

From THE DEPARTMENT OF NEUROSCIENCE  
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# **VOLTAGE SENSITIVITY OF DOPAMINE D<sub>2</sub>-LIKE RECEPTORS**

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**Karolinska  
Institutet**

Stockholm 2011

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ISBN 978-91-7457-475-3

## ABSTRACT

G protein coupled receptors (GPCRs) mediate a multitude of responses serving hormonal, neurotransmitter, and sensory functions. These receptors are important drug targets; in fact, about 27 % of prescribed drugs are GPCR ligands. The dopamine D<sub>2</sub> receptor is prominently expressed within the CNS as two distinct isoforms; D<sub>2L</sub> (long isoform) and D<sub>2S</sub> (short isoform). The former is mainly expressed postsynaptically, whereas the latter functions primarily as an inhibitory auto- and heteroreceptor. The D<sub>2</sub> receptor is of considerable pharmacological interest, as it constitutes the main target for most antiparkinsonian and antipsychotic drugs in clinical use. While many ion channels have long been known to be voltage sensitive, this property has not been attributed to GPCRs until quite recently. As a notable example, the muscarinic M<sub>2</sub> receptor was shown to display depolarization-induced decreases in agonist binding and functional potency. M<sub>2</sub> receptor voltage sensitivity has been implicated in the autoreceptor function of this GPCR, by permitting rapid control of neurotransmitter release kinetics by membrane voltage.

The present work investigated the voltage sensitivities of the three D<sub>2</sub>-like dopamine receptors; D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>. The bulk of the experiments were carried out in *Xenopus* oocytes heterologously expressing D<sub>2</sub>-like receptors with G protein-coupled inwardly rectifying potassium channel (GIRK) subunits. GIRK channels are activated by G<sub>βγ</sub> subunits from inhibitory G proteins and were used as readout of receptor activation. It was found that dopamine potency was reduced by depolarization to a similar extent at both isoforms of the D<sub>2</sub> receptor. However, at the dopamine D<sub>3</sub> receptor dopamine potency was not significantly affected, while a weak, albeit significant potency decrease was observed at the dopamine D<sub>4</sub> receptor. Moreover, in mammalian cells expressing fluorescent G protein subunits, changes in inter-subunit Förster Resonance Energy Transfer (FRET) were used as readout of D<sub>2S</sub> receptor activation. Determination of dopamine concentration-response relationships in single cells under simultaneous patch clamp revealed similar depolarization-induced potency shifts as when studying GIRK channel activation in oocytes. Furthermore, radioligand binding experiments carried out on oocytes in hyperpolarizing vs. depolarizing buffer established that dopamine binding is reduced by depolarization. Interestingly, the effect of voltage was different for different agonists at the D<sub>2S</sub> receptor, including efficacious, high-affinity antiparkinsonian agonists. This agonist-specificity did not reflect selective signalling via distinct G protein subtypes. However, contacts between agonist hydroxyl groups and receptor serine residues, as well as between the agonist amine group and a conserved aspartate residue, were found to be important for the voltage induced potency shift of phenethylamine agonists, such as dopamine.

In conclusion, the findings presented in this thesis suggest that the dopamine D<sub>2</sub>-like receptors are differentially affected by voltage. At the D<sub>2S</sub> receptor, specific agonist-receptor interactions determine the effect of the receptor's voltage sensitivity on agonist potency and efficacy. This information demonstrates the relevance of GPCR voltage sensitivity to dopaminergic signalling, reveals new details about the mechanism of voltage sensitive agonism, and points to the possibility of using differentially voltage-modulated agonists to investigate the relevance of this phenomenon in native tissue.

## LIST OF PUBLICATIONS INCLUDED IN THE THESIS

- I. **Sahlholm K**, Nilsson J, Marcellino D, Fuxe K, Århem P. 2008. Voltage-dependence of the human dopamine D2 receptor. *Synapse* **62**:476-480.
- II. **Sahlholm K**, Marcellino D, Nilsson J, Fuxe K, Århem P. 2008. Differential voltage-sensitivity of D(2)-like dopamine receptors. *Biochem Biophys Res Commun.* **374**:496-501.
- III. **Sahlholm K**, Marcellino D, Nilsson J, Fuxe K, Århem P. 2008. Voltage-sensitivity at the human dopamine D2S receptor is agonist-specific. *Biochem Biophys Res Commun.* **377**:1216-1221.
- IV. **Sahlholm K**, Barchad-Avitzur O, Marcellino D, Gómez-Soler M, Fuxe K, Ciruela F, Århem P. 2011. Agonist-specific voltage sensitivity at the dopamine D2S receptor –Molecular determinants and relevance to therapeutic ligands. *Neuropharmacology* **61**:937-949.

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## LIST OF ABBREVIATIONS

ADHD	Attention-deficit/hyperactivity disorder
CFP	Cyan fluorescent protein
CMV	Cytomegalovirus
DAT	Dopamine transporter
DPAT	Dipropyl-aminotetralin
FRET	Förster resonance energy transfer
GAP	GTPase activating protein
GIRK	G protein-coupled inward rectifier potassium channel
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
PTX	Pertussis toxin
RGS	Regulator of G protein signaling
TM	Transmembrane
VNTR	Variable number of tandem repeats
YFP	Yellow fluorescent protein

# 1 INTRODUCTION

## 1.1 G PROTEIN-COUPLED RECEPTORS AND G PROTEINS

G protein-coupled receptors (GPCRs) are a heterogeneous superfamily of transmembrane proteins, which share a common structural motif of seven transmembrane helices, and the capacity to activate heterotrimeric G proteins (Lagerström and Schiöth, 2008). However, the “GPCR” term has been suggested to be outdated, as these proteins have been shown also to signal *via* several other molecules independently of G protein activation; examples of this include activation of arrestins and receptor tyrosine kinases (Shukla *et al.*, 2011). Instead, “7-transmembrane (7-TM) receptors” has been suggested as a more appropriate name; nevertheless, the GPCR acronym remains the most widely accepted, and will be used in the following treatise, which will focus on G protein-dependent aspects of downstream signaling. GPCRs are important therapeutic targets; over 25 % of prescribed drugs, by number of drug compounds, are GPCR ligands (Overington *et al.*, 2006).

G proteins belong to the GTPase superfamily; proteins which bind GTP that is subsequently hydrolyzed to GDP and inorganic phosphate. These proteins are heterotrimers consisting of a large  $G_{\alpha}$  subunit, which contains the GTPase domain, and the smaller  $G_{\beta}$  and  $G_{\gamma}$  subunits (see Oldham and Hamm, 2006). When GDP-bound or empty, the G protein is considered to be in its inactive, non-signaling state. Upon GTP binding, this complex undergoes conformational rearrangement, which confers the ability to initiate downstream signaling, such as activation or inhibition of adenylate cyclase (typically mediated *via* the  $G_{\alpha}$  subunit) or ion channels (often *via* the  $G_{\beta\gamma}$  subunits; see below). When purified G proteins are investigated in solution, the GTP-induced conformational change in the G protein trimer leads to its dissociation into two separate  $G_{\alpha}$  and  $G_{\beta\gamma}$  entities. To what extent such dissociation also occurs in living cells is currently a matter of debate; this might be true only for some G protein subtypes (Lambert, 2008). In either case, both  $G_{\alpha}$  and  $G_{\beta\gamma}$  presumably remain associated with the plasma membrane throughout the G protein cycle, since  $G_{\alpha}$  and  $G_{\gamma}$  are covalently attached to lipids, which anchor them to the membrane’s inner leaflet. The signal is terminated by hydrolysis of the bound GTP, upon which the G protein trimer returns to its original, inactive conformation. To initiate a new cycle of activation, the GDP, which remains bound to the G protein in its inactive state, must be exchanged for a GTP molecule (Oldham and Hamm, 2006).

Agonist binding to a GPCR triggers a series of conformational changes which are transmitted *via* the transmembrane helices to the cytoplasmic face of the receptor protein. In particular, the second and third intracellular loops, as well as the C-terminus, have been strongly implicated in the interaction of GPCRs with G proteins. The conformational rearrangement of these receptor domains enables activation of GDP-bound G proteins. The active conformation of the GPCR stabilizes the empty state of the  $G_{\alpha}$  subunit; thus, GPCR activation favors release of GDP. Subsequently, GTP, which in living cells is present at several-fold higher concentrations compared to GDP, can enter the nucleotide binding pocket of the  $G_{\alpha}$  subunit and activate the G protein,

simultaneously destabilizing the agonist-receptor-G protein complex. (Oldham and Hamm, 2006).

