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ELECTROPHYSIOLOGY-BASED INVESTIGATIONS OF G PROTEIN- COUPLED RECEPTOR PHARMACOLOGY

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**Karolinska
Institutet**

Stockholm 2020

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Printed by US-AB, 2020

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ISBN 978-91-7831-816-2

ELECTROPHYSIOLOGY-BASED INVESTIGATIONS OF G PROTEIN-COUPLED RECEPTOR PHARMACOLOGY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Public defense at Torsten N Wiesel lecture hall, J3:04, Karolinska University Hospital, Solna
Friday 15th of May at 13.00 p.m.

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ABSTRACT

G protein-coupled receptors (GPCRs) constitute targets for ~34% of approved drugs. The muscarinic acetylcholine M₂ receptor (M₂R) activates G protein-coupled receptor inward rectifying potassium (GIRK) channels in the central nervous system and heart. Membrane potential modulates agonist potency at several GPCRs. However, the mechanism underlying the voltage sensitivity remains debated. A highly conserved aspartate residue (D^{2.50}69) has been proposed to mediate the voltage-sensitivity of the M₂R, although the low expression of D69 mutants has complicated further functional investigations.

Dopamine D₂ and D₃ receptors (D₂R and D₃R) are pre- and postsynaptic inhibitory receptors in the central nervous system, involved in locomotion, cognition and endocrine functions. D₂R antagonists and weak partial agonists are used clinically as antipsychotics but are associated with several side effects. Various strategies have been suggested to reduce the side-effect profile of novel antipsychotic drugs. One such strategy includes the selective targeting of non-canonical signaling pathways, e.g., the β -arrestin pathway, while leaving the classical, G protein pathway, undisturbed. Additionally, binding affinity and kinetics at the D₂R, as well as ligand lipophilicity, have been suggested to be of significance in determining the side-effect liability of antipsychotics.

In the thesis, M₂R, D₂R and D₃R were investigated using two-electrode voltage-clamp in *Xenopus laevis* oocytes co-expressing the respective receptor and GIRK channels. M₂R carrying a charge-neutralizing D69N mutation demonstrated a voltage-dependent shift of agonist-potency, similar to the wild type M₂R. This finding is in line with a recent alternative hypothesis, which implicates three tyrosine residues in the M₂R voltage sensor. The proposed β -arrestin-selective partial D₂R agonist, UNC9994, was found to be a weak partial- and almost full agonist at D₂R and D₃R mediated GIRK activation, respectively. These findings are incongruent with β -arrestin-selectivity and suggest that the promising effects of UNC9994 in animal models of psychosis may be related, at least in part, to involvement of the D₃R. Finally, the partial D₂R agonist positron emission tomography ligand, SV-III-130, demonstrated an insurmountable, yet competitive, binding mechanism at the D₂R. Mutations of residues in a secondary binding pocket, engaging the secondary pharmacophore, abolished the insurmountable binding. Kinetic models incorporating an irreversible, SV-III-130-bound state captured the experimentally observed data. Molecular dynamics simulations suggested that D₂R extracellular linkers participate in an induced-fit binding mechanism.

In summary, the thesis addresses the mechanism of voltage-dependent agonist-potency at GPCRs and contradicts earlier reports of a β -arrestin-selective action of the experimental antipsychotic, UNC9994, at the D₂R. Finally, a two-step induced-fit binding mechanism was demonstrated for the aripiprazole analogue, SV-III-130, at the D₂R. The findings may guide further mechanistic investigations and provide insights for the development of novel diagnostic and therapeutic GPCR ligands.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

- I. **Ågren R**, Sahlholm K, Nilsson J, Århem P. Point mutation of a conserved aspartate, D69, in the muscarinic M2 receptor does not modify voltage-sensitive agonist potency. *Biochem Biophys Res Commun*. 2018 Jan 29;496(1):101-104.
- II. **Ågren R**, Århem P, Nilsson J*, Sahlholm K*. The Beta-Arrestin-Biased Dopamine D2 Receptor Ligand, UNC9994, Is a Partial Agonist at G-Protein-Mediated Potassium Channel Activation. *Int J Neuropsychopharmacol*. 2018 Dec 1;21(12):1102-1108.
- III. **Ågren R**, Zeberg H, Stepniński TM, Reilly SW, Luedtke R, Århem P, Mach RH, Selent J, Nilsson J, Sahlholm K. A ligand with two modes of interaction with the dopamine D2 receptor – An induced-fit mechanism of irreversible binding (manuscript)

* Equal contributions

SCIENTIFIC PAPER NOT INCLUDED IN THE THESIS

Ågren R, Nilsson J, Århem P. Closed and open state dependent block of potassium channels cause opposing effects on excitability - a computational approach. *Sci Rep.* 2019 Jun 3;9(1):8175.

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LIST OF ABBREVIATIONS

5-HT ₁₋₇ R	Serotonin 1-7 receptors
AT ₁ R	Angiotensin II type 1 receptor
cAMP	Cyclic adenosine monophosphate
D ₁₋₅ R	Dopamine D ₁₋₅ receptors
EC ₅₀	Half maximal effective concentration
ECL1-3	Extracellular loops 1-3
E _{max}	Intrinsic activity
EPS	Extrapyramidal symptoms
ERK1	Extracellular signal-related kinase 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GABA _{B1-2} R	Metabotropic γ -aminobutyric acid receptor, subunits 1-2
GAP	Guanosine triphosphatase accelerating proteins
GPCR	G protein-coupled receptor
GIRK	G protein-coupled inward rectifier potassium channel
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GRK2	G protein-coupled receptor kinase 2
GSK3 β	Glycogen synthase kinase 3 β
IC ₅₀	Half maximal inhibitory concentration
IP ₃	Inositol 1,4,5-trisphosphate
K _d	Dissociation constant
K _i	Inhibition constant
k _{obs}	Observed association rate
k _{off}	Dissociation rate constant
k _{on}	Association rate constant
M ₁₋₅ R	Muscarinic acetylcholine M ₁₋₅ receptors
mGlu ₁₋₃ R	Metabotropic glutamate receptors 1-3
MS-222	Tricaine methanesulfonate
NMDA	N-methyl-D-aspartate
μ -OR	μ -Opioid receptor

OBP	Orthosteric binding pocket
PCP	Phencyclidine
PET	Positron emission tomography
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKB	Protein kinase B
PTX	Pertussis toxin
PTX-S1	Catalytic subunit of pertussis toxin
PPHT	2-(N-phenethyl-N-propyl)amino-5-hydroxytetralin
RGS	Regulator of G protein signaling
SBP	Secondary binding pocket
TEVC	Two-electrode voltage-clamp
WT	Wild type

1 INTRODUCTION

1.1 G-PROTEIN COUPLED RECEPTORS

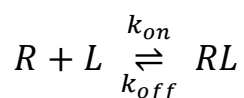
During the 20th century, theories of receptor-ligand interactions were derived from experiments on myocytes and glands. In 1972, the β -adrenergic G-protein coupled receptor (GPCR) was identified and purified by Lefkowitz et al. (1). This was followed by structural determination of the photon-sensitive visual rhodopsin, and later cloning of the β 2 adrenergic receptor (2). GPCRs, consisting of seven transmembrane helices, enable transmission and amplification of extracellular factors to intracellular signals by canonical, i.e., G-protein, and non-canonical, e.g., β -arrestin, pathways. The widespread expression of GPCRs in a multitude of cells and their involvement in disease support pharmacological targeting; currently 34% of all approved drugs target GPCRs (3).

1.2 DIMENSIONS OF RECEPTOR PHARMACOLOGY

Physiological, or pathological, receptor expression *in vivo* determines the target area of a receptor ligand. GPCRs involved in the modulation of neuronal signaling typically span the cell membrane and are located either pre- and/or postsynaptically. The ligand may either target one specific type of receptor (receptor-specific), mainly prefer one type of receptor (receptor-preferring) or target a whole span of different receptors (multireceptor, or “dirty”, targeting) (Fig. 1A). Most clinically used GPCR ligands interact with several receptors. For example, most antipsychotic drugs bind to dopamine, but also to serotonin, α -adrenergic, muscarinic acetylcholine and histaminergic receptors, each interaction associating with different clinical (side-)effects (see e.g. (4)).

Receptors may exist as monomers, homomers or heteromers, and only signal as obligate heteromers (Fig. 1B). This was first demonstrated for the G protein-coupled γ -aminobutyric acid receptor (GABA_BR), which requires co-expression of GABA_{B1}R subunits with GABA_{B2}R subunits to form functional receptors (5). Based on the assumption that heteromers are selectively expressed in regions of interest, ligands targeting specific heteromers may reduce the degree of side-effects (6).

When the ligand is in the vicinity of a targeted receptor, the probability of a binding event occurring is dependent on the ligand concentration (L), the receptor concentration (R), the ligand association rate constant (k_{on}) and the ligand dissociation rate constant (k_{off}),



where RL is the ligand-bound receptor (Fig. 1C). Assuming a one-step binding mechanism, the binding rate constants dictate the dissociation constant (K_d ; k_{off}/k_{on}). Both association and dissociation rate constants are critically dependent on the amino acid residues in the access pathway to the receptor binding site. An example illustrating the role of the first extracellular loop (ECL1) in regulating ligand entry and egress was illustrated by increased association and

dissociation rates of the antipsychotic drug risperidone at the dopamine D₂ receptor (D₂R) following mutation of W100 (7).

