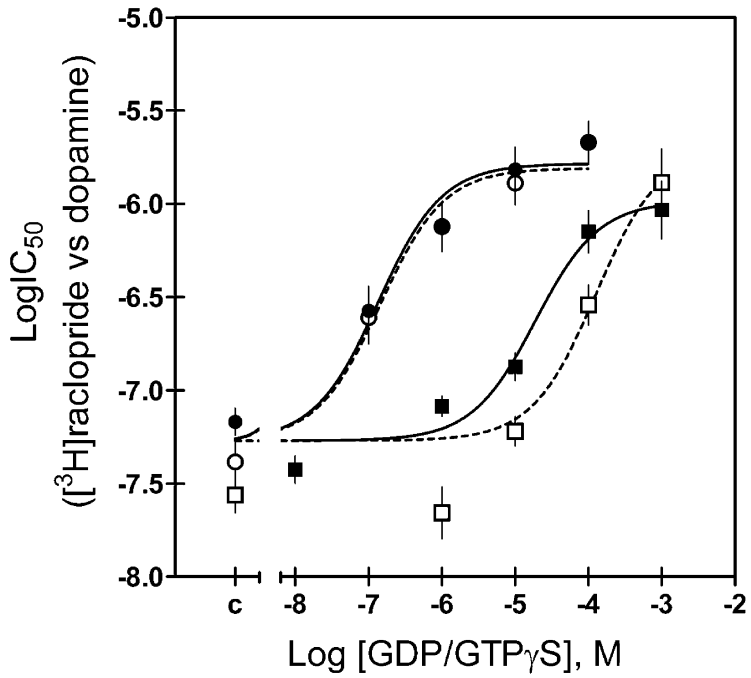

Figure 2 A reduction of affinity of GDP, but not of GTPγS to turn D_2 receptors into a low affinity state following 6-hydroxydopamine lesion of the dopamine pathways. Open symbols and broken lines for dopamine denervated and closed symbols and solid lines for intact side, respectively. Circles for GTPγS and squares for GDP.

Dopamine caused a concentration-dependent activation of [^{35}S]GTPγS binding to membranes from the striatum of both sides. There were no significant differences in the concentrations of dopamine causing a half-maximal stimulation of [^{35}S]GTPγS binding. However, the amount of dopamine stimulated [^{35}S]GTPγS binding was 2.8 times higher in membranes from dopamine denervated striatum in comparison with intact striatum, indicating a clear-cut dopamine receptor supersensitivity after dopamine denervation. This cannot be connected with changes in the density of G proteins, since there were no changes in the number [^{35}S]GTPγS binding sites. Also the affinity of [^{35}S]GTPγS binding was unchanged following dopamine denervation.

A significant influence of the dopamine denervation was found on the potency of GDP to displace [^{35}S]GTPγS binding. In membranes of intact striatum the pIC50 for GDP was 4.59±0.15, whereas in membranes of dopamine denervated striatum the corresponding value was 4.37±0.18 ($p<0.05$). On the other hand, no differences were found in the affinity of GTPγS.

PAPER 5

In the frontal cortex DSP-4 treatment had an overall dose related effect ($F_{2,41}=184.8$, $P<0.001$) on NA levels, and there was a significant dose and time interaction ($F_{2,41}=3.60$, $P<0.05$). Thus, 3 days after administration of the neurotoxin there was a dose-dependent reduction in NA levels, the effect of both doses of DSP-4 being significant compared to control animals. One month after the treatment, only the effect of the dose of 50 mg/kg was significant. In the striatum, the effect of DSP-4 was not significant.

Both dose and time variables had independent overall significant effects on dopamine levels in the frontal cortex ($F_{2,41}=4.32$ and $F_{1,41}=6.83$, respectively, $P<0.05$). However, according to post-hoc tests, the only significant difference was the higher cortical dopamine levels in DSP-4 (50 mg/kg) treated rats compared to the vehicle group 3 days after treatment. No effect on dopamine was found in the striatum. Significant overall effects of dose and time ($F_{2,41}=14.4$ and $F1,41=22.7$, respectively, $P<0.001$) and their significant interaction ($F_{2,41}=7.40$, $P<0.01$) were found on DOPAC levels in the frontal cortex. Similarly, significant overall effects of dose and time ($F_{2,39}=20.2$ and $F1,39=11.8$, respectively, $P<0.001$) and their significant interaction ($F_{2,39}=17.2$, $P<0.001$) were found on DOPAC levels in the striatum. Thus, DOPAC levels in the frontal cortex and striatum were dose-dependently reduced by DSP-4 3 days after administration of the neurotoxin, but had been recovered by 1 month after the neurotoxin treatment. No effect of DSP-4 treatment was found on HVA levels.