### 1.1.1 Dopamine receptors

Dopamine receptors belong to the “Class 1” (also known as “Rhodopsin family” or “amine-like”) group of GPCRs; thus, they share significant sequence similarities with, for example, serotonin, histamine, muscarinic, and adrenergic GPCRs (Lagerström and Schiöth, 2008; Foord *et al.*, 2005). Dopamine receptor function has been heavily implicated in physiological functions such as learning and memory, reward processes, attention and behavioral set-shifting, and the selection and execution of motor programs (Schultz, 2007). Aberrant dopaminergic signaling is also regarded as central to several common disease states (see below). There are five subtypes of dopamine receptors; D<sub>1</sub>-D<sub>5</sub>, which are typically subdivided into D<sub>1</sub>-like (D<sub>1</sub> and D<sub>5</sub>) and D<sub>2</sub>-like (D<sub>2</sub>-D<sub>4</sub>) based on their preferential coupling to G proteins which stimulate (G<sub>s</sub>) or inhibit (G<sub>i/o</sub>) adenylate cyclase, respectively (Stoof and Keibian, 1981). Of relevance for the present work, G<sub>i/o</sub> proteins can be inactivated by pertussis toxin (PTX); a bacterial peptide toxin which covalently ADP-ribosylates G<sub>i/o</sub> proteins. ADP-ribosylation mimics the effect of GDP occupancy on the G<sub>i/o</sub> proteins, thus rendering them permanently inactive and unable to interact with their cognate receptors. As will be discussed below, the specificity of PTX for G<sub>i/o</sub> proteins can be used experimentally to manipulate GPCR signalling pathways.

#### 1.1.1.1 D<sub>2</sub>-like subtypes

Besides adenylate cyclase inhibition, other effector actions of D<sub>2</sub>-like dopamine receptors include inhibition of calcium channels, increase of dopamine re-uptake *via* the dopamine transporter (DAT), and opening of G protein-coupled inward rectifier potassium channels (GIRK; De Mei *et al.*, 2009; Missale *et al.*, 1998). GIRK channel activating forms the basis for the main assay used in the papers in this thesis (see below).

The dopamine D<sub>2</sub> receptor is the main target for several drugs which find clinical use in some common neurological, neuroendocrinological, and psychiatric disorders, notably including Parkinson’s disease, hyperprolactinemia, restless legs syndrome, psychosis, and Tourette’s syndrome; D<sub>2</sub> agonists are used in the first three of these disorders, whereas antagonists or weak partial agonists are employed in treatment of the last two (Kvernmo *et al.*, 2008; Prabhakar and Davis, 2008; Seeman 2010). The dopamine D<sub>2</sub> receptor is expressed as two distinct splice variants; D<sub>2L</sub> and D<sub>2S</sub> (long and short, respectively), differing by a stretch of 29 residues in the third intracellular loop (Usiello *et al.*, 2000). Prominent D<sub>2</sub> receptor expression is found within the striatum, with lower density expression in thalamus and prefrontal cortex. D<sub>2S</sub> functions predominantly as an inhibitory auto- and heteroreceptor at dopamine, glutamate, and GABA terminals in the CNS, whereas D<sub>2L</sub> is considered to mediate the majority of postsynaptic responses to dopamine, notably on the dendrites of medium spiny neurons, which constitute the primary neurons in the striatum (Usiello *et al.*, 2000).

The D<sub>3</sub> receptor is also expressed in the striatum, although it displays a more restricted localization pattern than D<sub>2L</sub> and D<sub>2S</sub>, with predominant postsynaptic expression in the nucleus accumbens and the islands of Calleja (Suzuki *et al.*, 1998). Increased dopamine



release in the nucleus accumbens is widely believed to serve as a reward signal, and consistent with this notion, recent work implicates the D<sub>3</sub> receptor in addictive processes (Heidbreder and Newman, 2010). D<sub>3</sub> is also expressed in dopaminergic neurons and has been suggested to act as an autoreceptor, similar to D<sub>2S</sub>, but this remains a controversial issue (Koeltzow *et al.*, 1998). Whereas D<sub>2L</sub> and D<sub>2S</sub> can activate G protein of both the G<sub>oi</sub> and G<sub>oo</sub> subtypes, D<sub>3</sub> couples exclusively to G<sub>oo</sub> (Lane *et al.*, 2008).

The D<sub>4</sub> receptor, in contrast, is predominantly expressed on pyramidal cells of the prefrontal cortex and in the amygdala, but also shows some expression in the basal ganglia, both presynaptically on several types of nerve terminals, and postsynaptically on medium spiny neurons (Svingos *et al.*, 2000; Rivera *et al.*, 2002). The third intracellular loop of human D<sub>4</sub> contains a variable number of tandem repeats (VNTRs) of 16 amino acids, of which there exist three major polymorphisms; D<sub>4.2</sub>, D<sub>4.4</sub> and D<sub>4.7</sub>. Interestingly, the D<sub>4.7</sub> polymorphism has been associated with attention-deficit/hyperactivity disorder (ADHD) in several genetic linkage studies (Rondou *et al.*, 2010).

### 1.1.2 Ligand binding at GPCRs

Most GPCRs display states of high and low affinity binding to their agonists; the high-affinity state generally being regarded as the signalling, G protein-coupled form of the receptor. Specifically, the empty (nucleotide-free) G protein is believed to stabilize the high-affinity state of the receptor (see above). While such high- and low-affinity states are readily detected in competition binding experiments performed on purified cell membrane fractions, there is controversy in the literature as to whether these states can be observed in living cells. While Seeman (2008) reported on the existence of two affinity states for dopamine binding in competition with a radiolabeled antagonist, neither Sibley *et al.* (1983) nor Skinbjerg *et al.* (2009) found evidence of biphasic agonist binding to D<sub>2</sub> receptors in live cells. The structure of the antagonist used in these competition binding studies has been suggested to influence the ability to detect the two affinity states, potentially because the more lipophilic ligands, such as spiperone, readily cross the cell membrane and will thus also bind to internalized receptors, whereas relatively hydrophilic antagonists like domperidone will only access receptors expressed on the cell surface (Seeman *et al.*, 2006). However, another controversy regarding high-affinity live cell binding concerns the stability of the ternary complex of receptor, agonist, and guanine nucleotide-free G protein. Since the high intracellular GTP concentration (150 μM; Hatakeyama *et al.*, 1992) should promote its rapid breakdown, this complex presumably has a very short lifetime. Indeed, to visualize the two binding affinity components in membrane fractions, binding experiments are usually carried out in the absence of guanine nucleotides, and the addition of guanine nucleotides typically converts the biphasic dissociation curve into a monophasic one (the high affinity fraction is no longer observed). Even in membrane fractions, the exact mechanism giving rise to the two affinity states is controversial; under most conditions, the number of G proteins is assumed to exceed the number of GPCRs, suggesting that only one high-affinity fraction should be observed in the absence of guanine nucleotide (Sibley *et al.*, 1983).

The existence of homo- and heteromeric receptor complexes; the assembly of GPCRs into dimers, oligomers, or multimers (receptor mosaics) has gained increasing attention in recent years (Fuxe *et al.*, 2010). Considering dopamine D<sub>2</sub> receptors, recent evidence from experiments using engineered receptor constructs suggests that the high- and low-affinity binding states might result from negative cooperativity between two D<sub>2</sub> receptors forming a homodimer; agonist binding to one receptor decreases agonist potency at the other receptor in the dimer (Han *et al.*, 2009). Similarly, Ma *et al.* (2007) showed that muscarinic M<sub>2</sub> receptors oligomerize and display negative cooperativity of agonist binding when reconstituted together with (but not without) G protein. In agreement, it was suggested on the basis of modelling and crystallography data that for sterical reasons, only one of the protomers in a GPCR dimer could couple to G protein at a time (Han *et al.*, 2009). Thus, protomer competition for G protein coupling could potentially explain the appearance of the two binding fractions. However, adding to the controversy, it has been reported that monomeric  $\beta_2$  adrenergic receptors reconstituted into lipoprotein particles also display two affinity states (Whorton *et al.*, 2007). In this study, the fraction of high-affinity agonist binding increased from 0 -100 % with increasing concentration of the receptor's cognate G protein; G<sub>s</sub>, in the binding buffer, strongly suggesting that in this case, limited G protein binding to the GPCR indeed caused the partitioning of the receptor pool into high- and low-affinity fractions.