Typically, the ligands bind to amino acid residues in one or several receptor cavities, either by a single- or a multistep-process, respectively. An example of the latter is heterobivalent ligand binding (8); illustrated by a ligand composed of two (primary and secondary) pharmacophores, i.e. ligand fragments with affinities to macromolecules, which bind with different affinities to two distinct receptor sites (the primary or orthosteric binding pocket; OBP and the secondary binding pocket; SBP) respectively (Fig. 1D). The endogenous ligand typically binds to the OBP, activating the receptor. Depending on the affinities of the two pharmacophores to the OBP and SBP respectively, two different heterobivalent binding modes are possible; high affinity of the primary pharmacophore to the OBP would allow for an “abII” binding mode, whereas a high affinity of the secondary pharmacophore to the SBP would facilitate an “abI” binding mode (8)(illustrated in Fig. 1D).

Induced-fit binding is another multistep mechanism, where the ligand primary pharmacophore first binds to the OBP causing a conformational rearrangement of the receptor, which allows for subsequent binding of the secondary pharmacophore to the SBP (9)(illustrated in Fig. 1E). Such induced-fit binding may entail a prolonged drug residence time *in vivo* (10).

The receptor-bound ligand may elicit a spectrum of responses depending on its intrinsic activity (E_{max} ; Fig. 1F). An agonist could demonstrate supramaximal, full or partial efficacy; different amplitudes of responses, as compared to the response evoked by a reference full agonist (typically the endogenous ligand). A neutral antagonist has no efficacy and rather competes with agonists to abolish the response. Several antipsychotic drugs demonstrate partial agonist or antagonist features at the D₂R, while antagonizing serotonin 2 receptors (5-HT₂R_s) (11). In addition, inverse agonists reduce the constitutive receptor activity; i.e., inhibit the background signaling of the receptor in the absence of an agonist. β -blockers demonstrating inverse agonism, e.g. propranolol, act at β -adrenergic receptors in the heart and skeletal muscles, and are used to reduce tachycardia and essential tremor (12, 13).

Allosteric GPCR ligands may interact with separate, allosteric binding sites to modulate both the affinity and efficacy of ligands at the OBP (14, 15). For the GABA_BR, CGP7930 was one of the first reported positive allosteric modulators (16). Allosteric GPCRs modulators may affect endogenous ligand-receptor interactions only subtly, thereby allowing for additional strategies of targeting a range of conditions, including e.g. inflammation, psychosis, addiction and nociception (see (17)).

At the signaling level, a ligand could activate any of the various G protein subtypes, e.g. $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, G_z , G_{olf} , $G\alpha_{12/13}$, or the non-canonical β -arrestin pathway, with differential efficacies (Fig. 1G). Agonist-dependent G protein signaling bias has been demonstrated *in vitro* (18). Biased-signaling ligands, i.e. ligands preferentially activating either G protein or β -arrestin pathways, may be of importance in reducing side-effects and potentiating therapeutic

drug effects. Oliceridine is a G protein-biased μ -opioid receptor (μ -OR) agonist that has been suggested to reduce constipation and respiratory depression, while maintaining the antinociceptive effect (19).

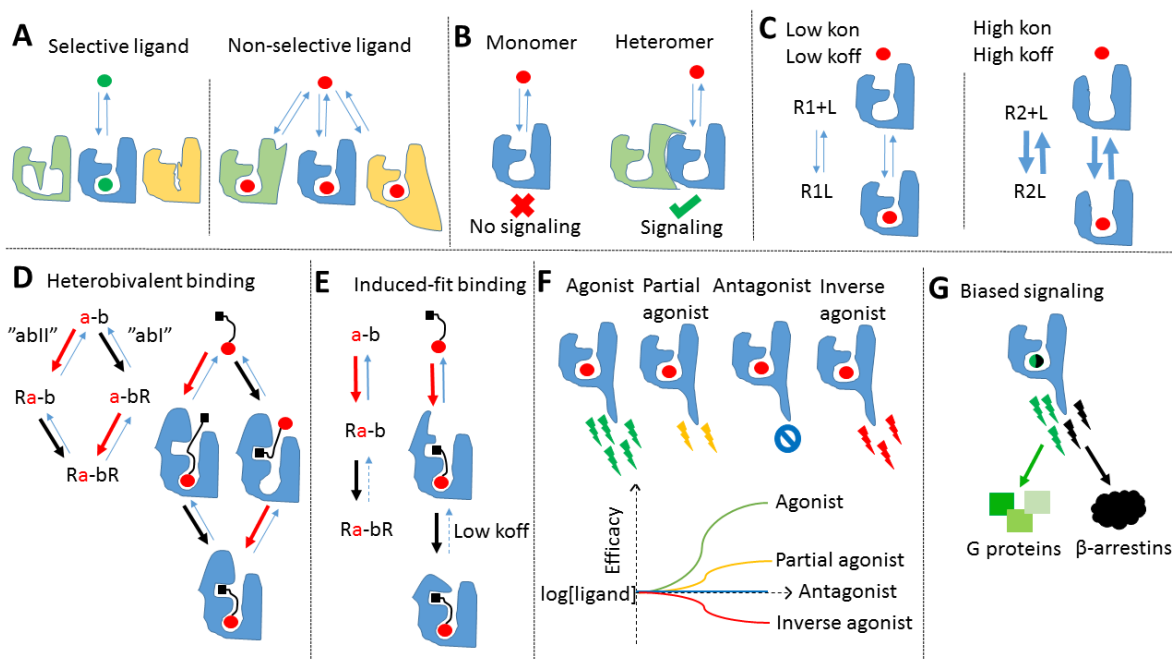


Figure 1. Summary of key dimensions of receptor-ligand interaction. A) Receptor selectivity. Left, the selective ligand (green) only binds to one receptor (blue). Right, the non-selective ligand (red) binds to all receptors. B) Heteromer-dependent signaling. Left, ligand binding to the monomer does not evoke a response. Right, ligand binding to the heteromer evokes a response. C) Association and dissociation rate constants dictate the dissociation constant K_d . Note that the K_d ratio could be identical for the low k_{off} /low k_{on} and the high k_{off} /high k_{on} examples. D) Heterobivalent ligand binding, with primary (a) and secondary (b) pharmacophores of a bitopic ligand (a-b) interacting with two distinct receptor binding sites; the OBP and the SBP respectively. Mechanistically, either the secondary (black arrow) or the primary pharmacophore (red arrow) may bind the receptor first, described as abI- and abII-mechanisms respectively (8). E) Induced-fit binding is viewed as an initial binding event between the primary pharmacophore (a) and the OBP (red arrow), followed by a conformational change allowing binding of the secondary pharmacophore (b) to the SBP (9)(black arrow). Note that the latter event reverses only slowly (low k_{off}). F) Agonist efficacy (or intrinsic activity). Following receptor-ligand interaction, the downstream signaling is fully induced by an agonist (green), partially induced by a partial agonist (yellow), abolished by an antagonist (blue) and reduced below the baseline (constitutive) level by an inverse agonist (red). G) Biased signaling. The ligand activates both G protein (green) and β -arrestin signaling pathways (black). Details are not drawn to scale.

1.3 G PROTEIN-SIGNALING PATHWAYS

1.3.1 Activation of G protein signaling pathways

GPCRs are recognized by their ability to induce G protein mediated signaling following activation. Ligand-activated GPCRs gain guanine-nucleotide exchange factor (GEF) properties, releasing guanosine diphosphate (GDP) from heterotrimeric G protein complexes of $G\alpha$ GDP/ $G\beta\gamma$ (see (20)). This promotes binding of guanosine triphosphate (GTP) to $G\alpha$, inducing an activating conformational change, and possibly separating the $G\alpha$ and $G\beta\gamma$ proteins (21). Depending on the G protein subtype, i.e. $G\alpha_s$ or $G\alpha_i/G\alpha_o$, $G\alpha$ GTP stimulates or

inhibits adenylate cyclase (AC) which converts ATP into 3',5'-cyclic AMP (cAMP). The $G\alpha$ protein stimulating AC, $G\alpha_s$, may be ADP-ribosylated and activated by the *V. Cholera* toxin (22, 23), whereas the AC inhibitory $G\alpha_i/G\alpha_o$ proteins may be ADP-ribosylated and inactivated by the *B. pertussis* toxin (PTX)(24, 25). Historically, this has facilitated the isolation and study of selected $G\alpha$ proteins. Downstream of the GPCR, cAMP binds to, and activates protein kinase A, in turn phosphorylating the cAMP-response element binding protein, which binds to genomic cAMP response elements and induces transcription. Also, cAMP may directly activate cyclic-nucleotide gated channels (26). In similar, $G\beta\gamma$ proteins modulate ion channel activities, e.g. of L- and T-type voltage-gated calcium channels, and activate G protein-coupled inward rectifying potassium 1-4 (GIRK1-4) channels (27, 28).

