UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Lakshmi Thoondee

Characterization of ligand binding to Dopamine D_3 receptor using fluorescence anisotropy and radioligand binding

Master's Thesis (30 ECTS)

Curriculum Bioengineering

Supervisors: Maris Johanna Tahk, MSc
Tõnis Laasfeld, MSc
Prof. Ago Rinken, PhD, MD

Tartu 2021

Characterization of ligand binding to Dopamine D_3 receptor using fluorescence anisotropy and radioligand binding

Abstract:

There are five subtypes of dopamine receptors that play a role in the dopaminergic system. Due to their limited distribution and involvement in cognitive and emotional functions, Dopamine D_3 receptors are attractive pharmacological targets for treatment of drug addiction and neuropsychiatric disorders. D_3 receptor ligands have been labelled with a fluorescent dye or a radioisotope for direct monitoring of ligand binding to the receptor. However, there are not many fluorescent ligands that are available for studying D_3 receptor. A2-TAMRA is a novel fluorescent ligand with high affinity for D_3 receptor. Binding of [^{3}H]-methylspiperone to D_3R was studied in parallel to validate results from A2-TAMRA binding to D_3R. The D_3 receptor ligands had similar affinities in inhibiting A2-TAMRA and [^{3}H]-methyl spiperone binding to D_3 receptor, since a very good correlation (R^2 = 0.94) was obtained between both methods. The affinities for the known antagonists had a good correlation with previously published data. Selectivity of A2-TAMRA towards two different subtypes was also studied and we found that A2-TAMRA prefers D_3 receptor over D_1 receptor.

Keywords: dopamine receptors, fluorescent ligand, fluorescence anisotropy assay, radioligand binding assay

CERCS: B740, P310

Ligandi seondumine dopamiin D_3 retseptoriga ja selle iseloomustus kasutades fluorestsentsanisotroopiat ja radioligandi seondumise meetodeid

Dopamiiniretseptorid on G-valguga seotud retseptorid, mis vahendavad oma toimet viie alamtüübi - D_1, D_2, D_3, D_4 ja D_5 - retseptorite kaudu. Arvestades, et D_3 retseptoritel on piiratud ekspressioon ja osalus kognitiivsetes ja emotsionaalsetes funktsioonides. Seetõttu on nad farmakoloogilised sihtmärgid narkomaania ja neuropsühhiaatriliste häirete raviks. Et jälgida ligandi seondumist retseptoriga on D_3 retseptori ligandeid märgistatud fluorestsentsvärvi või radioisotoopiga. A2-TAMRA on uus fluorestsentsligand, millel on kõrge afiinsus D_3 retseptorile. A2-TAMRA on D_3 retseptori suhtes selektiivsem kui D_1 retseptori suhtes. Antagonistide mõõdetud pKi oli heas vastavuses kirjandusega (R^2 = 0.88), kuid agonistide puhul erinevus mõõdetud pK_i ja kirjanduse pK_i vahel oli suurem.

Fluorestsentsanisotroopiat tulemusi võrreldi radioligandi seondumise meetodist tulemustega ning kahe meetodi vahel saadi tugev korrelatsioon ($R^2 = 0.94$).

Võtmesõnad: dopamiin, retseptorid, fluorestsentne ligand, fluorestsentsi anisotroopia katse, radioligandi seondumiskatse

CERCS: B740, P310

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

7TM: seven transmembrane

AC: adenylyl cyclase

BBV: budded baculovirus

B_{max}: concentration of available binding sites

cpm: counts per minute

D_3R: Dopamine D_3 receptor

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

ECL: extracellular loop

EDTA: Ethylenediamine tetraacetic acid

EGFR: epidermal growth factor receptor

FA: fluorescence anisotropy

GDP: guanosine diphosphate

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

ICL: intracellular loop

IC_{50}: molar concentration of an unlabeled ligand that inhibits binding of a labeled ligand by 50%