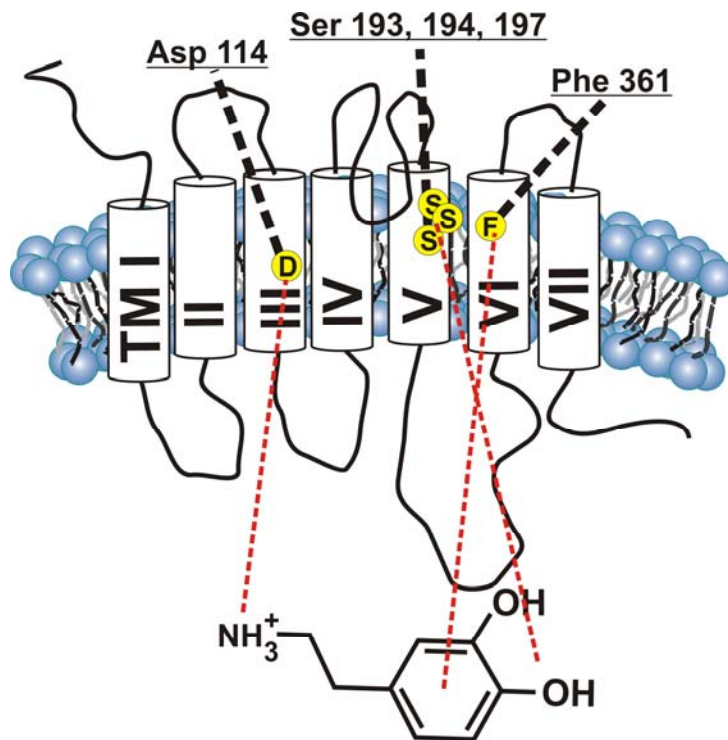
The *ex vivo* picture is further complicated by the presence of multiple D<sub>2</sub>-like receptor subtypes with similar affinities for the radioligands most commonly used in binding studies (see below). Moreover, in the case of dopamine D<sub>2</sub> receptors, the presence of heteromeric assemblies containing dopamine D<sub>1</sub>, D<sub>3</sub>, D<sub>4</sub>, and adenosine A<sub>2A</sub>, cannabinoid CB<sub>1</sub>, neurotensin NTS-1, serotonin 5-HT<sub>2A</sub>, have been compellingly demonstrated (Fuxe *et al.*, 2010). The presence of heterodimer partners frequently modulates the ligand binding and cooperativity characteristics of the other interacting receptors. Thus, in native tissue, or in heterologous expression systems where endogenous heteromeric partners might exist, the binding of selective radioligands to D<sub>2</sub>-like receptors will not reflect binding to a single receptor species, which frequently complicates the interpretation of binding data.

#### 1.1.2.1 Ligand-dopamine receptor interactions

The dopamine receptors share many features with the other amine-like GPCRs, especially other catecholamine receptors. Specifically, the primary amine of dopamine is envisaged to bind *via* a reinforced hydrogen-electrostatic bond to an aspartate residue in TM III. This aspartate residue is conserved between all amine-like receptors. The hydroxyl groups of dopamine are believed to form a network of hydrogen bonds with three serine residues in TM V (Floresca and Schetz, 2004). Serine or cysteine residues are located in the corresponding positions in adrenergic receptors, and have been shown to be involved in agonist binding also in these receptors. A  $\pi$  system of delocalized electrons (not necessarily aromatic; see Dörfler *et al.*, 2008) is regarded as essential for dopaminergic activity. This ligand  $\pi$  system is thought to interact with a hydrophobic cluster of aromatic residues in TM VI, which is likely to function as an important switch for receptor activation (Deupi and Kobilka, 2007). Synthetic dopaminergic D<sub>2</sub>-like ligands frequently feature hydrophobic substitutions, such as propyl chains, at the primary amine, which serve to increase receptor binding affinity. The existence of a

“propyl pocket” between TM III and TM VII, accommodating these substitutions, has been postulated (Malmberg *et al.*, 1994). Most clinically used dopamine agonist and antagonist show less than a 100-fold selectivity for one D<sub>2</sub>-like receptor subtype of another, and the contribution of individual subtypes to therapeutic response remains controversial (Löber *et al.*, 2011). While ligands reasonably (~150-fold) selective for the D<sub>3</sub> receptor over D<sub>2</sub> are just reaching clinical trials (e.g., for the treatment of drug addiction), D<sub>4</sub>-selective agonists and antagonists have been around for over a decade (Enguehard-Gueiffier and Gueiffier, 2006; Löber *et al.*, 2011). Whereas the initial hope that D<sub>4</sub>-selective antagonists would prove efficacious antipsychotics (due to the relative selectivity of the antipsychotic clozapine for D<sub>4</sub> over D<sub>2</sub>) turned into disappointment, it seems at present that proerectile D<sub>4</sub> agonists might have potential to reach the clinic (Löber *et al.*, 2011).

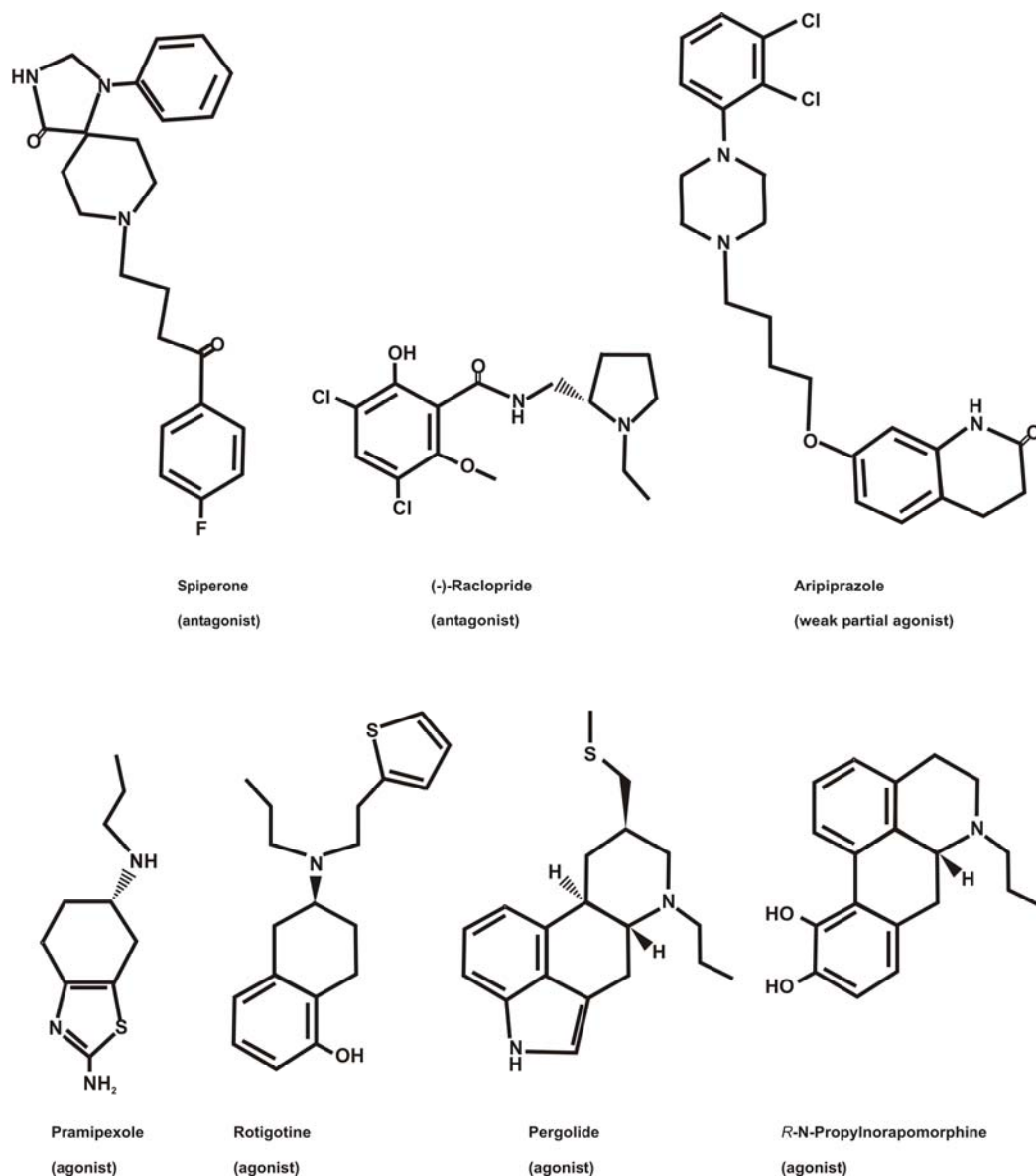
In competition binding experiments, both D<sub>2</sub> and D<sub>4</sub> receptors display high- and low-affinity agonist binding sites which are converted into monophasic displacement curves when guanine nucleotides are included in the binding buffer. However, when the corresponding experiments are carried out on dopamine D<sub>3</sub> receptors, the difference



between high- and low-affinity components often cannot be resolved, and when observed, is reported to be unusually low. Furthermore, most studies describe D<sub>3</sub> agonist binding as insensitive, or very weakly affected by, guanine nucleotides (Filteau *et al.*, 1999). In agreement, agonist binding at this receptor has been reported to be insensitive to the presence or absence of G protein (Vanhauwe *et al.*, 2000).

**Figure 1.**

Structural elements regarded as critical to agonist binding and efficacy at the dopamine D<sub>2S</sub> receptor: Asp114; conserved aspartate in TM III which binds the protonated amine of dopamine ligands *via* a reinforced electrostatic bond. Ser 193, 194, 197; conserved serines thought to form hydrogen bonding interactions with the hydrogen bond acceptors (such as hydroxyl groups) on the aromatic rings of agonist ligands. These interactions help position the ligand for interaction with the aromatic cluster in TM VI. Phe 361; phenylalanine in the aromatic cluster, important for binding to the catechol ring of dopamine agonists by a pi-bonding interaction. Tight interaction of an agonist with the aromatic cluster is believed to trigger one of the “switches” for receptor activation (Deupi and Kobilka, 2007). Adapted from Paper III.