1.3.2 G protein-coupled inward rectifying potassium channels

GIRK1-4 are expressed in the central nervous system and contribute to neuronal hyperpolarization (29, 30). Functional channels consist of GIRK1-4 subunit heterotetramers (with exception of GIRK2; able to form homotetramers), each consisting of two transmembrane helices (30, 31). For GIRK channel activation, phosphatidylinositol 4,5-bisphosphate (PIP_2) is required in addition to $G\beta\gamma$ (32). Also, GIRK channels are modulated by sodium ions, which might serve as a mechanism to hyperpolarize the cell following depolarization-induced sodium influx (33, 34). The specificity between G protein and GIRK channel activation is believed to be due to the higher rate of G protein activation, and thereby $G\beta\gamma$ generation, by $G\alpha_i/G\alpha_o$ -coupled receptors as compared to G_q - and G_s -coupled receptors (35).

1.3.3 Termination of the G protein signaling cascade

Signal termination is evoked by the GTPase activity intrinsic to $G\alpha$ proteins, hydrolyzing GTP into GDP and subsequently releasing inorganic phosphate. This is followed by sequestration of $G\beta\gamma$ proteins, which reform with $G\alpha$ proteins. By stabilization, GTPase accelerating proteins (GAPs), including the regulator of G protein signaling (RGS) family, augment the catalytic activity of the $G\alpha$ GTPase domain and thereby decrease the life time of the active, GTP-bound complex (see (20)). Structurally, the RGS domain consists of nine α -helices, although there are functional differences between the GAPs; e.g. RGS4, being pre-coupled to GIRK1/2 channels, potently accelerates G protein dependent gating, whereas the free cytosolic RGS3 only accelerates G protein dependent gating with 100-fold lower potency (36). Beyond modulation of AC by $G\alpha_s$ and $G\alpha_i/G\alpha_o$ pathways, the $G\alpha_q$ protein activates phospholipase $C\beta$, hydrolyzing PIP_2 to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Following activation of the IP_3 receptor, located in the membrane of the endoplasmatic reticulum, Ca^{2+} is released to the cytosol, inducing transcriptional effects through Ca^{2+} /calmodulin-dependent protein kinase II. Furthermore, a diverse array of G proteins have been characterized with a multitude of functions; e.g. activation of $G\alpha_{12/13}$ proteins have been related to cell structure functions, e.g. actin remodeling (37). Finally, the differential activation of G proteins, and the kinetics within the signaling pathways, vary between GPCRs (18).

1.4 NON-CANONICAL GPCR SIGNALING BY BETA-ARRESTINS

Active GPCRs recruit GPCR kinases, e.g. GPCR kinase 2 (GRK2), responsible for GPCR phosphorylation and signal termination. GPCR phosphorylation enhances the recruitment of β -arrestin 1 and 2 to the GPCR, which induces GPCR desensitization (see (38)). In addition, β -arrestins activate downstream signaling pathways, separate from those activated by G-proteins, e.g. nuclear factor kappa-light-chain-enhancer of activated B cells and tumor suppressor protein p53, regulating cell metabolism, mitochondrial function and synaptic plasticity in neurons (39-41). In addition, both G protein and β -arrestin signaling activate the extracellular signal-related kinase 1 (ERK1) pathway albeit with different kinetics; the former induces a rapid activation and deactivation, whereas the latter induces a slow activation and prolonged response (42, 43). Also, the β -arrestin-activated ERK1 remains cytoplasmic, as compared to the G protein-induced ERK1, which translocates to the nucleus (42, 43). G protein-independence of β -arrestin downstream signaling has been proposed (44, 45), although an investigation using mammalian cell lines with genetically deleted $G\alpha_s/G\alpha_q/G\alpha_{12}$ in conjunction with the $G\alpha_{i/o}$ -inactivating PTX demonstrated G protein-dependence of β -arrestin signaling through ERK1 (46).

Antipsychotic effects of D_2R antagonists have been related to β -arrestin signaling, whereas extrapyramidal symptoms (EPS; i.e. dystonia, akathisia, tremor and tardive dyskinesia) have been suggested to result from interference with G protein signaling (45, 47). The mood stabilizer lithium has been proposed to inhibit glycogen synthase kinase 3 β (GSK3 β) signaling via modulation of a signaling complex consisting of β -arrestin, protein phosphatase 2A and protein kinase B (PKB) (44). At cardiomyocyte angiotensin II type 1 receptors (AT_1R), activation of β -arrestin signaling conferred positive inotropic effects, although these were counteracted by G protein-signaling activation (48). Thus, further development of β -arrestin-selective AT_1R and D_2R ligands for cardiac failure and schizophrenia may prove valuable. In contrast, at the μ -OR, G protein signaling was related to analgesic effects, whereas β -arrestin signaling was related to gastrointestinal and respiratory adverse effects (19).

1.5 MUSCARINIC ACETYLCHOLINE RECEPTORS

Acetylcholine is the endogenous agonist at metabotropic muscarinic acetylcholine M_{1-5} receptors ($M_{1-5}R$), which are structurally highly homologous, but differ in G protein signaling; M_1R , M_3R and M_5R activate $G\alpha_q$, whereas M_2R and M_4R activate $G\alpha_i$, (49). $M_{1-5}R$ are expressed in the peripheral and central nervous system in varying degrees (see (50) for review). M_1R is expressed in the cortex and the striatum, and is involved in regulating dopamine release from substantia nigra. $M_1R^{-/-}$ mice display basal and amphetamine-induced hyperlocomotion as compared to wild type (WT) mice (51)). M_2R and M_3R are involved in parasympathetic nervous system responses (52). For example, cardiac acetylcholine release, which induces M_2R activation, $G\beta\gamma$ release and subsequent GIRK channel activation, contributes to membrane repolarization and a negative chronotropic effect (53). The inhibitory M_4R is present in cholinergic interneurons in the striatum, reducing dopaminergic tone and inhibiting movement (54). For hypodopaminergic conditions, e.g. Parkinson's disease, pharmacological inhibition of the M_4R may prove valuable (55). The M_5R is expressed in dopaminergic neurons in the substantia nigra and ventral tegmental area, and has

been suggested involved in opioid reward; $M_5R^{-/-}$ mice displayed reduced reward from morphine administration (56). Although high degrees of homology between the $M_{1-5}R$ have complicated the development of receptor subtype-specific ligands, recently reported crystal structures of M_1R and M_4R (57), M_2R (58), M_3R (59) and M_5R (60) will likely facilitate *in silico* ligand development.

1.6 VOLTAGE-SENSITIVITY OF GPCRS

Voltage-gated and, to some extent, ligand-gated ion channels functionally depend on voltage-sensitivity (61). Interestingly, in 2003, also GPCRs were demonstrated to be voltage-sensitive; acetylcholine potency at the M_2R was reduced at depolarized potentials in GIRK activation- and radioligand binding assays (62). In contrast, the M_1R was found to possess an inverse voltage-dependence, binding acetylcholine with increased affinity at depolarized potentials (62). Experiments on metabotropic glutamate 1-3 receptors ($mGlu_{1-3}R$) revealed differential voltage-dependent agonist potencies. Following depolarization, glutamate potency at the $G\alpha_q$ -coupled $mGlu_1R$ increased, whereas glutamate potency decreased at the $G\alpha_i$ -coupled $mGlu_3R$ (63). In search of a voltage-sensor, gating-charges were recorded in the M_2R , and were consistent with the voltage-sensitivity of ligand binding (64). The voltage-sensitivity of ligand potency was subsequently demonstrated in D_2R , histamine H3 and H4 receptors, generalizing the concept to include numerous GPCRs (65-67). Further investigations revealed ligand-specificity of the voltage-dependent shift in agonist potency at the D_2R ; in contrast to aporphines, which retained potency and efficacy at -80 mV and at 0 mV, several other D_2R agonists demonstrated reduced potencies and, for some agonists, also reduced E_{max} at depolarized potentials (68).

In search for the GPCR voltage-sensor(s), a well-conserved aspartate residue, $D^{2.50}69$ (Ballesteros-Weinstein nomenclature; (69)), was proposed to confer the voltage-sensing properties of the M_2R , as D69 is involved in coordinating a charged sodium ion within the seven transmembrane helices (70). M_2R D69A demonstrated no gating currents, although surface expression of the mutant receptor was reduced (71). Additional investigations, based on molecular dynamics simulations, supported the role of the D69 residue in regulating M_2R voltage-sensitivity (72). Recently, tyrosine-phenylalanine substitution of three residues ($Y^{3.33}104$, $Y^{6.51}403$ and $Y^{7.39}426$) surrounding the OBP, were demonstrated to eliminate the voltage-dependent agonist potency (73). However, the involvement of the $D^{2.50}69$ in the voltage-sensing of the M_2R , and other GPCRs, still remains unclear.

The voltage-dependent agonist potency at GPCRs explains a cardiac phenomenon; the depolarization-induced decrease of acetylcholine-dependent potassium current in sinoatrial node cardiomyocytes (74). Based on electrophysiological investigations of atrial cardiomyocytes, this finding is mechanistically related to the lower potency of acetylcholine at M_2R in depolarized cells (75).