K_d: equilibrium dissociation constant of a labelled ligand-receptor complex

K_i: inhibition constant, used to refer to the equilibrium dissociation constant of an unlabelled ligand-receptor complex

k_{off}: dissociation rate constant

k_{on}: association rate constant

MAPK: mitogen-activated protein kinase

MSN: medium spiny neuron

PIC: protease inhibitor cocktail

RET: resonance energy transfer

Sf9: *Spodoptera frugiperda* cell line

TAMRA: tetramethylrhodamine

TFI: total fluorescence intensity

Tris: 2-amino-2-(hydroxymethyl)propane-1,3-diol

TIRF: total internal reflection fluorescence

INTRODUCTION

Cell signalling is a crucial process for normal growth and development of a cell. It enables cells to respond to several environmental cues or stimuli (Tuteja, 2009). The stimulus can be a physical or chemical signal which is received by specific proteins and converted to a cellular response through a series of molecular events. This process is known as signal transduction (Nelson and Cox, 2017). Proteins that are responsible for detecting these signals and relaying the effects of the signal are known as receptors and they bind to specific chemical compounds called ligands. Receptors can be divided into intracellular or transmembrane receptors. G protein - coupled receptors (GPCRs) constitute the largest and most diversified transmembrane receptor family in the mammalian genome (Luttrell, 2006). They recognize several endogenous as well as exogenous cues such as odorants, light, ions, metabolites, and neurotransmitters (Farooqui and Farooqui, 2016).

Dopamine receptors (DRs) belong to the family of GPCRs and they are divided into five different subtypes namely, $D_{1\text{-}5}$ receptors. Dopamine receptors play important roles in the central nervous system as well as in the periphery where they are involved in crucial functions such as voluntary movement, reward, hormonal regulation (Beaulieu and Gainetdinov, 2011). Pharmacological agents targeting dopaminergic neurotransmission have been clinically used for treating various neurological and psychiatric disorders, such as Parkinson's disease, schizophrenia, bipolar disorder, Huntington's disease, attention deficit hyperactivity disorder (ADHD1), and Tourette's syndrome (Beaulieu and Gainetdinov, 2011). Interactions of ligand binding to dopamine receptors can be studied through different ligand binding assays.

In this thesis, we characterized ligand binding to Dopamine D_3 receptor through fluorescence anisotropy assay and radioligand binding assay. We used a novel fluorescent ligand, A2-TAMRA, for fluorescence anisotropy assay and compared the results from fluorescence anisotropy assay with radioligand binding assay. We also studied selectivity of A2-TAMRA for D_3 receptor compared to D_1 receptor. Budded baculoviruses obtained from Sf9 cells were used as receptor source in all the experiments.

1 LITERATURE REVIEW

1.1 G protein - coupled receptor structure and classification

Most GPCRs possess seven transmembrane (7TM) α helices connected by alternating intracellular and extracellular loop (ICL and ECL) regions, an amino terminus and a carboxyl terminus (Figure 1) (Rosenbaum *et al*., 2014). The greatest similarity among the various GPCRs is observed in the transmembrane regions while the most variable structures are the amino terminus, followed by carboxyl terminus and intracellular loop between transmembrane helices five and six (Kobilka, 2007). GPCRs in the human genome are generally classified into five families according to similarities in their sequence: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion (family D) and frizzled/taste 2 (family E) (Fu *et al*., 2014). The rhodopsin family containing four main groups (α, β, γ and δ) with 13 subbranches, is the largest family (Fu *et al*., 2014). Individual GPCRs have distinct signal transduction pathways, involving several G protein subtypes as well as G protein independent signaling pathways, thus it has been suggested to use the appellation 7TM receptors instead of GPCRs (Kobilka, 2007).