**Figure 2.**

Ligands showing selectivity for D<sub>2</sub>-like receptors, and commonly used in *in vitro* pharmacology experiments.

## 1.2 G-PROTEIN-COUPLED INWARDLY RECTIFYING POTASSIUM CHANNELS

GIRK channels (also known as Kir3) are composed of four subunits, which can be either of four subtypes; GIRK1-4. GIRK1-3 expression is high in neuronal tissue, whilst GIRK4 is found mainly in the heart and in a subset of central neurons. GIRK1 subunits occur both in the heart and the nervous system, forming heteromers with GIRK2, GIRK3 and GIRK4. GIRK1 cannot form functional channels on its own, but must assemble with the other subunits (see Mark and Herlitze, 2000, for a thorough review on GIRK channels). Each subunit also consists of two transmembrane domains, flanking the intramembrane pore region, which contains the glycine-tyrosine-glycine signature characteristic of potassium channels; this stretch of amino acids forms the narrowest part of the channel, called the selectivity filter, as it only lets potassium ions through.

The inward rectification, meaning that the GIRK channels predominantly pass current flowing into the cell, is caused by intracellular cations, notably  $Mg^{2+}$  and the polyamines spermine, spermidine and putrescine, which block the ion conducting pore at positive voltages. When the membrane potential is stepped from depolarized to hyperpolarized potentials, the GIRK current increases in a biphasic manner; a large, instantaneous current increase, which is believed to result from the unbinding of  $Mg^{2+}$  from the channel pore, is followed by a smaller and slower increase in current, which has been found to reflect the unbinding of polyamines (Lancaster *et al.*, 2000).

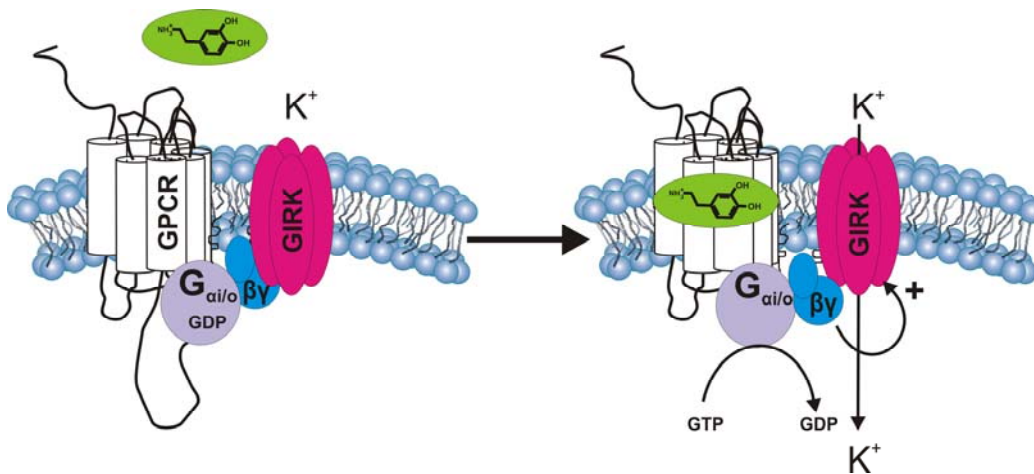
Thus, the voltage dependence of the conductance of these channels is not caused by a gating mechanism intrinsic to the channel protein itself, as is the case for example with members of the Kv family of voltage-gated potassium channels. However, the voltage dependence is also affected by the extracellular  $K^+$  concentration, in a way such that increasing the extracellular potassium concentration will shift the voltage dependence of the channel in the positive direction. This shift is not simply a reflection of the shift in potassium reversal potential, as alterations of the intracellular potassium concentration do not appear to affect channel voltage dependence. Rather, evidence suggests that extracellular  $K^+$  favours channel opening by preventing collapse of the pore region (Claydon *et al.*, 2004; For detailed references on GIRK channel kinetics, see Hille, 2001.)

GIRK channels are gated by binding to the  $G_{\beta\gamma}$  subunits of trimeric G proteins (Logothetis *et al.*, 1987; Fig. 2). The  $G_{\beta\gamma}$  subunits are believed to bind to the N- and C-termini of the subunit peptide chains, binding of separate  $G_{\beta\gamma}$  subunits to each of all four channel subunits being necessary for channel opening. Although the  $G_{\beta\gamma}$  subunits are the ones activating the channel, only G proteins in which the  $G_{\alpha}$  subunit is of the PTX-sensitive  $G_{i/o}$  class couple efficiently to GIRK channels (Leaney *et al.*, 2000). This is most likely because receptors, G proteins and effectors (in this case, GIRK channels) are arranged in close spatial proximity at the membrane, and the  $G_{\alpha}$  subunit mediates at least part of the binding specificity in the formation of such signalling complexes (Lavigne *et al.*, 2002). Thus, only those GPCRs coupled to  $G_{i/o}$  proteins can efficiently exert their actions *via* GIRK channels.

When expressed in *Xenopus* oocytes, GIRK channels exhibit basal activity in the absence of activated receptors. This activity is believed to stem from channel binding of  $G_{\beta\gamma}$  subunits, released by low-level spontaneous cycling of G proteins in the oocytes. This notion is supported by the observation that exogenous expression of  $G_{\alpha}$  subunits greatly reduces the amplitude of the basal GIRK current, and also causes a corresponding increase in agonist-evoked current, when a  $G_{i/o}$ -coupled receptor is coexpressed. Conversely, increasing the expression of  $G_{\beta\gamma}$  subunits increases the basal current while diminishing the amplitude of the current response to receptor activation. (see Mark and Herlitze, 2000)

Electrophysiological measurement of GIRK channel currents or fluorescence-based measurements of G protein conformational change in response to GPCR activation (see below) offer a means of studying the time course of G protein activation with high temporal resolution, as opposed to, e.g., biochemical assays measuring the production

of cAMP by adenylate cyclase or GTP $\gamma$ S binding (Doupnik *et al.*, 2004; Bünemann *et al.*, 2003). Measurements on the finer time scale afforded by the present technique are of advantage, since the rates of G protein activation and deactivation following GPCR activation are very likely physiologically important (Rahman *et al.* 2003). These rates can be regulated by, for example, RGS proteins (regulators of G protein signalling) which accelerate the rate of GTP hydrolysis by G $\alpha$  subunits (i.e., the RGS proteins are GTPase activating proteins) and, although not studied here, form part of many GPCR-G protein-effector signalling complexes *in vivo* (see Doupnik *et al.*, 2004). Furthermore, the G protein/GIRK channel population kinetics can give information about ligand binding kinetics - e.g.; the population rate of GIRK deactivation upon agonist removal has been found to reflect the rate of agonist unbinding from the GPCR (Benians *et al.*, 2003).



**Figure 3.**  
GIRK activation by G $_{i/o}$ -coupled GPCRs.

### 1.3 GPCR VOLTAGE SENSITIVITY

Whereas GPCRs have not traditionally been regarded as sensitive to membrane potential, increasing evidence to this effect has accumulated in recent years (see Mahaut-Smith *et al.*, 2008; Parnas and Parnas, 2010). Decreases in agonist potency and binding at G $_{i/o}$ -coupled M $_2$  muscarinic receptor and glutamate mGluR $_3$  upon membrane depolarization were shown by electrophysiological assays in *Xenopus* oocytes, using GIRK opening as readout of receptor activity, as well as by radiolabeled agonist binding experiments performed on intact oocytes (Ben-Chaim *et al.*, 2003; Ohana *et al.*, 2006). Conversely, for G $_q$ -coupled M $_1$  muscarinic receptor and mGluR $_1$  depolarization caused an increase in agonist potency and binding (Ben-Chaim *et al.* 2006, Ohana *et al.*, 2006). In other studies of lysophosphatidic acid receptors expressed in oocytes, as well as P2Y $_1$  and other G $_q$ -coupled receptors in rat megakaryocytes, increases in agonist potency were observed upon depolarization (Martinez-Pinna *et al.*, 2010; Gurung *et al.*, 2008).

As pointed out by Bolton and Zholos (2003), these observations suggest that GPCRs with opposing effects on membrane excitability (e.g., G $_{i/o}$ -coupled receptors activating

GIRK channels and inhibiting P/Q- and N-type calcium channels, G<sub>q</sub>-coupled receptors closing a number of different potassium channels *via* phosphatidylinositol 4,5-bisphosphate [PIP<sub>2</sub>] hydrolysis) are affected by membrane potential in opposite ways, so as to stabilize cell excitability; possibly helping to define “up- and down-states”, as has been described for principal cells of the cortex and the striatum. Up- and down-states of principal cells of the striatum and the prefrontal cortex last several hundreds of milliseconds up to seconds, respectively, (Murer *et al.*, 2002; O’Donnell, 2003); enough time for GPCR voltage-dependence to have an impact on downstream effectors, such as ion channels, especially when considering that the kinetics of native GPCR-G protein coupling are accelerated by RGS proteins (see Doupnik *et al.*, 2004).