1.7 DOPAMINE RECEPTORS

In the 1950s, Arvid Carlsson discovered the ability of dopamine to reverse loss of motor functions following reserpine-induced depletion of synaptic vesicles (see (76) for an overview). This finding distinguished dopamine from the related monoamine serotonin and paved the way for understanding the role of dopamine in modulating cognition, mood,

learning, memory, motor and endocrine functions (77, 78). Dopamine receptors are classified by their main signaling pathways; D₁-like receptors (D₁R and D₅R) activate stimulatory G_{α_{s/olf}} and D₂-like receptors (D₂R, D₃R and D₄R) activate inhibitory G_{α_{i/o}} proteins (79). The regional expression of dopamine receptors differs; postmortem autoradiography studies indicated high expression levels of D₁R and D₂R in the caudate nucleus and putamen, with D₁R concentrated to the medial caudate nucleus in contrast to the more evenly distributed D₂R (80). Additionally, D₁R, more densely expressed in cortical regions as compared to D₂R (80), has been related to physiological and pathological cognitive functioning (81-83). D₁R is mainly postsynaptically expressed, in contrast to D₂R, which is present both pre- and postsynaptically (77). Prominent heterodimerization of dopamine receptors, e.g. D₁R-D₃R, D₁R-D₂R, and D₂R-D₃R provides additional complexity to receptor subgroup signaling (84, 85). The reported crystal structures of D₂-like receptors; D₂R (7), D₃R (86) and D₄R (87), allow for *in silico* design of selective ligands.

Dopaminergic signaling is mediated by three separate pathways, the nigrostriatal, the mesocorticolimbic and the tuberoinfundibular pathway (see (88) for review). Briefly, the nigrostriatal pathway consists of dopamine neurons in substantia nigra pars compacta projecting to the caudate nucleus and putamen, modulating motor functions but also memory, and to some extent reward. The mesocortical and mesolimbic pathways consist of dopamine neurons in the ventral tegmental area projecting to the cortex, modulating executive functions, and to the nucleus accumbens, mediating reward and positive reinforcement. The tuberoinfundibular pathway consists of dopamine neurons of the hypothalamic arcuate nucleus projecting to the hypophysis.

1.8 DOPAMINE RECEPTORS AS TARGETS

1.8.1 Dopaminergic disorders

Numerous pathological conditions, e.g. Parkinson's disease, hyperprolactinemia, Huntington's disease, schizophrenia, bipolar disorder and addiction are related to hypo- or hyperactive dopaminergic signaling (89-94). For schizophrenia, the dopamine hypothesis has been revised multiple times. Hyperactive mesolimbic dopamine signaling has been proposed to be responsible for positive symptoms (hallucinations, delusions and thought disorder) while hypoactive mesocortical dopamine signaling would explain negative (flattened affect, avolition, alogia) and cognitive symptoms (95). Accumulating evidence from neuroimaging, genome-wide association- and epidemiological studies has updated the dopamine hypothesis. Genetic variants and mutations are thought to drive a presynaptic increase in dopamine levels, which increase the probability of developing psychotic disorders, including schizophrenia, and to disturb the perception of external stimuli (96). An alternative hypothesis of schizophrenia, glutamatergic dysregulation, was based on N-methyl-D-aspartate (NMDA)-antagonists, e.g. phencyclidine (PCP) and ketamine, replicating the positive, negative, and cognitive symptoms seen in schizophrenia (97). Finally, the kynurenine hypothesis involves glutamatergic dysregulation related to increased concentrations of kynurenic acid, an endogenous NMDA-antagonist, in patients with schizophrenia (98).

1.8.2 Clinically used antipsychotics

The mainstay of pharmacological treatment of schizophrenia is currently D₂R blockade, by antagonists, or weak partial agonists. First-generation or typical antipsychotic drugs, based on the first agent chlorpromazine discovered in 1952, are recognized as ligands with high D₂R potency, prone to evoke EPS at high concentrations (99). In positron emission tomography (PET) studies of schizophrenic patients treated with typical antipsychotic drugs, the D₂R occupancy was 70-89%, with higher receptor occupancy related to EPS (100). This seemingly narrow therapeutic interval stimulated investigations to explain- and decrease the EPS propensity of antipsychotic drugs. The second-generation or atypical antipsychotics, initially based on clozapine (first discovered in 1958), demonstrated reduced EPS propensities (101). The typical/atypical drug classification has been questioned, as this classification does not fully explain the EPS frequency (102). In addition, third-generation antipsychotics refer to drugs demonstrating partial D₂R agonism, e.g. aripiprazole, and more recently also brexpiprazole, cariprazine and lumateperone (103). Due to more pronounced presynaptic D₂R receptor reserves, a partial agonist may activate autoreceptors to a larger extent than postsynaptic receptors, thereby decreasing dopamine synthesis and release (103). Alternatively, assuming increased synaptic dopamine concentrations in the mesolimbic pathway, partial agonists may functionally antagonize D₂Rs, based on their low E_{max}. Oppositely, the partial agonists may activate D₂Rs in the mesocortical pathway, where low dopamine concentrations prevail (103-105). This concept of functional selectivity motivates further development of partial D₂R agonists as therapeutic “dopamine stabilizers”.

In 2000, experimental radioligand binding studies of antipsychotics at the D₂R suggested that atypical antipsychotics dissociate up to 100 times more rapidly as compared to typical antipsychotics; this “fast-off” feature was proposed to facilitate dopamine rebinding at D₂R following drug dissociation, thus preserving the physiological dynamics of dopamine signaling (106). In addition, this hypothesis opposed the theory of 5-HT₂R antagonism reducing the EPS propensity, as D₂R occupancy had the highest correlation with EPS propensity (107). The “fast-off” hypothesis was reinvestigated by analysis of binding kinetics of 17 antagonists at the D₂R, using the Gβγ-mediated GIRK activation assay. Rapid chlorpromazine dissociation from the D₂R and a mere twofold difference in dissociation kinetics between chlorpromazine and clozapine were demonstrated (108), in stark contrast to previous findings by Kapur and Seeman (106). Extended analyses of antipsychotic drug dissociation rates from the D₂R and validation of the GIRK assay results continued to challenge the “fast-off” hypothesis (109), instead proposing the variation in dissociation kinetics and extents of reversibility possibly related to D₂R antagonist lipophilicity (110). A following investigation, using a kinetic assay observing D₂R antagonist competition with the fluorescent D₂R agonist 2-(N-phenethyl-N-propyl)amino-5-hydroxytetralin hydrochloride (PPHT)-red, correlated antipsychotic drug association rates at the D₂R with EPS odds ratios and dissociation rates with the risk of hyperprolactinemia. Pronounced drug-D₂R rebinding due to high association rates, in the context of limited drug diffusion, was proposed to be mechanistically related to a high EPS propensity (111). These findings have been questioned, based on the slow association rate of the used agonist PPHT and the generalization of k_{on} dictating EPS propensities (112). Different determinants of association rates have been suggested, including ligand lipophilicity, diffusion and ligand conformation (113-115).

1.8.3 β -arrestin-biased partial agonists as experimental antipsychotics

The observation that a range of GPCRs were able to affect both canonical G protein, and non-canonical β -arrestin, signaling pathways opened the field of biased signaling (88). Numerous antipsychotic drugs are able to antagonize β -arrestin signaling at the D₂R, in addition to antagonizing G protein signaling (47). A reduction of the β -arrestin downstream effector PKB has been suggested in patients with schizophrenia and the mood stabilizer lithium has been proposed to modulate PKB-GSK3 β -signaling (44, 116). At this point, development of β -arrestin-biased ligands opened for further probing of non-canonical GPCR signaling pathways *in vivo*.

In 2011, a series of β -arrestin-selective aripiprazole analogues; UNC9994, UNC9975 and UNC0006, was synthesized at the University of North Carolina, and demonstrated to be devoid of G-protein signaling activity at the D₂R (45). Furthermore, UNC9994 elicited antipsychotic properties in a mouse model of PCP-induced locomotion; this feature was fully reversed in β -arrestin-2^{-/-} mice, supporting the hypothesis that β -arrestin-2-dependent D₂R signaling is involved in mediating antipsychotic drug effects (45). UNC9994 was reported to recruit β -arrestin-2 to D₂R only in the presence of GRK2, especially relevant for cortical tissue with high expression levels of β -arrestin-2 and GRK2 as compared to the striatum, where UNC9994 is an antagonist at β -arrestin-2 D₂R signaling. The resulting cortical β -arrestin-2-selective D₂R agonism increased fast-spiking interneuron activity and was hypothesized to potentially counteract negative symptoms of schizophrenia (117).

2 AIMS OF THE THESIS

The thesis investigates GPCR pharmacology using GIRK current recordings. Specifically, molecular features of ligand binding sites and implications for signaling were explored. Details regarding GPCR-ligand interactions will inform future drug development.

The aims are:

- To determine if the conserved residue D^{2.50}69 in the M₂R constitutes a part of the voltage-sensor, mediating voltage-dependent agonist-potency (paper I).
- To determine if the aripiprazole-derived, putatively β -arrestin-selective, D₂R ligand UNC9994 couples to G protein signaling pathways at D₂R and D₃R (paper II).
- To explore binding properties and kinetics of aripiprazole radioanalogues of varying lipophilicity at the D₂R (paper III).
- To experimentally and computationally characterize how a secondary binding pocket in the D₂R may mediate insurmountable ligand binding (paper III).