DSP-4 treatment had no significant effect on 5-HT levels in the frontal cortex , but a significant time effect ($F1,41=15.1$, $P<0.001$) and dose and time interaction ($F2,41=3.55$, $P<0.05$) were found regarding 5-HIAA levels. Both doses of DSP-4 significantly reduced frontal cortical 5-HIAA levels 3 days but not 1 month after

administration of the neurotoxin. No effect of DSP-4 on these measures was present in the striatum.

Three days after administration of the neurotoxin there were no significant differences in the number of [^{3}H]raclopride binding sites, but the binding affinity was decreased in rats treated with 50 mg/kg of DSP-4. DSP-4 treatment caused an increase of [^{3}H]raclopride binding sites 1 month after treatment already at the dose of 10 mg/kg without a significant influence on the affinity of the antagonist.

Displacement of 1.1 nM [^{3}H]raclopride binding by dopamine was heterogeneous and best described with a two-site binding model, with K_H=40 nM and K_L=54.1 µM for high and low affinity sites, respectively. The proportion of high-affinity binding sites was 65±3% for the control and 66±8% and 62±8% for the DSP-4 10 mg/kg and 50 mg/kg treated rats, respectively. The addition of 30 µM GTPγS to the incubation medium turned receptors into a low affinity state and shifted the displacement curves to IC_{50} values of 3.6±0.6 µM, 4.0±0.8 µM and 4.3±0.9 µM for control and DSP-4 10 mg/kg and 50 mg/kg treated rats, respectively. No significant influence of the DSP-4 treatment was found on the ability of GDP to displace [^{35}S]GTPγS binding. The GDP/[^{35}S]GTPγS competition curves were heterogeneous with 62% of the sites with high affinity for GDP (0.13 µM). The affinity of GDP to low affinity sites decreased in the presence of dopamine from 81 to 120 µM, but none of these parameters depended on the treatment of animals with DSP-4. There were no significant differences between effective concentrations of dopamine causing a half-maximal stimulation of [^{35}S]GTPγS binding, the apparent pEC_{50} values being 5.02±0.09, 5.00±0.05 and 4.98±0.06 for control, DSP-4 10 mg/kg and DSP-4 50 mg/kg groups, respectively.

PAPER 6

An antibody against calmodulin detected a single band at 17 kDA in Western blots from solubilized naïve CHO cell preparations (data not shown).

Ca^{2+} (100 µM) and dopamine (100µM, the concentration giving maximal responses) had no effect on the basal level of [^{35}S]GTPγS binding in membranes prepared from naïve or A_{2A} alone transfected CHO cells.

Ca^{2+} (100 µM) showed a trend to decrease the baseline level of [^{35}S]GTPγS binding (-3%) in D_2 alone transfected CHO cells as well as a trend for reduction in

the 100 μM dopamine activated [^{35}S]GTPγS binding (-4%) in these cells. Ca^{2+} (100 μM) increased levels of basal and 100 μM dopamine stimulated [^{35}S]GTPγS binding in membranes prepared from A_{2A}/D_2 co-transfected CHO cells. Treatment with Ca^{2+} produced a 18% increase in basal and a 23% increase in the dopamine activated [^{35}S]GTPγS binding in A_{2A}/D_2 CHO cell membranes.

DISCUSSION

CHO cells were used as a model system to study D_2 receptor signaling. The use of D_2 transfected CHO cell lines made it possible to estimate efficacies of serotonin and serotoninergic agonists at the D_2 receptor. Binding of radiolabelled dopamine in membrane preparations from rat brain is very heterogeneous and thus not very useful as an experimental tool. In contrast, all [^{3}H]dopamine binding in D_2 transfected CHO cells could be inhibited by specific D_2 antagonists. Thus, it was possible to use agonist, antagonist and [^{35}S]GTPγS binding to investigate the cross regulation between G proteins and D_2 dopamine receptors expressed in CHO cells. The obtained results indicate that not only analogues of GTP but also GDP and GMP turned D_2 dopamine receptors into a low affinity state for dopamine. Activation of the D_2 receptor by dopamine caused a decrease in the binding affinity for GDP, but not for analogues of GTP. According to these results, the high-affinity state of agonist binding can be achieved only when no nucleotides are bound in the agonist receptor-G protein complex.