Ben-Chaim *et al.* (2006) recorded charge movement within the M<sub>2</sub> receptor which correlated with the voltage-dependent shift in binding affinity, and Kupchik *et al.* (2011) as well as Navarro-Polanco *et al.* (2011) further demonstrated that this charge movement is affected by the presence of receptor ligands in a concentration-dependent, ligand-specific manner. These findings suggest that parts of the receptor itself move upon changes of the membrane potential; presumably, one or several charged, voltage-sensing residues move in response to voltage changes, and these movements are relayed to the ligand binding site of the receptor.

Ben-Chaim *et al.* (2003; 2006) and Ohana *et al.* (2006) suggested that depolarization affects the coupling of receptor to G protein, and that the depolarization-induced decreases in agonist binding and potency at G<sub>i/o</sub>-coupled receptors reflects shifts in the fractions of receptors configured in the high- and low-affinity states. E.g., in the case of the M<sub>2</sub> receptor, depolarization was envisaged to uncouple a fraction of the receptor pool from G<sub>i/o</sub> protein, thereby shifting this fraction from high to low agonist affinity. In support of this hypothesis, Ben-Chaim *et al.* (2006) presented data showing that depolarization had no effect on the binding of radiolabeled acetylcholine to M<sub>2</sub> receptor-expressing oocytes treated with PTX.

An increase in the fraction of high-affinity binding of D<sub>2</sub> receptors has been consistently reported in animal models of psychosis (Seeman *et al.*, 2006). The putative voltage-sensitivity of the dopamine D<sub>2</sub> receptor could provide a link between the observed alterations in receptor affinity and functional disturbances in basal ganglia synaptic plasticity and neuron firing patterns. Furthermore, comparison between the voltage-sensitivities of different pairs of receptors and ligands might provide insight into the mechanistic basis of GPCR voltage sensitivity, and of receptor affinity states in general. Thus, we were interested in determining the potential impact of membrane voltage on the pharmacology of dopamine D<sub>2</sub>-like receptors.

## **2 AIMS OF THE THESIS**

The general aim of the present work has been to investigate the putative impact of transmembrane voltage on the pharmacology of dopamine D<sub>2</sub>-like receptors. The initial goal was:

**To determine whether agonist potency at the dopamine D<sub>2L</sub> receptor is affected by transmembrane voltage**

As work progressed, further specific aims were defined:

**To compare the voltage sensitivities of dopamine potency at the different dopamine D<sub>2</sub>-like receptors**

**To compare the effects of voltage on the potencies of different agonists at the dopamine D<sub>2S</sub> receptor**

**To explore the ligand-receptor interactions underlying agonist-specific effects of voltage on dopamine D<sub>2S</sub> receptor pharmacology**



### 3 METHODOLOGICAL CONSIDERATIONS

Here, some basic principles and practical considerations regarding the experimental methods are presented. For details on the specific experimental protocols used, please refer to the methodological sections of the papers.

#### 3.1 MOLECULAR BIOLOGY

DNA encoding the relevant receptors and channel subunits was obtained in plasmids suitable for *in vitro* transcription of RNA. The plasmids were linearized 3' to the coding sequence of the insert using restriction enzymes of the appropriate specificity. RNA was subsequently transcribed *in vitro* from the linearized plasmids using T7 or SP6 RNA polymerase kits (Ambion, Austin, TX, USA). Some of the receptor constructs used were cloned into the pXOOM vector. This dual-purpose vector contains both a cytomegalovirus (CMV) promoter that enables mammalian expression as well as untranslated regions from the *Xenopus*  $\beta$ -globin gene, which are located downstream the T7 promoter and 5' and 3' to the coding sequence. These untranslated regions are thus included in RNA transcribed *in vitro* from this plasmid, and often increase expression of the RNA in oocytes as they bind to proteins which prevent RNA degradation and enhance the efficiency of its translation by ribosomes (Jespersen *et al.*, 2002). This vector was a gift from Dr. Søren-Peter Olesen, University of Copenhagen, Denmark.

#### 3.2 THE *XENOPUS* OOCYTE EXPRESSION SYSTEM

*Xenopus* oocytes are obtained from the African clawed toad, *Xenopus laevis*. The oocyte expression system has long been a popular system for electrophysiological testing of drug candidates in the pharmaceutical industry, as well as being used in several other types of electrophysiological and molecular biological investigations in the academic setting. Advantages of using *Xenopus* oocytes for heterologous expression of ion channels include their capacity for synthesizing large amounts of protein, their huge size (1mm diameter) enabling microinjection of individual oocytes with mRNA, assuring expression in virtually every cell, and their relatively low expression of endogenous ion channels. Potential drawbacks of the *Xenopus* system include the fact that it is a non-mammalian expression system; for example, protein glycosylation and intracellular trafficking might differ from mammalian cells. For an extensive review on the use of *Xenopus* oocytes in ion channel research, see Dascal (1987).

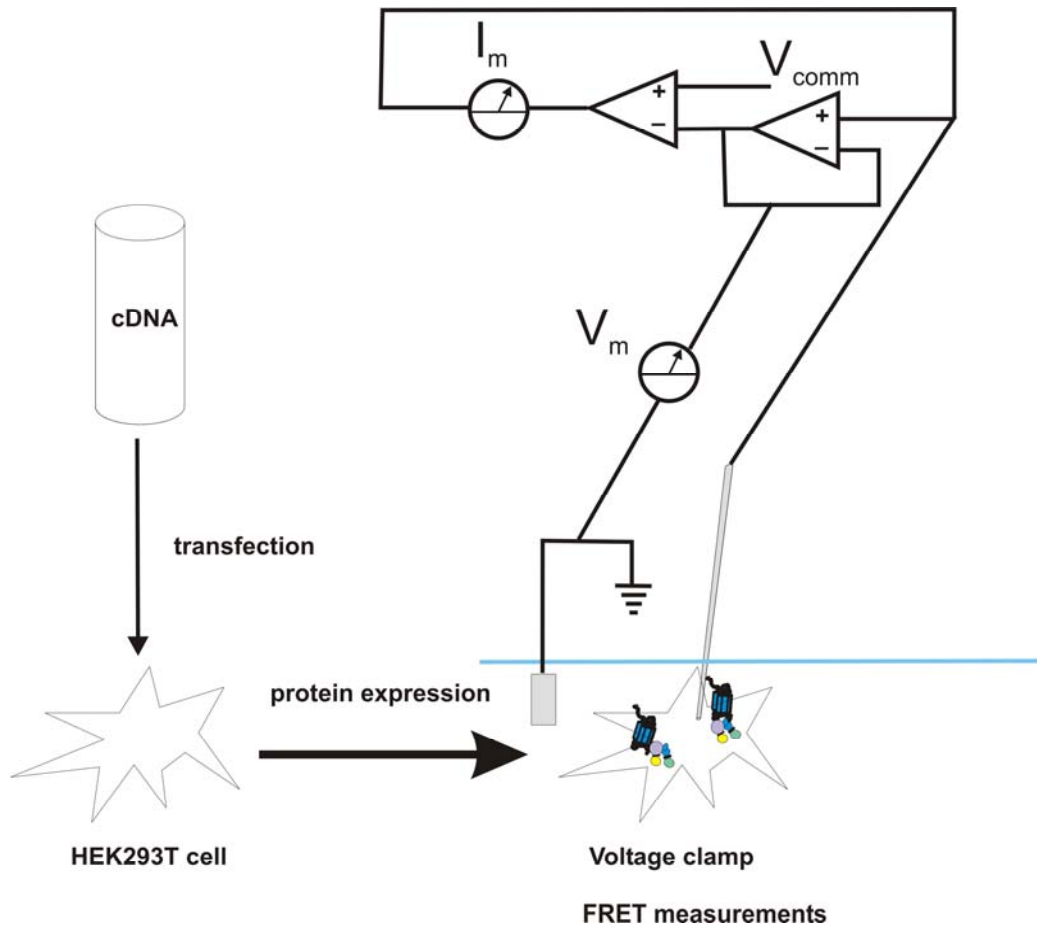
#### 3.3 ELECTROPHYSIOLOGY

Electrophysiology is the measurement of electrical signals from a biological specimen. In the present context, we will consider electrophysiology experiments performed on single cells. Electrical contact with the cells under study is usually achieved using micropipettes, made from glass capillaries by simultaneous heating and pulling on the glass. The micropipette contains a conductive salt solution, which is in contact with an electrode, typically a silver-silver chloride electrode.