3 METHODS

3.1 MOLECULAR BIOLOGY

Receptor, accessory protein and ion channel cDNAs were acquired from Genscript (Piscataway, NJ). cDNAs encoding the long-isoform human D₂R (including WT, V91A, L94A and E95A), D₃R and β -arrestin-2 were in the pXOOM vector, which includes the *Xenopus* globin gene (118). All mutations were verified by sequencing. cDNAs encoding RGS4 (from the Missouri cDNA Resource Center; www.cdna.org), GIRK1 and GIRK4 (provided by Dr. Terence Hebert, University of Montreal, Canada) were in pCDNA3 (Invitrogen). cDNAs encoding M₂R (from Dr. Hanna Parnas, Hebrew University of Jerusalem, Israel) and the catalytic subunit of PTX (PTX-S1) were in pGEM-HE. The D69N point mutation was introduced to the WT M₂R using the QuickChange (Agilent technologies) kit according to the manufacturer's instructions. Plasmids were linearized using restriction enzymes (D₂R WT, V91A, L94A and E95A, D₃R, RGS4; XhoI, GIRK1, GIRK4, M₂R WT and D69N; NotI, PTX-S1; NheI) and the DNA product was purified using the PureLink™ kit (Invitrogen; see Fig. 2A). Linearized DNA was transcribed *in vitro* using the mMessage mMachine™ T7 kit (Ambion, Austin, TX). DNA and RNA products were quantified using spectrophotometry (Nanodrop, ThermoFisher).

3.2 XENOPUS LAEVIS OOCYTES AS EXPRESSION SYSTEM

Oocytes from the female African clawed toad, *Xenopus laevis*, were surgically isolated according to the procedure described in the ethical permit (N245/15), approved by the Swedish National Board for Laboratory Animals and the Stockholm Ethical Committee. Briefly, the *X. laevis* was immersed for 15 min in 5.4 mM tricaine methanesulfonate (MS-222; Sigma) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma; buffered to pH 7.4 using NaOH). Following verification of anesthesia, a minor laparotomy was performed to allow for extraction of ovarian tissue, which was transferred to a modified Barth's solution (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂ and 0.92 mM MgSO₄, 2.5 mM sodium pyruvate, 25 U/ml penicillin and 25 μ g/ml streptomycin, buffered to pH 7.6 using NaOH). The abdominal and skin layers were closed separately using 6-0 silk sutures (Ethicon), followed by transfer of the *X. laevis* to a separate tank for observation during the following 24 hours.

Isolated ovarian tissue was treated with 1.5 mg Liberase DH (Roche) for 90 minutes, to separate the individual oocytes, which were subsequently manually screened for adequate staging (stages V-VI (119)) and quality (see Fig. 2A). Oocytes were incubated for 24 hours at 12°C, and thereafter injected with 50 nl of aqueous RNA solution using Nanoject II (Drummond Scientific). The RNA amounts per oocyte were: M₂R (WT), 0.2 ng; M₂R (D69N), 0.7 ng; D₂R (WT, V91A, L94A and E95A), 0.2 ng; D₃R, 0.2 ng; β -arrestin-2, 5.6 ng; PTX-S1, 3 ng; RGS4, 40 ng; GIRK1, GIRK4, 1 ng of each. RGS4 is one of several GAPs

expressed in native tissues, which speed up the G protein cycle such that GIRK channel activity more closely follows receptor occupancy by agonist (120).

3.3 TWO-ELECTRODE VOLTAGE-CLAMP

X. laevis oocytes of maturation stages V-VI have a diameter around 1 mm, substantially larger as compared to neurons or mammalian cell lines. The two-electrode voltage-clamp (TEVC) method allows for precise control of the membrane potential, using separate current-conducting and voltage-following electrodes (121). Activation of GPCRs evoked GIRK currents that were recorded using the TEVC technique (Fig. 2B).

Electrophysiological recordings were performed at room temperature (22°C), 5 to 7 days after RNA injection using the CA-1 amplifier (Dagan, Minneapolis, MN). Data were acquired at 134 Hz using pCLAMP 8 (Molecular Devices) software. A high-potassium solution (64 mM NaCl, 25 mM KCl, 0.8 mM MgCl₂, 0.4 mM CaCl₂, 15 mM HEPES and 1 mM ascorbic acid, adjusted to pH 7.4 with NaOH), giving a K⁺ reversal potential of about -40 mV, was used for GIRK current recordings. Ascorbic acid was added to prevent the oxidation of dopamine. To increase the GIRK currents, oocytes were clamped at -80 mV during registrations. Ligands were added to the 20 µl recording chamber by superfusion at 1.5 ml/min using a computer-controlled, pressure-driven perfusion system (SmartSquirt, AutoMate Scientific, Berkeley, CA).

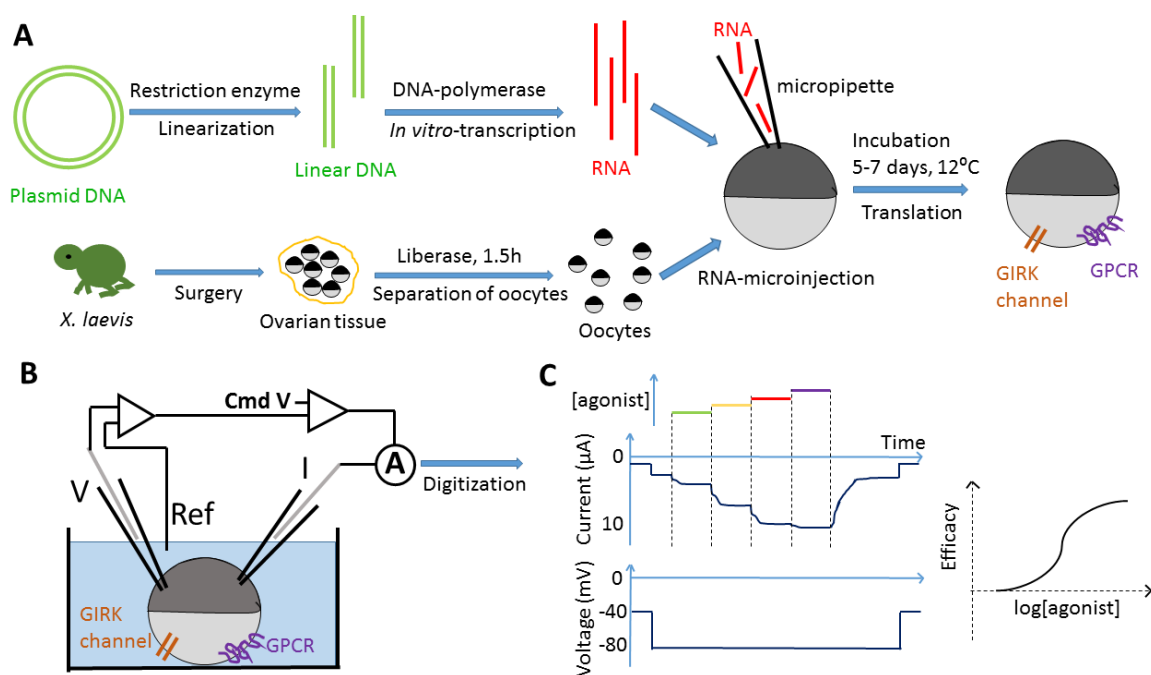


Figure 2. Overview of molecular biology, oocyte preparation, electrophysiology and GIRK recordings. A) Converging pipeline of molecular biology and *X. laevis* oocyte harvesting, followed by RNA-microinjection and membrane expression of GPCRs and GIRK channels. See Methods 3.1-3.3 for details. B) General principle of two-electrode voltage-clamp of *X. laevis* oocytes. The voltage-sensing electrode (V) registers the intracellular voltage (by comparison with the bath electrode, Ref), which is compared to the clamped voltage (Cmd V), and if needed a compensatory current is injected into the oocyte via the current-passing electrode (I). The current is registered by an amperometer (A), digitized, and analysed. C) Example of dose-response data, e.g. from oocytes

co-expressing D₂R and GIRK channels. The oocyte is initially clamped to -40 mV and lowered to -80 mV to increase the GIRK currents during the recordings. Increasing agonist concentrations (green-yellow-red-purple) evoke increasing GIRK currents, which saturate around the purple concentration. Following halted agonist administration, the current returns to baseline. The steady-state currents for each concentration may generate dose-response data, here plotted semi-logarithmically. Details are not drawn to scale.

3.4 RECORDING PROTOCOLS

3.4.1 Agonist dose-response curves

For evaluation of agonist ligands, 3 to 4 increasing concentrations were applied at 35-, 50- or 100-second intervals to oocytes expressing the respective receptor (see Fig. 2C). The interval was determined to ensure maximal or pseudomaximal agonism (for ligands demonstrating slow association rates). The agonist-evoked current response was determined by subtracting the basal (agonist-independent) current from the agonist-evoked current. Concentration-responses were subsequently normalized to the mean responses of maximal agonist-evoked responses, either in the same oocytes (expressing D₂R or M₂R; paper I and III) or in other oocytes from the same batch (expressing D₂R and D₃R, the latter desensitizes after agonist application; paper II). For dopamine concentration-response data, 4 to 5 increasing concentrations of dopamine were applied at 25-second intervals, ending with a response-saturating concentration (100 μ M) of dopamine.