In the rat model of Parkinson's disease, the coupling between the D_2 receptor and the Gi/o protein is altered leading to a decreased affinity of GDP at the Gα subunit. The decrease in the GDP affinity is clearly connected with the D_2 receptor as the ability of GDP to turn D_2 receptors into a low affinity state is reduced. Following striatal dopamine denervation and chronic haloperidol treatment the expression of RGS proteins is altered which could be a mechanistic basis of the D_2 receptor supersensitivity. Levels of RGS9-2 protein are increased in caudate putamen from patients with Parkinson's disease (Tekumalla *et al.*, 2001). Acute administration of the D_2 antagonist enhances the level of RGS2 mRNA in the striatum, indicating a tonic inhibition of RGS2 expression by the D_2 receptors (Burchett *et al.*, 1999; Robinet *et al.*, 2001; Geurts *et al.*, 2002). RGS proteins accelerate intrinsic GTP-ase activity of the Gα-subunit, thus reducing the life span of the active conformation of GTP-bound Gα subunit (Ross & Wilkie, 2000). Such a mechanism makes it possible for the G protein complex to reassociate before the agonist is dissociated from the GPCR and therefore ready for the next activation cycle by the agonist bound receptor. The overall effect is an increase in the GDP/GTP exchange rate (Ross & Wilkie, 2000) without a decrease in the effective concentration of activated G

proteins in the presence of an activated receptor, but allows a fast termination of the signaling following dissociation of the agonist (Ross & Wilkie, 2000). In line with this model, recovery of the rod photoresponse in mice lacking the RGS9-1 is strongly reduced (Chen *et al.*, 2000). However, according to the model proposed by Ross, an increase in the GDP/GTP exchange is achieved by increasing the amount of available GDP bound forms of G proteins, as a result of an increased GTP-ase activity of Gα in the presence of RGS. Also, facilitation of D_2 receptor $G_{\alpha o}$ protein coupling by endogenous RGS protein in CHO cells has been reported (Boutet-Robinet *et al.*, 2003). Therefore, a change in RGS protein activity would have a major impact on the signaling by the GPCR and a possible role of RGS2 in the dopamine supersensitivity should not be underestimated.

There are several proteins that interact directly with the third intracellular loop of the D_2 receptor (Bergson *et al.*, 2003) and potentially interfere with the activation of G proteins by the receptor. In fact, calmodulin has been shown to interact with the third intracellular loop in a Ca^{2+} dependent manner, leading to inhibition of activation of the G proteins by the D_2 receptor (Bofill-Cardona *et al.*, 2000). The domain of the D_2 receptor involved in the interaction with calmodulin (AA208-226) (Bofill-Cardona *et al.*, 2000) overlaps with the domain being part of the A_{2A}/D_2 heterodimeric interface (AA215-224) (Canals *et al.*, 2003; Ciruela *et al.*, 2003). Therefore, a possible competition between Ca^{2+}/calmodulin and the adenosine A_{2A} receptor at the third intracellular loop of D_2 receptor was postulated. Indeed, it was found that Ca^{2+} substantially enhanced the basal and dopamine induced [^{35}S]GTPγS binding in A_{2A}/D_2 CHO cotransfected cells in contrast to the case with D_2 alone transfected CHO cells. Thus, Ca^{2+} increases $D_2/G_{i/o}$ protein coupling when D_2 mainly exists as an A_{2A}/D_2 heterodimer. Such an increase in the basal level of [^{35}S]GTPγS binding at a constant concentration of [^{35}S]GTPγS and GDP reflects a decrease in the GDP affinity. Thus, it is possible that calmodulin, by inhibiting formation of A_{2A}/D_2 heterodimers is involved in the observed decrease of the GDP affinity following striatal dopamine depletion.

Increased glutamatergic transmission in the basal ganglia has been implicated in the pathophysiology of Parkinson's disease (Klockgether & Turski, 1993) and degeneration of dopaminergic striatal afferents in Parkinson's disease may result in an increase of striatal glutamate levels (Lannes & Micheletti, 1997). Furthermore,

changes in the interactions of the dopamine and glutamate transmission in striatal medium spiny neurons appear to contribute to symptom production in Parkinson's disease (Chase & Oh, 2000). The balance between kinase and phosphatase signaling modifies the phosphorylation state of glutamate receptors and thus their synaptic activity. In fact, sensitization of spiny-neuron NMDA and AMPA receptors occurs in models of Parkinson's disease and alters cortical glutamatergic input to the striatum and modifies striatal GABAergic output (Chase & Oh, 2000). This may lead to an enhancement of Ca^{2-} influx via NMDA receptor channels, and increase of intracellular Ca^{2+} levels may also be mediated via mGluR5 with activation of PLC. Thus, calmodulin can become translocated to the D_2 receptor located in the medium spiny neurons of striatum in Parkinson' disease. Such possibilities and the present findings on the role of Ca^{2+} in dopamine receptor signaling opens up a new perspective to the complexity of regulation of D_2 receptor signaling where A_{2A}/D_2 heterodimers dynamically interact with Ca^{2+}/calmodulin. It may be speculated that Ca^{2+} signaling is capable of enhancing D_2 signaling by reducing the amount of A_{2A}/D_2 heterodimers or at least by interfering with the inhibitory action of A_{2A} receptors on $D_2R/G_{i/o}$ protein coupling within this heterodimer. Ca^{2+} via a postulated increase in the calmodulin binding to the D_2 receptor increases D_2 receptor signaling in the presence of the A_{2A} receptor but not in the absence of A_{2A} receptor. Such events may contribute to the increase in strital D_2 receptor G protein coupling seen in a model of Parkinson's disease. Future studies are necessary to unravel the precise molecular mechanism involved.