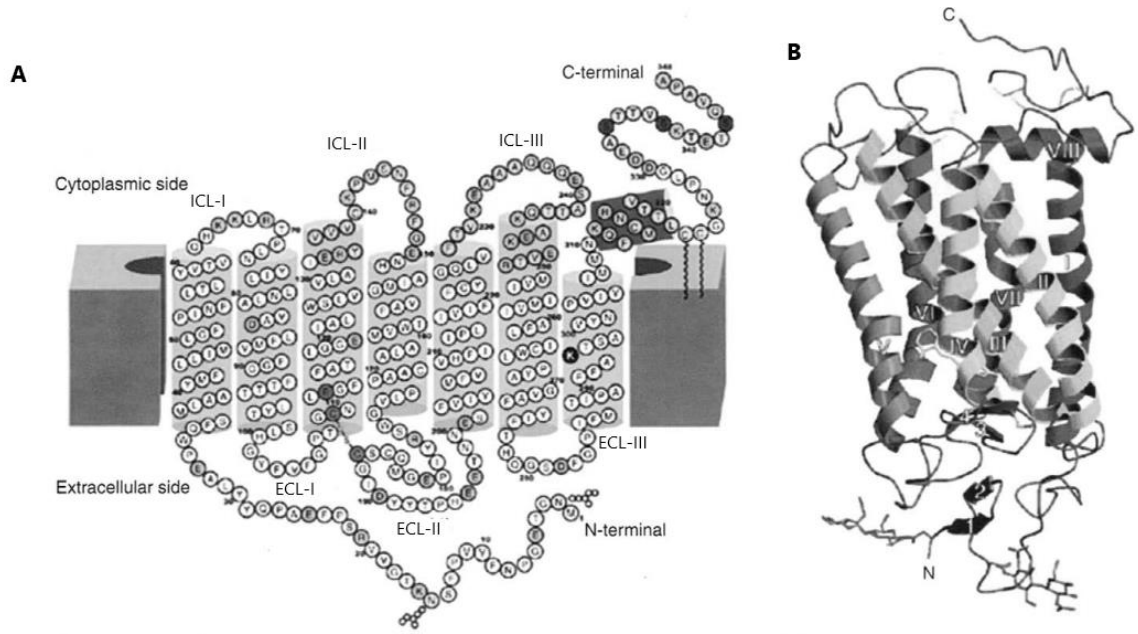

Figure 1. Structure of bovine rhodopsin. (A) Two-dimensional model of bovine rhodopsin. Grey cylinders represent the transmembrane helices. The ICLs and ECLs are shown. (B) The three-dimensional model of rhodopsin (Jacob and Bunnett, 2006).

1.1.1 GPCR signalling through G proteins

G proteins are heterotrimeric guanine nucleotide-binding proteins, consisting of α, β, and γ subunits (Luttrell, 2006). Guanosine diphosphate (GDP) is bound to the α subunit of a G protein when it is in an inactivated state (Jackson, 1991). Upon agonist binding to the GPCR, it undergoes conformational changes and activates the G protein, catalyzing dissociation of GDP from the G protein and association of guanosine triphosphate (GTP) with the α subunit (Jackson, 1991). This results in the dissociation of the G protein subunits from each other and from the receptor, generating a monomeric α subunit and a βγ dimer (Jackson, 1991). These can then regulate the activity of specific effectors which include second-messenger generating enzymes or specific ion channels (Gainetdinov *et al*., 2004). The subunits become reassociated together and thus, deactivated after hydrolysis of GTP to GDP and inorganic phosphate (Jackson, 1991). G proteins can be classified into four subtypes on the basis of the structure and function of alpha subunits: G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$. The G_s class has a role in activation of adenylyl cyclase (AC), the $G_{i/o}$ class inhibits AC and regulates ion channels, the $G_{q/11}$ activates phospholipase C and $G_{12/13}$ is involved in activation of the Na^+/H^+ exchanger pathway (Li *et al*., 2012).

Ligands that bind to the GPCR active site can be classified based on the effect that they have on receptor function. An agonist is a ligand that binds to the receptor, altering its state and producing a biological response (Neubig *et al*., 2003). Agonists are divided into full agonists and partial agonists, depending on the extent of the response produced. A full agonist produces maximal response, whereas a partial agonist produces submaximal response and it cannot produce maximal response even at high concentrations (Neubig *et al*., 2003). The maximal response is the maximal response that can be obtained by the endogenous ligand for that particular receptor. The effect of an agonist can be reduced by an antagonist, which binds to the receptor without mediating cellular response (Berg and Clarke, 2018). Receptors can also be active without an activating ligand and thus, they display constitutive activity (Benovic *et al*., 1985; Berg and Clarke, 2018). Inverse agonists are ligands that can decrease the constitutive activity of a receptor (Berg and Clarke, 2018).