3.4.2 Antagonist dose-response curves

For dopamine receptor antagonists, 100 nM dopamine was first applied to provide a baseline response, followed by 3 to 4 applications of increasing concentrations of antagonist at 50-100 second intervals in the continued presence of 100 nM dopamine (paper II and III). This allowed for calculation of inhibition constants (K_i) using the Cheng-Prusoff relationship (122). For each oocyte, the current amplitude at the end of each antagonist application interval was normalized to the control response to 100 nM dopamine obtained at the start of the protocol.

3.4.3 Evaluation of ligand binding kinetics

Dissociation rates for acetylcholine (paper I) were determined by fitting a monoexponential function to the decay following agonist washout. Observed association rates (k_{obs}) for antagonists (paper III) were determined by 40 s applications of 100 nM dopamine, followed by varying antagonist concentrations in 100 nM dopamine. A monoexponential function was fitted to the antagonist phase, and the inhibition time constant was converted to k_{obs} (see Methods 3.5.2).

Dissociation rates and extents of recovery for ligands that antagonize the receptor-evoked GIRK channel current (paper III) were determined by application of 1 μ M dopamine, followed by ligand in 1 μ M DA (antagonizing the response), and subsequent application of either 1 or 100 μ M dopamine. Surmountability was defined as an increased agonist response recovery following application of higher agonist concentrations (e.g. 100 μ M dopamine vs. 1 μ M dopamine).

3.5 DATA ANALYSIS

3.5.1 Dose-response analysis

Electrophysiological data were analyzed in Clampfit (Axon™ Instruments). Dose-response curves were calculated using the variable-slope sigmoidal functions in GraphPad (Prism software). Antagonist data were fitted to the equation:

$$Y = bottom + \frac{1}{1 + 10^{(X - \log_{10} IC_{50})n}}$$

where Y is the response as a fraction of 1, bottom is the maximal response inhibition evoked by the antagonist, X is the logarithm of ligand concentration, IC₅₀ is the half maximal inhibitory concentration and n is the Hill slope. For agonist data, the equation used was:

$$Y = \frac{1}{1 + 10^{-(X - \log_{10} EC_{50})n}}$$

where EC₅₀ is the half maximal effective concentration of the agonist.

The inhibition constant, K_i, was calculated as

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[agonist]}{EC_{50}}\right)^n}$$

according to Cheng-Prusoff (122). Data points were shown as mean ± SEM.

3.5.2 Estimation of rate constants

GIRK current activation or deactivation kinetics were quantified by fitting a monoexponential decay function

$$q = q_0 + A \times e^{-\frac{(t-t_0)}{\tau}}$$

to data, to estimate the time constant of response decay upon agonist washout. q₀ is the initial and q the final current amplitude, t₀ is the initial and t is the final time point, A is the current amplitude at the start of the fit, and τ is the time constant of current increase or decay.

The association rate constant k_{on} was previously related to the observed association rates, k_{obs}, determined using the GIRK assay (109). Briefly, the calculation is based on a three-state model of agonist-bound receptor (RA), unbound receptor (R) and ligand-bound receptor (RL; see Methods 3.6.1 below), in which the transition between RA and R (agonist dissociation) is assumed to be swift compared to the transition from R to RL (ligand binding). At very high ligand concentrations, a kinetic roof of the k_{obs} may be reached (109).

By plotting k_{obs} at varying antagonist concentrations, followed by a linear regression, k_{on} was calculated in accordance with previous descriptions (109)

$$k_{on} = \frac{\Delta k_{obs}}{\Delta [antagonist] \times R_0}$$

where the antagonist concentration was known and the fraction of unoccupied receptors, R_0 , prior to antagonist application was derived from the dose-response curve of agonist at the relevant receptor. k_{off} was estimated separately as

$$k_{off} = \frac{\ln(2)}{T_{1/2}} = \frac{1}{\tau}$$

where \ln is the natural logarithm and $T_{1/2}$ is the time for half-maximal response recovery.

Kinetic K_d was calculated as

$$K_d = \frac{k_{off}}{k_{on}}$$

3.5.3 Curve shift assay

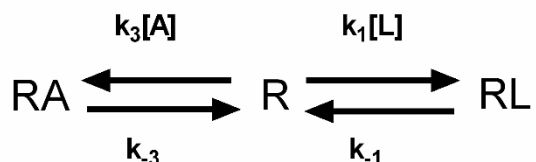
For curve shift experiments, a maximal response was first evoked by application of 1 μ M dopamine, which was subsequently washed out, followed by the concomitant application of dopamine in the presence (or absence, to generate the control EC_{50} ; EC_{50C}) of SV-III-130. The current amplitude following 500 s of co-application of dopamine and SV-III-130 was normalized to the initial response elicited by 1 μ M dopamine. To extract EC_{50} estimates, normalized responses to the various concentrations of dopamine applied in the presence of each concentration of SV-III-130, or in its absence, were fit by the equation given above to yield EC_{50L_z} or EC_{50C} , respectively, where EC_{50L_z} is the EC_{50} in the presence of a given concentration, z , of antagonizing ligand. A Schild plot was generated by plotting; $\log_{10}(EC_{50L_z}/EC_{50C} - 1)$ against SV-III-130 concentration, and linear regression was used to assess the competitiveness of the agonist and ligand interactions. For competitive ligands, the slope is expected to be 1, whereas a ligand with negative allosteric effects typically would demonstrate a slope <1 (123).

3.6 KINETIC BINDING MODELS

Response recovery following D_2R antagonism was simulated based on a three-state model and experimental k_{on} and k_{off} values for ligands and dopamine. For antagonists, k_{on} were derived from k_{obs} , and k_{off} from response recovery experiments using supramaximal dopamine concentrations (100 μ M). For dopamine, k_{off} was derived from previous reports using the GIRK assay (124), and k_{on} calculated from the EC_{50} value. k_{on} to irreversibly bound states was calculated for the insurmountable ligand SV-III-130, based on the fraction of response recovery observed after 125 s, assuming that the insurmountable fraction represents the irreversibly bound state.

3.6.1 Three-state competitive binding model

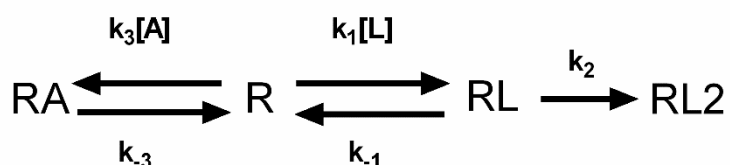
Competitive ligand binding (see e.g. Fig. 1A or C), in the presence of an agonist, was simulated using a three-state model. RA represents agonist-bound receptor, mediating GIRK activation, in the competitive binding model



where R, RA and RL denote unbound receptor, agonist-bound receptor and (competing) ligand-bound receptor, k_1 , k_{-1} , k_3 and k_{-3} , association and dissociation rate constants for competing ligand and dopamine respectively, L denoting ligand and A agonist (dopamine).

3.6.2 Four-state irreversible binding model of induced-fit type

Irreversible ligand binding was simulated by an induced-fit binding model lacking a dissociation from the second binding step (see e.g. Fig. 1E),



where RL2 denoted the irreversibly bound receptor and k_2 the association rate to the RL2 state. For SV-III-130, k_2 was set to 0.01 s^{-1} , based on the observation that approximately 36% ($=1/e$) recovery would be observed after 100 s application of $1 \mu\text{M}$ SV-III-130 in $1 \mu\text{M}$ DA.

Kinetic binding models were evaluated using Matlab 2018a (MathWorks). The RA fraction, representing the fraction coupled to GIRK channels, was plotted as a function of time (response recovery simulations) or concentration (dose-response simulations).

3.7 MOLECULAR DYNAMICS

Receptor-ligand complexes were generated based on docking of SV-III-130 to the crystal structure of the D₂R (PDB code: 6CM4 (7)). The complexes were placed in a lipid bilayer, with aqueous solution surrounding the membrane and receptor-ligand complex. Using the Accelerating bio-molecular dynamics simulation package (125), the complex was minimized, equilibrated and followed by production runs of $3.2 \mu\text{s}$, which were used for analysis. Ligand receptor contacts were quantified using the `get_contacts` script (126). The computed ratio of residue contacts was quantified by dividing the stability of W100 contacts by I184 contacts.

3.8 STATISTICAL ANALYSIS

Current amplitudes, extents of recovery and dissociation half-lives were compared using the Student's t-test. Concentration-response curves were compared using analyses of variance (F-test), to assess differences in pEC₅₀. Observed association rates were plotted against antagonist concentrations, and linear regressions were used to determine the slope and k_{on}. p<0.05 was considered significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

In this study, we investigated the putative voltage-sensing residue in the M₂R by expression of the uncharged D69N mutant and GIRK channels in oocytes. Expression levels of the M₂R D69N allowed for functional characterization of agonist-induced currents at -80 mV and 0 mV using the TEVC technique. The results suggested similar reductions of WT ($pEC_{50} = 7.82 \pm 0.04$ at -80 mV and $pEC_{50} = 7.52 \pm 0.05$ at 0 mV) and D69N receptor potencies ($pEC_{50} = 6.80 \pm 0.07$ at -80 mV and $pEC_{50} = 6.47 \pm 0.03$ at 0 mV) at depolarized potentials, i.e.; a ~2-fold increased EC_{50} at 0 mV compared to -80 mV.