Voltage-clamp electrophysiology provides a measure of the ionic current flow across a biological membrane at a given membrane potential. This is accomplished by a negative feedback amplifier, which continuously measures the membrane potential (i.e., the potential inside the cell minus the potential in the extracellular solution) and compares it to a desired command voltage. As soon as the measured potential deviates ever so slightly from the command voltage, current will be injected by the feedback circuitry so as to bring the measured voltage back to the value of the command voltage. During optimal conditions, any charge transfer across the membrane (which would tend to alter the membrane potential if left unopposed) will be compensated for by an opposing charge transfer induced by the feedback amplifier. Thus, the current injected to maintain the membrane voltage at its desired value is a measure of the current flowing across the membrane at that voltage, and can thus be used to study the opening and closing of ion channels.

### **3.3.1 Whole-cell voltage clamp using the patch-clamp technique**

When using the patch-clamp technique in the whole-cell configuration, which is a popular mode of electrophysiological recording from small mammalian cells, a glass microelectrode is advanced toward the cell until it is just above or slightly touches the membrane. At this point, negative pressure is carefully applied to the pipette (typically by pulling on a syringe attached to the electrode holder, the interior of which is continuous with the micropipette). When performed correctly, this maneuver will cause the cell membrane to adhere tightly to pipette, forming what is known as a “gigaohm seal”; referring to the high electrical resistance between the pipette and the membrane. Next, stronger negative pressure is applied to the pipette, causing the membrane patch just under pipette opening to rupture. The interior of the cell is now continuous with the interior of the pipette, and all currents passing through the cell membrane can (under ideal conditions) be measured by clamping the cell membrane voltage, as just described. In a typical experiment, the pipette solution will rapidly equilibrate with the intracellular solution, a fact which should be considered when preparing the pipette solution. The equilibration of pipette and intracellular solutions can be useful in case one wants to modify the internal milieu of the cell; e.g. by delivering pharmacological agents.

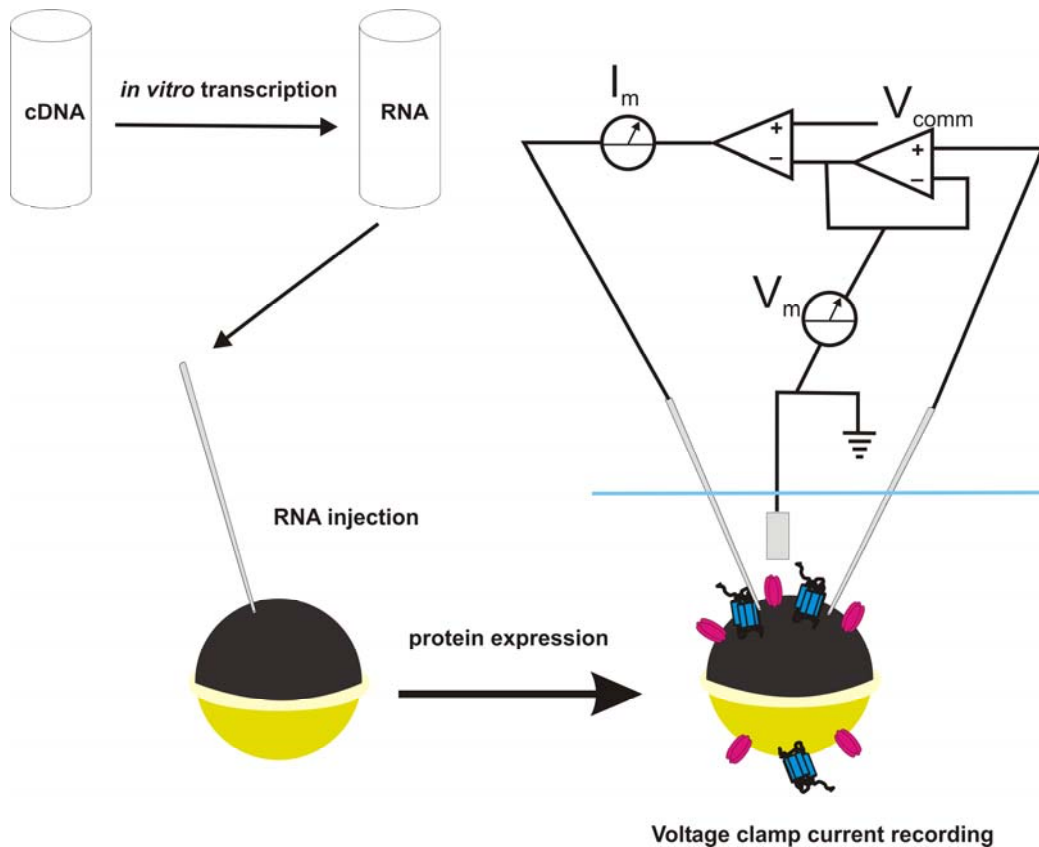


**Figure 4.**  
Schematic illustration of the patch clamp technique, as explained in the main text, above.

### 3.3.2 Whole-cell voltage clamp using the two-electrode technique

The large size and efficient protein expression of the oocyte also means that the currents recorded from these cells will frequently be very large –usually on the order of  $\mu\text{As}$ . Injection of such big currents through a high-resistance pipette would lead to a large voltage drop over the pipette, leading to a falsely low estimate of the membrane potential, if the current-passing electrode was to be used also as a voltage-sensing electrode. The two-electrode system overcomes this potential problem, by (as the name implies) using two separate electrodes for current injection and voltage-sensing. Furthermore, to lower the resistance of the pipettes, thus easing the injection of large currents by the amplifier, saturated salt solutions (e.g., 3 M KCl), are commonly used in the pipettes instead of physiological intracellular solutions. As opposed to patch-clamp experiment, in two-electrode experiments the microelectrodes are pushed through the cell membrane, rather than forming a gigaohm seal before gaining access to the cell interior. This is possible because the vitelline layer, a thin sheet of protein enveloping the oocytes, and the large size of the oocyte provide mechanical stability, preventing the cell from collapsing when the electrodes are inserted.

Two-electrode experiments are easier to perform than patch-clamping, but do not allow for control of the internal environment of the cell, as does whole-cell patch-clamping of mammalian cells, in which the pipette solution equilibrates with the solutes in the cell cytoplasm. In fact, in two-electrode experiments, influx of pipette solution into the oocyte interior is to be avoided, because of the non-physiological nature of the saturated salt solution. In the present work, influx of pipette solution was prevented by filling the pipette tips with a molten agarose-KCl gel, which was allowed to solidify before backfilling the pipette with 3 M KCl solution, as originally described by Schreibmayer *et al.* (1994). Also, the large capacitance of the oocyte membrane means that it is slower to clamp compared to that of a small cell; i.e., the kinetics of fast currents such as those passed by voltage-gated sodium channels cannot be accurately resolved. However, for the present purposes this is not a problem, given the relatively slow kinetics of GPCR-induced GIRK channel activation. Finally, the vitelline layer has been shown to impede the diffusion of some pharmaceutical compounds, slowing the rate of onset of their action and skewing their concentrations of action toward higher values (Madeja *et al.*, 1997). The large size of oocytes also increases the time required for a drug to reach all areas of the cell membrane, which might give a “false” slowing of response kinetics of agonist-activated channels under certain circumstances (Doupnik *et al.*, 2004).



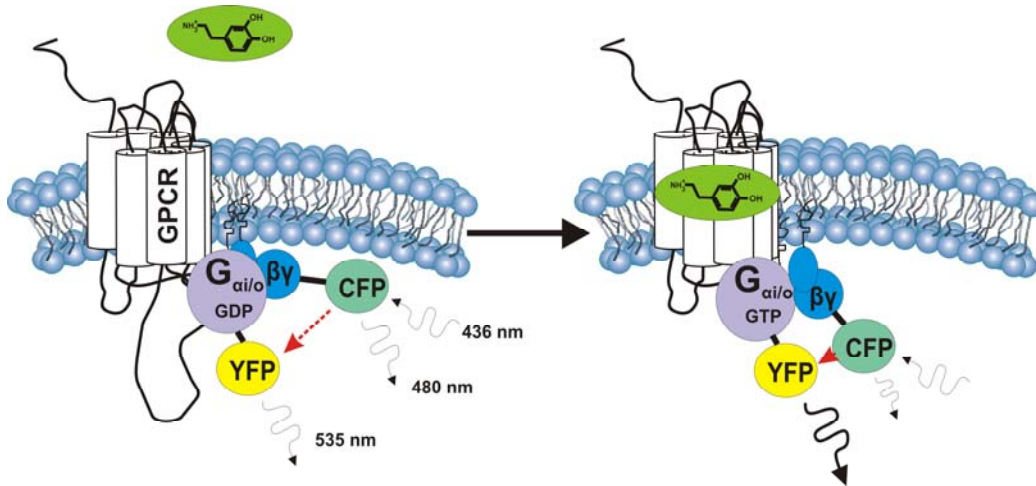
**Figure 5.**

Principles of the *Xenopus* oocyte expression system and two-electrode voltage clamp, as explained in the main text, above.