1.1.2 GPCR desensitization and internalization

Continued or overstimulation of GPCRs can be harmful to cells and can result in uncontrolled cell growth (Rajagopal and Shenoy, 2018). The desensitization of a GPCR response can be described as the loss of response subsequent to prolonged or repeated administration of an agonist (Hausdorff *et al*., 1990). The term `prolonged´ can be quite

misleading as experimentally, it can refer to a time duration ranging from seconds to hours (Kelly *et al.*, 2008). Desensitization of the receptor involves phosphorylation of the receptor followed by β-arrestin binding. Desensitization can be homologous or heterologous. In the case of heterologous desensitization, the receptor does not need to be bound by an agonist, it just requires kinase activation by different stimuli (Pierce and Lefkowitz, 2001). Second messenger-dependent protein kinases, such as cAMP-dependent protein kinase and protein kinase C phosphorylate the receptor in this case (Pierce and Lefkowitz, 2001). GPCR kinases (GRKs) are involved in homologous receptor desensitization. It is known as homologous desensitization since the only substrates for GRKs are agonist-occupied receptors, thus ensuring the desensitization of only those receptors that have been activated (Pierce and Lefkowitz, 2001). Binding of arrestins is involved in both fast desensitization of the receptor as well as receptor internalization (Krupnick and Benovic, 1998; Pitcher *et al.*, 1998). β-arrestin binding is crucial for the internalization of many GPCRs as they act as adaptors linking the receptors to clathrin-coated pits (Ferguson *et al*., 1996).

GRKs and arrestins can also act as signaling switches, giving rise to signaling pathways that are G protein-independent (Hall *et al*., 1988; Luttrell *et al*., 1999). For instance, arrestins can act as adaptors that induce the scaffolding of several signaling proteins including, mitogen-activated protein kinases (MAPK) and protein kinase B (Akt) (Beaulieu *et al*., 2009; Shenoy and Lefkowitz, 2003, 2005).

1.2 The dopaminergic system

Dopamine forms part of a group of neurotransmitters called catecholamines (Vallone *et al*., 2000). Catecholamines are characterized by a benzene ring with two adjacent hydroxyl groups, a single amine group and a side chain of ethylamine or one of its derivatives (Rondou *et al*., 2010) (Figure 2). The brain dopaminergic system comprises dopamine containing nuclei (substantia nigra pars compacta, ventral tegmental area, and arcuate nucleus) and the target areas (cortex, basal ganglia, thalamus, limbic structures, and pituitary gland) (Prieto, 2017). Within the central nervous system, dopamine synthesis occurs in the cytosol of dopaminergic neurons and begins with hydroxylation of L-tyrosine by tyrosine hydroxylase to produce L-3,4-dihydroxyphenylalanine (L-DOPA), followed by its decarboxylation to dopamine by aromatic amino acid decarboxylase (Meiser *et al*., 2013).

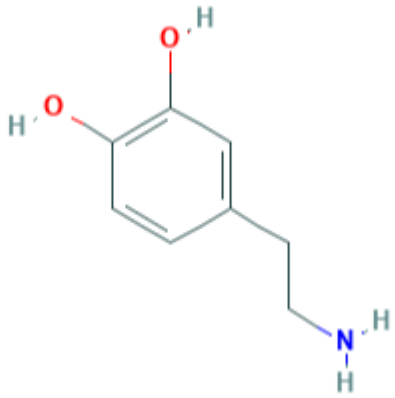

Figure 2. Dopamine structure. (Pubchem, 2021)

1.2.1 Dopamine receptors

The effects of dopamine are mediated by dopamine receptors (DRs), which are a subclass of GPCRs. DRs are further divided into two classes: D_1-like and D_2-like, based on their structural, pharmacological and biochemical properties.