For the M₂R, two prevailing hypotheses regarding voltage-sensing residues have been suggested; either D69 or three tyrosine residues, Y104, Y403 and Y426 (see Introduction, 1.6). Our results contradict previous experimental and computational studies, which have attributed a role to D69 in the voltage-sensing of M₂R and other GPCRs (71, 72, 127). Instead, our results are in agreement with the hypothesis that Y104, Y403 and Y426 are responsible for voltage-dependent agonist potency of the M₂R (73). These findings have implications for understanding the mechanism underlying the depolarization-induced reduction of acetylcholine-evoked GIRK currents in sinoatrial node cardiomyocytes. In the central nervous system, e.g. for dopaminergic projections, the role of voltage-dependent agonist potency and efficacy in regulating presynaptic transmitter release, as well as postsynaptic transmission and neuronal firing patterns, remains largely unexplored.

Reduced M₂R WT and D69N potencies at depolarized potentials were related to increased agonist dissociation rates. Due to small GIRK current amplitudes observed at depolarized potentials for the M₂R D69N, agonist association kinetics were difficult to characterize. The small currents may be related to the pronounced constitutive activity of the M₂R D69N, which reduces the fraction of inactive receptors available to be activated by an agonist. In all, the implications of membrane potential on agonist binding kinetics may provide details regarding which receptor region that is involved in voltage-sensing and inform future studies on voltage-sensing residues in GPCRs.

4.2 PAPER II

In this study, we investigated a potential G protein-coupling of the proposed fully β -arrestin selective D₂R ligand, UNC9994 (45, 117). In oocytes expressing D₂R and GIRK, potent partial agonist efficacy of UNC9994 was observed at D₂R-evoked GIRK currents ($pEC_{50} = 6.73 \pm 0.4$, $E_{max} = 14.5 \pm 2.8\%$), indicating G protein-coupled signaling. The partial agonist efficacy of UNC9994 on GIRK activation was abolished by co-expression of PTX-S1 but retained in oocytes co-expressing D₂R and β -arrestin-2. These findings contradict previous investigations (45, 117), where no G protein agonist or antagonist activity of UNC9994 was observed at the D₂R.

UNC9994 demonstrated higher potency and efficacy at the D₃R, eliciting almost full agonism ($pEC_{50} = 7.21 \pm 0.55$, $E_{max} = 89.1 \pm 24.3\%$). Possibly, such D₃R interaction may address the proposed beneficial antipsychotic effects of UNC9994 in rodents (45, 117). Interestingly, the D₃R-preferring ligand cariprazine has demonstrated superior efficacy against negative symptoms in patients with schizophrenia as compared to the atypical antipsychotic risperidone, thereby addressing a previously untargeted symptom domain (128).

The suggested binding of UNC9994 at the OBP of the D₂R suggests an ability to compete with dopamine binding (129), and thereby interfering with endogenous activation of G protein signaling pathways. Additionally, the UNC9994 binding mode raises questions regarding the interactions underlying β -arrestin signaling bias. For lysergic acid diethylamide, both the residence time and the β -arrestin signaling efficacy at the 5-HT_{2A}R were reduced following a mutation in the second extracellular loop (ECL2); L209A (130). Structure-functional selectivity-relationship investigations at the D₂R suggested ligand interactions with the ECL2, specifically with I184, over interactions with serine residues of transmembrane helix 5, to bias towards β -arrestin signaling (131). The broader picture of binding kinetics and ligand-receptor interactions governing β -arrestin bias is continuing to be revealed.

4.3 PAPER III

In this study, we investigated a series of aripiprazole (Abilify®) radioanalogues, with varying aliphatic linker lengths that connects the primary and secondary pharmacophores, at the D₂R. SWR-1-8, SV-III-130 and SWR-1-14 have aliphatic linkers of 3, 4 and 5 carbons respectively, with increasing lipophilicity (Fig. 3). Interestingly, SV-III-130 demonstrated potent interactions ($pK_i = 8.57 \pm 0.05$) and an insurmountable binding at the D₂R (fraction of response recovery following antagonism, agonist concentration: 0.20 ± 0.18 , $1 \mu\text{M DA}$, and 0.23 ± 0.03 , $100 \mu\text{M DA}$), effects not observed for SWR-1-8 ($pK_i = 8.01 \pm 0.18$, fraction of response recovery following antagonism, agonist concentration: 0.27 ± 0.06 , $1 \mu\text{M DA}$, and 0.91 ± 0.09 , $100 \mu\text{M DA}$) or SWR-1-14 ($pK_i = 7.70 \pm 0.13$, fraction of response recovery following antagonism, agonist concentration: 0.37 ± 0.04 , $1 \mu\text{M DA}$, and 0.98 ± 0.12 , $100 \mu\text{M DA}$). Thus, the findings are not in agreement with a strict relationship between lipophilicity and insurmountable binding.

The higher potency of SV-III-130 was mediated by both increased association and decreased dissociation rates. Insurmountable binding may be consistent with either a competitive or a non-competitive (e.g. allosteric) binding mechanism. Adaptation of the GIRK activation assay provided curve-shift data of SV-III-130 at varying agonist concentrations. The resulting Schild plot demonstrated a slope close to unity (1.07 ± 0.19 , $R^2 = 0.97$), suggesting a competitive binding mechanism.

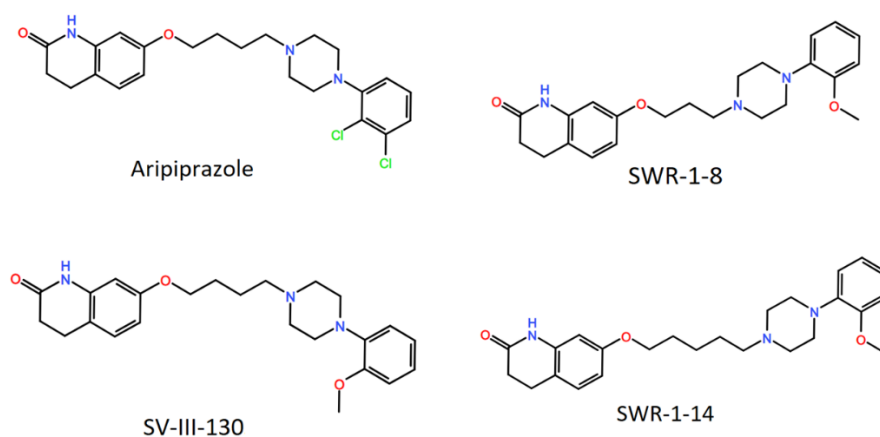


Figure 3. Structures of aripiprazole, SWR-1-8, SV-III-130s and SWR-1-14. Note the shared aliphatic 4 carbon linker in aripiprazole and SV-III-130s. The piperazine moiety constitutes the primary pharmacophore and the tetrahydroquinolinone moiety constitutes the secondary pharmacophore.

The role of the 4 carbon linker was further investigated by alanine mutation of SBP residues V91, L94 and E95, presumed to contact the secondary pharmacophore of SV-III-130 (132). D₂R V91A and E95A reduced the potency ($pK_i = 7.63 \pm 0.16$ for V91A and $pK_i = 7.97 \pm 0.13$ for E95A) and abolished the insurmountability of SV-III-130, whereas the D₂R L94A mutation retained the potency ($pK_i = 8.95 \pm 0.17$ for L94A) and abolished the insurmountability (fraction of response recovery following antagonism, agonist concentration: 0.32 ± 0.05 , 1 μ M DA, and 0.64 ± 0.11 , 100 μ M DA). Interestingly, the time to half-maximal response recovery of SV-III-130 from D₂R L94A ($T_{1/2} = 107.7 \pm 11.4$ s, 100 μ M DA) resembled that from D₂R WT ($T_{1/2} = 110.7 \pm 14.8$ s, 100 μ M DA), although a pronounced surmountability (agonist competition) was observed, suggesting a similar dissociation rate of SV-III-130 from the L94A mutant and from (at least a fraction of) WT D₂R. These findings support a crucial role of V91, L94 and E95 for maintaining the stability of the D₂R SBP and allowing secondary pharmacophore interactions.

To further characterize the competitive, but insurmountable, binding mechanism of SV-III-130 at the D₂R, binding models were adapted based on experimental data (see Methods 3.6 and (9)). A competitive, three-state, ligand-agonist-receptor binding model (109), captured the behavior observed in response recovery experiments with SWR-1-8 and SWR-1-14. Based on the seemingly irreversible binding of SV-III-130 to a fraction of D₂R, as observed during our experimental timescale, two ligand binding steps were included in a second model: First, a reversible step followed by an irreversible one. This four-state model replicated the experimental findings from response recovery experiments with different agonist concentrations. To evaluate the induced-fit binding model of SV-III-130 at the D₂R, an inductive approach was undertaken; prolonged application of SV-III-130 at the D₂R would be expected to extinguish the response recovery, according to the model. In the corresponding experiments, a prolonged (400 s) application of SV-III-130 yielded no response recovery, in

agreement with the model. Additionally, the four-state model replicated the experimental curve-shift assay for SV-III-130 at D₂R WT.