### 3.4 FRET ASSAY PRINCIPLES

Förster Resonance Energy Transfer (FRET), also known as Fluorescence Resonance Energy Transfer, is the radiationless (i.e., without photon emission) transfer of energy from the excited electronic system of one dye molecule (for present purposes; a fluorophore) to the electronic system of another dye molecule in close proximity (see Lohse *et al.*, 2008). The efficiency of this energy transfer is dependent both on the extent of overlap between the emission spectrum of the first dye and the excitation spectrum of the second, as well as on the distance between the two molecules involved; The efficiency of energy transfer decreases with the sixth power of the distance between the molecules, enabling the use of FRET-based assays to report on inter- and intramolecular motion on the scale of a few nm. Two genetically encoded fluorophores forming a suitable FRET pair are Cyan fluorescent protein (CFP) and Yellow fluorescent protein (YFP).

In the present experiments, an immortalized mammalian cell line (HEK 293T) was transiently transfected with the constructs of interest (the human dopamine D<sub>2</sub> receptor, G<sub>αi1</sub>-YFP, CFP-G<sub>β1</sub>, G<sub>γ2</sub>) and grown on coverslips for 3-5 days prior to experiments. During an experiment, a healthy-looking cell is selected on the basis of its membrane-associated expression of the fluorescent constructs (as judged using a fluorescence microscope; Zeiss Axio Observer). The CFP tag is excited at 436 nm, upon which it emits light with an intensity maximum at 480 nm. The excitation light is generated using a monochromator (Polychrome V, Till Photonics). In the monochromator, white light from a Xenon lamp is split into its component wavelengths using a diffraction grating. As just described, when the two fluorophores are sufficiently close together, part of the energy absorbed by CFP is donated to YFP (whose excitation maximum is about 505 nm), which then emits at 535 nm. Since the amount of energy transferred between two fluorophores is dependent on their distance, conformational change in the G protein trimer upon its activation by the dopamine D<sub>2S</sub> receptor can be studied by recording the emission ratios of CFP and YFP (i.e., the ratios of intensities at 480 and 535 nm; Bünemann *et al.*, 2003). In the experimental setup used in the present experiments, the emitted light is separated into CFP and YFP channels using a dichroic mirror, and subsequently measured using photodiodes connected to an amplifier (Till Photonics; see Bünemann *et al.*, 2003). By selecting which part of the field of view is transmitted to the photodiodes, FRET data can be collected from single cells expressing the fluorescent constructs. The equipment used in the present experiments also minimizes the extent of photobleaching; during prolonged excitation, fluorophores tend to undergo chemical decomposition due to oxidative processes during repeated excitation and emission. By using a galvanometer mirror, the monochromator is able to turn the light delivered to the specimen on and off at high frequencies, thus providing intermittent excitation of the fluorophores. This reduces the photobleaching of the fluorophores over time, as compared to continuous excitation.



**Figure 6.**

Schematic showing the principles of the FRET assay of G protein activation, as explained in the main text, above.

### 3.5 RADIOLIGAND BINDING EXPERIMENTS

Radioligand binding assays are based on the principle of using radioactively labelled molecules with affinity for a receptor of interest to investigate the distribution, density, or ligand binding properties of that receptor. In the present experiments, the binding of [<sup>3</sup>H]-labelled dopamine to *Xenopus* oocytes coexpressing dopamine D<sub>2S</sub> receptors with GIRK channels was investigated under hyperpolarizing and depolarizing conditions. The oocytes were incubated for 30 s with 40 nM or 80 nM of [<sup>3</sup>H]dopamine, in a physiological buffer solution or in a buffer solution in which Na<sup>+</sup> had been replaced with K<sup>+</sup>. In GIRK-expressing oocytes, the resting membrane potential is determined largely by the reversal potential for K<sup>+</sup>, and thus elevating extracellular K<sup>+</sup> to near the intracellular concentration brings the membrane potential up from about -80 mV in physiological solution to about 0 mV (Ben-Chaim *et al.*, 2003). Following incubation with radioligand, the oocytes were washed for 1 s, again in physiological or high- K<sup>+</sup> buffer, and the bound radioactivity was subsequently measured using a scintillation counter. Non-specific binding was determined by measuring [<sup>3</sup>H]dopamine binding to oocytes expressing GIRK channels without D<sub>2S</sub> receptors, and specific binding was determined by subtracting the average non-specific binding from the total binding in each oocyte coexpressing D<sub>2S</sub> receptors with GIRK channels.

## 4 RESULTS AND DISCUSSION

### Paper I:

The putative voltage sensitivity of the dopamine D<sub>2L</sub> receptor was examined by determining the potencies of dopamine and the widely used D<sub>2</sub>-like agonist quinpirole in the GIRK activation assay described under “Methodological considerations”. It was found that the potencies of both dopamine and quinpirole to activate GIRK channels is reduced by about tenfold when the membrane is held at +40 mV, as compared to -80 mV. It was also found that this reduction in potency was not an artifactual result of altered current-voltage relationships of the GIRK channel. Since G protein activation and G<sub>βγ</sub>-induced GIRK channel activation have been shown to be voltage insensitive processes (Ben-Chaim *et al.*, 2003), it was concluded that the voltage-sensing mechanism behind the observed potency shift is likely to reside within the dopamine D<sub>2L</sub> receptor.

### Paper II:

The potencies of dopamine to activate GIRK channels *via* dopamine D<sub>2S</sub>, D<sub>3</sub>, and D<sub>4.4</sub> receptors at 0 mV and at -80 mV were studied. As opposed to the previous study, 0 mV was used as the depolarized potential in most experiments, since it was easier to obtain stable recordings at this potential than at +40 mV. Furthermore, using 0 mV rather than +40 mV prevents the opening of an endogenous, slowly activating Na<sup>+</sup> conductance (Baud and Kado, 1984), which in some oocytes is large enough to considerably contaminate GIRK current measurements. It was found that the D<sub>2S</sub> receptor was similarly voltage sensitive as the D<sub>2L</sub> receptor isoform (here, depolarization to +40 mV was used as well, to allow comparison). The potency shift between -80 mV and 0 mV was about 4.5-fold for the D<sub>2S</sub> receptor, whereas the dopamine D<sub>3</sub> did not show any significant potency shift between these two potentials. Considering the proposed link between G protein coupling and voltage-sensitive potency (Ben-Chaim *et al.*, 2003) and the previous observation of G protein-independent high-affinity agonist binding at the dopamine D<sub>3</sub> receptor (Vanhouwe *et al.*, 2000), we also investigated a chimeric D<sub>3</sub>/D<sub>2</sub> receptor, which had previously been shown to display G protein-sensitive dopamine binding (Filteau *et al.*, 1999). However, this chimera did not show any significant depolarization-induced shift. Finally, the dopamine D<sub>4.4</sub> receptor was shown to display a significant loss of dopamine potency with depolarization, which was about 2-fold; smaller than that of the D<sub>2S</sub> receptor. This study thus demonstrated differences in voltage-sensitivity between D<sub>2</sub>-like receptors, but was unable to demonstrate a clear link between G protein-sensitivity of agonist binding and sensitivity to voltage.

### **Paper III:**

The voltage sensitivities of a range of structurally related agonists at the dopamine D<sub>2S</sub> receptor were investigated. It was found that neither the potencies nor the efficacies of phenethylamine agonists lacking the two hydroxyls of dopamine were significantly altered by depolarization from -80 mV to 0 mV. However, agonists of the dipropyl-aminotetralin (DPAT) series were similarly voltage sensitive, regardless of hydroxylation. Differential engagement of G protein subtypes did not underlie the agonist-specific differences in voltage sensitivity, since both voltage-sensitive and insensitive behaviour (by dopamine and *p*-tyramine, respectively) was observed when PTX was used to constrain D<sub>2</sub> receptor signalling so as to occur exclusively *via* exogenous PTX-insensitive G<sub>αo1</sub> proteins. Thus, it appears that specific agonist-receptor interactions determine the effect of voltage on agonist potency, rather than a general decoupling of G protein, as suggested by Ben-Chaim *et al.* (2003; 2006), and later by Kupchik *et al.*, (2011).

### **Paper IV:**

The agonist-specific effects described in Paper III were examined in more detail by using site-directed mutagenesis of the D<sub>2S</sub> receptor ligand binding pocket, as well as a wider selection of agonists as compared to Paper III.

It was also established that the depolarization-induced decrease of dopamine potency is reflected in decreased binding of radiolabeled dopamine, thus supporting the notion that the reduced functional potency of dopamine at depolarized voltages reflects lower binding affinity. Furthermore, voltage sensitive dopamine potency was observed in a mammalian cell line using a FRET-based G protein activation assay, demonstrating that D<sub>2S</sub> receptor voltage sensitivity is not dependent on GIRK coexpression, nor is it only observed when the receptor is expressed in *Xenopus* oocytes.