Members of the same class of DR have significant homology within their transmembrane regions but distinct pharmacological properties (Beaulieu and Gainetdinov, 2011). D_1-like receptors, consisting of D_1 and D_5 receptors couple to stimulatory G protein alpha subunits G_s, activating AC, while D_2-like receptors couple to inhibitory G protein alpha subunits $G_{i/o}$ inhibiting AC (Chien *et al*., 2010). The different dopamine receptors have different affinities for dopamine, ranging from nanomolar to micromolar range (Beaulieu and Gainetdinov, 2011). D_1-like receptors are found only post synaptically on dopamine-receptive cells, such as GABAergic medium spiny neurons (MSNs) in the striatum while D_2-like receptors are found both pre- and post-synaptically (Rondou *et al*., 2010; Sokoloff *et al*., 2006). Structurally, D_1-like and D_2-like receptors have the same number of amino acids in the NH_2 terminal domain but the COOH terminal domain for D_1-like receptors is longer than for D_2-like receptors (Missale *et al*., 1998). At the genetic level, the two classes of DRs differ primarily in the presence of introns in the dopamine receptor gene coding sequence (Gingrich and Caron, 1993). There are no introns in the D_1 and D_5 receptor genes, but the genes encoding D_2-like receptors have several introns (Gingrich and Caron, 1993). DR subtypes also differ in their expression pattern in the brain and in the periphery. D_1Rs are the most widely expressed in the human central nervous system (CNS), followed by D_2, D_3, D_5 and D_4Rs (Mishra *et al*., 2018). In the periphery, all subtypes of DRs have been found in varying proportions in the kidney, adrenal glands, sympathetic ganglia, gastrointestinal tract,

blood vessels, and heart (Li *et al.*, 2006; Missale *et al.*, 1998; Svennilson and Aperia, 2000; Villar *et al.*, 2009; Witkovsky, 2004).

All DR subtypes can form homo and heterodimers in vivo, just like many GPCRs (Martel and McArthur, 2020). These impact the signal transduction pathway of the dopamine receptor (Martel and McArthur, 2020). The regions involved in DR dimerization are transmembrane domains 5 and 6 (Martel and McArthur, 2020). This interaction can be a transient process and can be stabilized with agonists like dopamine or quinpirole (Kasai *et al.*, 2018). DR heterodimers which are most commonly observed in vivo are D_1-D_2, D_1-D_3, D_1- Histamine H_3 and D_2- Adenosine A_{2A} (Borroto-Escuela and Fuxe, 2019). They all have an effect on the MAPK response of these receptor systems, and D_1-D_3 can also modify the mechanism of β-arrestin recruitment and heterodimer internalization (Martel and McArthur, 2020).

Dopamine receptors mediate a lot of important functions such as locomotion, cognition, affect, attention, impulse control, decision making, motor learning, sleep, reproductive behaviors, and the regulation of food intake (Di Chiara and Bassareo, 2007; Iversen and Iversen, 2007; Koob and Volkow, 2010; Missale *et al.*, 1998; Rondou *et al.*, 2010). Other functions mediated by dopamine receptors that are localized outside the central nervous system include olfaction, vision, and hormonal regulation, such as the pituitary D_2 dopamine receptor-mediated regulation of prolactin secretion, kidney D_1R-mediated renin secretion, adrenal gland D_2R-mediated regulation of aldosterone secretion, the regulation of sympathetic tone, D_1, D_2, and D_4 receptor- mediated regulation of renal function, blood pressure regulation, vasodilation, and gastrointestinal motility (Iversen and Iversen, 2007; Li *et al.*, 2006; Missale *et al.*, 1998; Svennilson and Aperia, 2000; Villar *et al.*, 2009; Witkovsky, 2004).

1.2.1.1 D_3 receptor

D_3R was first cloned in 1990 (Sokoloff *et al.*, 1990). Compared to other subtypes of the D_2-like receptors, D_3R has a limited pattern of distribution in the brain, the highest level of expression being observed in the limbic areas, such as in the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja (Missale *et al.*, 1998; Sokoloff *et al.*, 2006). D_3R is also detectable at considerably lower levels in the striatum, the substantia nigra pars compacta, the ventral tegmental area, the hippocampus, the septal area, and in various cortical areas (Beaulieu and Gainetdinov, 2011).

At postsynaptic level in MSN, D_3R modulates Ca^{2+} channels through Phospholipase C, and protein phosphatase 2B (Martel and McArthur, 2020). At extra-synaptic location (in cell bodies), D_3Rs are involved in selective modulation of Ca^{2+} influx through low-voltage activated (CaV3, T-type) Ca^{2+} channels, in a β-arrestin-dependent pathway (Martel and McArthur, 2020). D_3R turnover is regulated by the epidermal growth factor receptor (EGFR) tyrosine kinase signaling cascade (Sun *et al*., 2018). This is achieved by phosphorylation of GRK2 by EGFR, the GRK2 then phosphorylates the intracellular domain of the D_3R to promote D_3R intracellular receptor degradation (Sun *et al*., 2018). Compared to D_2R, D_3R undergoes limited agonist mediated receptor internalization (Kim *et al*., 2001).