To further investigate the role of L94 in the SBP interaction with the secondary SV-III-130 pharmacophore, molecular dynamics simulations were conducted based on the previously published crystallographic D₂R structure (7). The simulations suggested interactions between the secondary pharmacophore of SV-III-130 and W100 (in the ECL1) and I184 (in the ECL2) of the WT D₂R. Similarly, at the L94A D₂R, the secondary pharmacophore interacted with W100 and I184, although W100 relocated into the cavity previously formed by L94, thereby not sealing the SBP, and possibly increasing the probability of SV-III-130 egress.

The results suggest an insurmountable binding of SV-III-130 at the WT D₂R, which might address the slow and limited displacement of [¹¹C]-SV-III-130 following induced endogenous dopamine release in non-human primates (133). Similarly, the reduced displacement of other D₂R ligands in PET studies may potentially be related to an induced-fit binding mechanism (9). In general, reduced, and slow, drug dissociation could be beneficial in the design of a PET radiotracer or long-acting drug.

Mechanistically, the proposed binding mechanism implies that the primary pharmacophore of SV-III-130 first binds to the OBP and also to the SBP in an open conformation. In the next step, the SBP undergoes a conformational change which closes the extracellular loops 1 and 2 over the ligand through an induced-fit mechanism (9). For ligands with two pharmacophores, a heterobivalent binding mode may be possible (8). However, for SV-III-130, the experimental data on recovery from D₂R antagonism, recorded during a short timeframe (400 s), was recapitulated by an irreversible binding model of induced-fit type. Response recoveries from antagonism by SWR-1-8 and SWR-1-14 were captured by three-state, competitive binding models.

Based on the L94A D₂R mutation, which increased the response recovery following SV-III-130 antagonism, and *in vivo* observations of limited agonist-mediated displacement of [¹¹C]-SV-III-130 (133), an induced-fit binding mechanism was proposed and experimentally tested. Previous descriptions of non-covalent irreversible GPCR-ligand interactions have been described for risperidone at the 5-HT₇R (134, 135).

4.4 LIMITATIONS AND ETHICAL CONSIDERATIONS

Experimentally, papers I-III rely on TEVC investigations of *X. laevis* oocytes, with several shared methodological limitations. Heterologous expression systems demonstrate differences compared to *in vivo* conditions, e.g. in lipid constitution of the cell membrane, co-expression of proteins, post-translational modifications and trafficking mechanisms, and should be regarded as models. The GIRK assay implies that GPCR activation is transmitted to GIRK channels. The coupling efficiency between GPCR and GIRK is increased using a GAP (here RGS4), thereby providing binding and kinetic values in agreement with radioligand binding

studies (109). Batch-dependent variations in GPCR and GIRK expression were reduced whenever possible by normalizing data to the same oocytes, or in some cases (paper II), to oocytes from the same batch. The use of simple kinetic models to explain experimental data (paper III) might not provide a complete picture of the *in vivo* pharmacology, but rather a theoretical model. The explanatory value of such models should be carefully evaluated; for SV-III-130s, we first simulated the expected response recovery following a prolonged SV-III-130 application to D₂R, and thereafter conducted the corresponding experiment.

The use of *X. laevis* oocytes for the present two-electrode voltage-clamp require consideration of the 3Rs; Replacement, Reduction and Refinement (136). Replacement would imply using different biological cells or methods for analysis of receptor-ligand interactions, e.g. assays based on mammalian cell lines. RNA injection of oocytes provides a precise control of stoichiometry and a ground for reproducibility, as compared to corresponding transfections of multiple constructs in mammalian cell lines. Alternative methods may be radioligand binding or fluorometric assays, although these have specific drawbacks with regard to functional measurements and kinetic investigations.

Reduction is fundamental in the use of *X. laevis* oocytes; e.g. by reusing the *X. laevis*. Additionally, multiple experiments should be conducted for each oocyte extraction, to optimize the use of the oocytes. Refinement is conducted by using MS-222, a preferred anesthetic, with analgesic properties (137).

5 CONCLUSIONS

In this thesis, GPCR-ligand interactions in the M₂R and D₂R were explored using electrophysiology and computations.

Voltage-sensing properties of the M₂R were not mediated by D^{2.50} (paper I). Instead, the findings may support the hypothesis of three tyrosine residues (Y104, Y403 and Y426) acting as voltage sensors in the M₂R, as proposed by Barchad-Avitzur et al. (73).

The proposed β -arrestin-selective D₂R ligand, UNC9994, activated GIRK channels via D₂R and D₃R, in a G protein-dependent manner, and in the presence of β -arrestin (paper II). UNC9994 seemed more potent and efficacious at the D₃R, as compared to D₂R. In the light of other investigations suggesting an inability of ligands to evoke β -arrestin signaling without concomitant G protein activation (46), the evidence for completely β -arrestin-selective ligands is currently weak.

The bitopic ligand, SV-III-130, displayed an insurmountable binding at the D₂R (paper III). The two homologues, SWR-1-8 and SWR-1-14 were fully surmountable, which is incongruent with a strict relationship between lipophilicity and insurmountable binding. The competitive binding observed with SV-III-130 at the D₂R was crucially dependent on the integrity of the secondary binding pocket. A two-step, irreversible binding model captured the experimentally observed binding mechanism of SV-III-130. Additionally, molecular dynamics suggested a role of the extracellular loops in modulating SV-III-130 binding to the D₂R.

6 FUTURE DIRECTIONS

GPCR voltage-sensing mechanisms have mainly been investigated in the M₂R. Based on the hypothesis that Y104, Y403 and Y426 act as voltage sensors in the M₂R, the generalizability to other G $\alpha_{i/o}$ -coupled GPCRs, e.g. the D₂R, should be considered. Prospective studies investigating inactivating mutations of the corresponding residues in voltage-dependent G $\alpha_{i/o}$ -coupled GPCRs, e.g. the D₂R or mGlu₁R, would address this hypothesis. Additionally, based on previous findings of agonist-specific voltage-dependency (probe dependence) (68), additional molecular dynamics investigations may provide information regarding the structural network involved in GPCR voltage-sensing.

β -arrestin-selective signaling in the absence of G protein activation remains a controversial but highly interesting topic; ligands with strong β -arrestin preference would allow for the selective study of non-canonical GPCR signaling. Recently, an experimental ligand was reported to be a β -arrestin-selective melatonin 1 receptor agonist, but a mixed G protein/ β -arrestin-agonist at the melatonin 2 receptor (136). Further structural-activity relationship investigations of the mechanistic underpinnings of G protein/ β -arrestin signaling at various GPCRs will provide insights for the *in silico* development of functionally selective ligands. A truly β -arrestin-selective D₂R ligand would be of substantial value in the research on and development of novel therapeutics for psychotic disorders. Based on the physiological relevance of β -arrestin-biased ligands at several GPCRs, further investigations of receptor-ligand interactions conferring signaling bias is of importance.

Demonstration of induced-fit binding of SV-III-130 at the D₂R indicates that additional ligands could demonstrate similar binding mechanisms. For example, a PET study using the radiotracer [¹⁸F]-N-methyl-benperidol reported low displaceability from the D₂R following amphetamine-induced DA competition (138). Based on structural similarities with SV-III-130, N-methyl-benperidol may bind D₂R by an induced-fit binding mechanism. This hypothesis could be addressed using the electrophysiology-based GIRK-assay. Further investigation of bitopic ligands acting at GPCRs may reveal additional induced-fit binding ligands. Also, based on the assumption that the prominent ECL2 of the D₂R is a key mediator in encapsulating the SBP, it would be valuable to investigate whether an induced-fit binding mechanism could be observed at GPCRs with smaller ECL2, e.g. D₃R and D₄R (7). Finally, PET investigations evaluating endogenous dopamine competition with [¹¹C]-SWR-1-8 or [¹¹C]-SWR-1-14 may clarify whether the observed *in vitro* surmountability holds *in vivo*.

7 ACKNOWLEDGEMENTS

This thesis is crucially dependent on the hard work and support of numerous people, to whom I am very thankful. Especially, I wish to express my gratitude to:

Dr. Johanna Nilsson, main supervisor, for giving me the freedom to pursue my own interests in neuroscience.

Professor Peter Århem, co-supervisor, for the endless transmission of scientific knowledge in all possible forms and the interest in computational neuroscience.

Dr. Kristoffer Sahlholm, co-supervisor, for introducing me to electrophysiology, molecular biology and GPCRs.

Dr. Hugo Zeberg, for broad scientific advice, especially regarding theoretical aspects of electrophysiology.

Dr. Jana Selent and Dr. Tomasz Maciej Stępniewski, for productive collaboration and *in silico* contributions.

Dr. Sean W. Reilly, Dr. Robert R. Luedtke and Professor Robert H. Mach, for all work on SWR-1-8, SV-III-130, SWR-1-14, and paper III.

Dr. Matthew Kirkham, for providing theoretical knowledge and practical guidance regarding *X. laevis* husbandry, thereby enabling the research presented above.

The Department of Clinical Neurophysiology, Karolinska University Hospital, for scientific discussions and valuable support.

The administrative staff at the Department of Clinical Neuroscience, for kind helpfulness and physical availability.

All colleagues in Bioclinicum and Biomedicum, Karolinska Institutet, including members of the Svenningsson, Chergui, Holmin, Brundin and Svensson research groups, for interesting discussions, scientific input, collaboration, and for providing a stimulating research environment.

Finally, I wish to express my deepest gratitude to my family and friends for all unconditional and endless support.

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