The question arises, if the observed decrease in the GDP affinity following 6-hydroxydopamine lesion is caused by altered activity of Gα-subunit interacting proteins (such as RGS) and/or by altered activity of D_2 receptor interacting proteins. In the first case, there should be an increased signaling also by other Gi/Go coupled GPCRs expressed in the striatopallidal GABAergic neurons. In the latter case, the effect of dopamine denervation should be specific for the D_2 receptor.

CONCLUSIONS

Changes in D_2 receptor G protein coupling are associated with the development of D_2 receptor supersensitivity as studied using a model of Parkinson's disease. The D_2 receptor interacting protein calmodulin and the A_{2A} receptor modulate D_2 receptor G protein coupling as studied in *in vitro* cell models. The following observations were made:

1. Striatal dopamine denervation results in a reduced potency of GDP to turn D2 receptors into a low affinity state and in a reduced affinity of GDP for GTPγS binding sites as seen in competition experiments with [^{35}S]GTPγS vs GDP. This probably reflects the increased dissociation rate of GDP from its complex with the Gα subunit, since the affinity of GTPγS was unchanged by dopamine denervation.

2. The role of dopamine D_2 receptor interacting proteins calmodulin and the A_{2A} receptor in the regulation of the D_2 receptor G protein coupling was studied. Ca^{2-} increases D_2 receptor signaling in the presence of the A_{2A} receptor but not in the absence of A_{2A} receptor via a postulated increase in the calmodulin binding to the D_2 receptor. This finding opens a new perspective to the complexity of regulation of D_2 receptor G protein coupling, where A_{2A} receptors and calmodulin dynamically interact with the D_2 receptor at a common binding domain. Such a mechanism may be involved in the development of D_2 receptor supersensitivity following striatal dopamine denervation.

ACKNOWLEDGEMENTS

This work has been done at the Prof. Kjell Fuxe's laboratory at the Department of Neuroscience, Karolinska Institutet. I would like to thank:

Professor Kjell Fuxe for great enthusiasm, never ending ideas, support and scientific guidance.

My co-supervisor Prof. Ago Rinken, for sharing all the written and unwritten knowledge about G proteins and receptors.

Prof. Jaak Järv, my very first tutor in scientific world, for showing me the inner beauty of chemical kinetics and teaching how to see complex things in a simple terms.

I thank all present and former members of the laboratory of cellular and molecular neurochemistry for creating a very special place – the Fuxe's lab, especially Dr. Sergi Ferré for many spontaneous and passionate discussions that we have had about science and other matters. Joelle Hillion for valuable advises about how to drive a car in the right way and for a wine, Beth Andbjer for preparing the best denervated rats and for teaching me how to place the earbars. Anita Hansson, Wolfgang Sommer and Roberto Rimondini for friendship. Ulla-Britt Finnman for great help during my first years in Sweden. Anne Edgren for always being helpful and for being able to read Kjells hand writing, Malin Höistad for all the help and for the nice drawing of basal ganglia. Niklas Lindgren for making fun out of the everyday lab work. Meritxell Canals for helping us out during the last weeks. Zaida Diaz, Alicia Riviera, Sonia Trias, Alberto del Arco, José Big and José Small and many others for bringing piece of Spain to the cold, dark and rainy part of the world. Haleh Razani for all the fun that always follows you, wherever you are.

All my Estonian friends, Arno Ruusalepp, Ursel Soomets, Margus Pooga, Annika Krüüner, Peeter Tähepõld, Peep Talving, Tiit and Kadri Land, Kersti Talving and many others.

Bill Staines, Barbro Tinner-Staines, Kirsten Jacobsen and all people at your lab for hospitality during my visit to Ottawa last year.

Ida Engqvist for help regarding computers and network.

Former and present PhD students, colleagues and friends at the department of neuroscience, thank you all.

This work was supported by a grant from Swedish Research Council (04X-715) and by a grant from European community (QLG3-CT-2001-01056), Wallenberg foundation and KEMP

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