D_3Rs are a target of interest for treatment of neuropsychiatric disorders and drug addiction. D_3R has similar signal transduction mechanisms to D_2R, they both couple to inhibitory G protein alpha subunits $G_{i/o}$. Inhibition of D_2R signalling pathways is important for obtaining antipsychotic effects, but is associated with harmful effects on motor functions, causing extrapyramidal effects (Joyce and Millan, 2005). Thus, DR antagonists which are selective for D_3R over D_2R are not expected to produce these effects and this has been demonstrated with several D_3R selective antagonists (Silverdale *et al*., 2004). The link between drug addiction and D_3R has been established through evidence showing neuroplasticity changes in drug addicted subjects, such as the increase in D_3R density in cocaine addicts and methamphetamine polydrug users (Le Foll *et al*., 2014).

1.2.2 D_3R ligands

Several D_3R ligands have been developed, with therapeutic potential for treatment of drug addiction and neuropsychiatric disorders. Some of the early D_3R selective ligands that have attracted interest include BP897, NGB2904, and FAUC 365 (Bettinetti *et al*., 2002; Xi and Gardner, 2007; Yuan *et al*., 1998). Studies done with NGB2904 have shown that it inhibited cocaine self administration, cocaine-seeking behaviour and other addictive drug-enhanced brain stimulation reward (Xi and Gardner 2007). RGH-188, another D_3R selective ligand, also known as cariprazine, was shown to diminish the reward effect of cocaine and decreased cocaine relapse (Román *et al*., 2013). It was approved in 2015 in the USA for the treatment of schizophrenia and bipolar disorders under the brand name Vraylar (Maramai *et al*., 2016).

D_3R ligands can also be labelled with a radioisotope or a fluorescent dye to study receptor-ligand binding interactions. Radioligands have been used for a long period of time for studying ligand binding to D_3R. Radioligands have been used for characterizing receptors in their natural environment and those transfected into cell lines; studying receptor dynamics

and localization; identifying novel chemical structures that interact with receptors; and for defining ligand activity and selectivity in normal and diseased tissues (McKinney and Raddatz, 2006). However, there are safety concerns with radioligands and studying receptor-ligand binding kinetics with radioligands is very labour intensive. Thus, fluorescent ligands have proven to be more promising to study receptor- ligand binding interactions (Stoddart *et al*., 2015). Fluorescent ligands can be used together with microscopy for studying cellular and tissue localisation of receptors (Sabirsh *et al.*, 2005). Additionally, fluorescent ligands can be used for studying receptor ligand binding kinetics, to probe receptor organization in the membrane and to study dimerization (Tabor *et al*., 2017). However, there are very few fluorescent ligands available for studying D_3R (Allikalt *et al.*, 2020, 2021).

1.3 Receptor ligand binding assays

The aim of receptor ligand binding assays is to study interactions between ligands and receptors. The principal aspects of receptor-ligand binding interactions include binding affinity and kinetics, conformations of targets, binding thermodynamics, and ligand efficiency. Each aspect can be analysed using a different ligand binding assay (Yakimchuk, 2011).

For labelled ligand binding assays, it is crucial to ensure that the labelled ligand is actually bound to the receptor, irrespective of the type of experiment performed (Flanagan, 2016). Most labelled ligands bind, to some extent, to cell membranes, proteins, plastic or glassware present in the assay, in addition to the receptor (Flanagan, 2016). Binding to sites other than the receptor active binding site is known as nonspecific binding. The total binding measured in the experiment is the sum of specific and nonspecific binding of the labelled ligand. In general, nonspecific binding of the labelled ligand is considered to be linear and non-saturable, thus, nonspecifically bound labelled ligand is not displaced even if excess of unlabeled ligand is used (Bylund and Toews, 2011; Hulme and Trevethick, 2010). Therefore, nonspecific binding is usually measured by adding excess of unlabelled competitive ligand so that it occupies all the receptor binding sites, and prevents the labelled ligand from binding to the receptor, but does not affect interactions of the labelled ligand with nonspecific binding sites (Flanagan, 2016). This measure of nonspecific binding is then subtracted from total binding to calculate specific binding of the labelled ligand to the receptor.