Site-directed mutagenesis data supported the hypothesis from Paper III that interactions between agonist hydroxyls and the conserved TM V serine residues of the D<sub>2S</sub> receptor are important for voltage sensitivity of phenethylamine agonists. Specifically, mutation of serine 193 to alanine (S193A) drastically decreased the voltage sensitivity of dopamine potency; only a 1.5-fold shift was observed, whereas S194A did not have a pronounced effect in this regard. Voltage sensitivity of dopamine at the S197A mutant could not be assessed, since dopamine failed to evoke quantifiable GIRK currents *via* this mutant. The unhydroxylated agonist DPAT showed depolarization-induced shifts of between 2.3–fold to 3.5–fold at all three mutant receptors (at the *wt* receptor, DPAT showed a 3.2-fold shift), suggesting that serine mutation did not grossly perturb receptor voltage-sensitivity.

The agonist structure-function analysis was also extended to include the amine head group frequently present in amine receptor ligands. Removing the ethylamine side chain of dopamine leaves catechol, a compound which has been shown to act as a weak partial agonist at the β<sub>2</sub> adrenergic receptor (Swaminath *et al.*, 2005). Catechol was shown to be a partial agonist also at the D<sub>2S</sub> receptor, although it was impossible to obtain complete concentration-response relationships for this molecule because of its



GIRK channel-blocking effects at concentrations above 1 mM. Interestingly, the fractional responses for catechol (relative to a maximally effective concentration of dopamine) was higher at 0 mV than at -80 mV, suggesting that the potency or efficacy of catechol was increased by depolarization. Reciprocally, mutating the conserved aspartate residue, D114, to alanine (D114A), resulted in a receptor at which dopamine potency was increased at 0 mV, relative to -80 mV.

Finally, a number of therapeutic agonists were investigated; three ergoline compounds (bromocriptine, lisuride, and pergolide), two aporphines (apomorphine and N-propyl-norapomorphine) and four unrelated compounds (piribedil, pramipexole, ropinirole, and rotigotine). Whereas the aporphines showed no significant voltage-induced shifts in potency nor efficacy, the ergolines all showed a significant decrease in efficacy, and about 2-fold shifts in potency (significant for pergolide and bromocriptine) at 0 mV vs. -80 mV. Decreased efficacy was observed also for piribedil and rotigotine, and decreased potency for piribedil, pramipexol, and ropinirole.

This study corroborated our earlier findings regarding agonist-specific voltage sensitivity, and strengthened our hypothesis that agonist-receptor interactions determine the effect of the D<sub>2S</sub> receptor's voltage sensitivity on agonist potency and efficacy. Furthermore, the finding that highly affine and efficacious dopamine agonists differ widely in terms of how they are affected by voltage points to the possibility of using differentially voltage-modulated agonists to investigate the relevance of dopamine D<sub>2</sub> receptor voltage sensitivity in native tissue.

## 5 CONCLUSIONS

The present work provided the first reports on voltage sensitivity of the dopamine D<sub>2</sub>-like receptors, showing that both D<sub>2</sub> isoforms, D<sub>4</sub>, but not D<sub>3</sub>, are regulated by membrane voltage (Paper I and II). Furthermore, for the first time, it was demonstrated that different agonists acting at the same receptor can be affected by the receptor's voltage sensitivity in qualitatively different ways (Paper III and IV). For example, while potency and efficacy of the antiparkinsonian agonist piribedil are diminished by depolarization, neither parameter is affected in the case of apomorphine (another antiparkinsonian), and the fractional responses to the "agonist fragment", catechol, were greater at depolarized potentials. Moreover, it was found that the diverse effects of voltage on agonist potency correlated with certain agonist-receptor interactions (Paper IV). These results contribute evidence that the effects of voltage on agonist potency are indeed brought about by voltage-induced changes in agonist-receptor interactions.

Together with recent findings by Kupchik *et al.* (2011) and Navarro-Polanco *et al.* (2011) that agonists affect GPCR gating charge movements, the present evidence supports the notion that a (yet-to-be-identified) voltage sensor within a voltage sensitive GPCR moves within the transmembrane voltage field, which in turn causes allosteric changes in the agonist binding pocket, as suggested by Ben-Chaim *et al.* (2003; 2006). For the G<sub>i/o</sub>-coupled receptors investigated so far, these changes seem to decrease the binding affinity for the native GPCR agonist (with the exception of the D<sub>3</sub> receptor, at which dopamine potency was unchanged upon depolarization). Since many G<sub>i/o</sub>-coupled receptors are autoreceptors, these findings are in line with the suggested role for GPCR voltage sensitivity in the regulation of neurotransmitter release kinetics (see Parnas and Parnas, 2010). Whilst being in overall agreement with the pioneering work of the Parnas group, the present data argue against the idea that voltage changes the overall degree of G protein coupling of a voltage sensitive GPCR, since such a mechanism would be expected to produce similar effects on all agonists at the receptor in question. Rather, it appears that the equilibrium between certain agonist-bound receptor conformations is shifted by membrane potential changes, such that, at a given GPCR, voltage has distinct effects on the affinities and efficacies of different ligands.

The results presented in paper IV further suggest that the degree of voltage sensitivity of a receptor-ligand interaction is not simply related to its affinity; both some of the largest and some of the smallest (non-significant) potency shifts are observed with agonists which show EC<sub>50</sub>s in the nanomolar range, and which are reported to show correspondingly high affinities in radioligand binding studies (see Seeman, 2007). The present findings that dopamine D<sub>2</sub> and D<sub>4</sub> receptors are regulated by membrane voltage might have important implications for the function of these receptors in regulating transmitter release and in dopamine-mediated synaptic plasticity.

## 6 FUTURE DIRECTIONS

The present results demonstrate the effect of voltage on dopamine D<sub>2</sub> receptor pharmacology at the ligand binding, G protein, and effector levels. However, all of these studies were carried out in heterologous expression systems, which might not faithfully reproduce all aspects of the *in vivo* situation, e.g., in terms of receptor-interacting proteins. Thus, future studies should examine the relevance of dopamine D<sub>2</sub>-like receptor voltage sensitivity under more physiological conditions; e.g., in acute *ex vivo* preparations.

Another crucial aspect deserving future attention concerns the temporal aspects of depolarization. The voltage-sensitive GPCR regulation of transmitter release described above occurs at a sub-millisecond time scale, and has been suggested to proceed *via* direct interaction between the receptor and vesicle-associated proteins (Kupchik *et al.*, 2008). Thus, this process is fast enough to be affected by a single action potential. However, in order for voltage to impact G protein activation, altered requires several hundreds of milliseconds. However, since depolarization has been proposed to impact ligand binding affinity on a sub-millisecond time scale (Kupchik *et al.*, 2011), and the process from agonist binding to GPCR and G protein activation has been shown to be slower by about two and three orders of magnitude, respectively (Vilardaga, 2011), it is conceivable that also intermittent depolarizations, applied over a few seconds (as during a train of action potentials) might significantly impact the level of G protein activity induced by voltage sensitive GPCRs.

## 7 ACKNOWLEDGEMENTS

Thanks to:

My supervisor, Peter Århem, for giving me the freedom to choose my own way.

Cosupervisors Kjell Fuxe, Daniel Marcellino and Johanna Nilsson for your support and encouragement during my work. Special thanks to Daniel Marcellino for mentorship in molecular biology and cell culture techniques.

Past and present members of the Århem and Fuxe labs; Hugo Zeberg, Göran Klement, Dasiel Borroto-Escuela, Malgorzata Frankowska, and Wilber Romero Fernandez, for friendly sharing of offices and lab space.

Dr. Francisco Ciruela, Laia Bahima, Viktor Fernández-Dueñas, Jorge Gandía, Maricel Gómez Soler, Aroa Soriano, and all the others at the University of Barcelona, Campus Bellvitge, Barcelona, Spain, for your hospitality, generosity, and productive collaboration.

Professors Hanna and Itzhak Parnas, Ofra Barchad-Avitzur, and Noa Dekel, at the Hebrew University, Jerusalem, Israel, for collaboration and fruitful discussions.

Dr. Kathleen van Craenenbroeck, University of Ghent, Belgium, and Dr. Gunnar Schulte, Karolinska Institutet, for collaboration and scientific discussions.

Professor Fredrik Elinder, Linköping University, for advice and instructions on technical aspects of oocyte electrophysiology. Past and present members of the Elinder lab; Amir Broomand, Sara Börjesson, Ulrika Englund, Johan Brask, and Nesar Akanda, for comradeship in the lab and during conferences.

Professor Örjan Wrangé, Karolinska Institutet, for invaluable advice concerning *Xenopus laevis* husbandry.

The technical and administrative staff at the Department of Neuroscience and the Neuroscience Course Secretariat, for your kind helpfulness with technical equipment, economy, and teaching duties.

All colleagues and friends with whom I have had the pleasure to share the Retzius building through the years; For friendship, moral support, interesting discussions, and for making this building such a stimulating place to work.

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