1.3.1 Kinetic aspects of receptor-ligand binding assays

The relationship between the labelled ligand L, the free receptor R, and the receptor-labelled ligand complex RL, can be described by the simple reversible reaction (Jong *et al.*, 2005):

$$[R] + [L] \underset{k_{OFF}}{\overset{k_{ON}}{\rightleftarrows}} [RL] \quad (1)$$

where k_{ON} and k_{OFF} are the association and dissociation rate constants respectively.

At equilibrium, the rate of formation of receptor-ligand complex is equal to the rate of dissociation of receptor-ligand complex:

$$k_{ON} \times [R] \times [L] = k_{OFF} \times [RL] \quad (2)$$

Rearranging equation (2) gives the equilibrium dissociation constant, K_d:

$$K_d = \frac{k_{OFF}}{k_{ON}} = \frac{[L] \times [R]}{[RL]} \quad (3)$$

Introduction of an unlabelled ligand which is competing for the receptor binding site (competitor ligand) leads to the formation of two types of receptor complexes: receptor-labelled ligand complex, and receptor-competitor complex. The competitor displaces certain amount of the labelled ligand and this depends on both the concentration of the competitor and its affinity for the receptor (Jong *et al.*, 2005).

Incubation of a series of concentration of the competitor in the presence of fixed concentrations of the receptor and the labelled ligand, generates inhibition curves from which IC_{50} values can be found. IC_{50} values describe the concentration of competitor which displaces 50 % of bound labelled ligand from the receptor (Jong *et al.*, 2005).

The IC_{50} value is related to the affinity constant K_i of the competitor by the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$IC_{50} = K_i \times \left(1 + \frac{[L]}{K_d}\right) \quad (4)$$

where K_d is the equilibrium dissociation constant of the labelled ligand-receptor complex, and [L] is the concentration of the labelled ligand used in the assay.

Since Cheng-Prusoff equation is derived from enzymatic reactions, it is based on some assumptions when used in the context of ligand binding assay: the system should be at equilibrium, binding should be reversible, the competitor and labelled ligand should bind to

the same site and there is no significant depletion of labelled ligand nor competitor upon binding to the target (Newton *et al*., 2008).

Radioligand binding assay is done with large excess of the radioligand compared to the receptor. Thus, the free radioligand concentration is almost equal to the total concentration of radioligand $[L]_{total}$. The total concentration of the radioligand used in each experiment is a known value, as it is measured during each experiment.

Thus, $K_d = \frac{[R]\times[L]}{[RL]} = \frac{([R]_{total}-[RL])\times[L]_{total}}{[RL]}$, where $[R]_{total}$ is the concentration of receptor added.

Saturation of the receptor binding sites with high amount of labelled ligand enables us to find the concentration of specific binding sites on the receptor. This parameter describes the concentration of available binding sites and is denoted as B_{max}.

$$[RL] = \frac{B_{max}\times[L]_{total}}{K_d+[L]_{total}} \quad (5)$$

When $[L]_{total}$ is equal to K_d, then $[RL] = B_{max}/2$, thus, K_d describes the concentration of the radioligand at which 50% of the receptors are occupied by it.

1.3.2 Radioligand binding assay

Radioligand binding assay, first developed by Paton and Rang (Paton and Rang, 1965) has been thereafter used extensively in GPCR studies. Radioligand binding assay usually involves incubation of a receptor preparation with a radiolabeled ligand for a specific period of time at a specific temperature. The receptor bound radioligand is then separated from the free radioligand, usually by filtration (McKinney and Raddatz, 2006). The amount of bound radioligand to the receptor is then measured using liquid scintillation spectroscopy (McKinney and Raddatz, 2006) (Figure 3). The radioactivity count obtained from the receptor bound radioligand describes the total binding.

The choice of radioisotope is a crucial aspect in the synthesis of the radioligand to be used in radioligand binding assay. Radio isotopes such as ^{3}H, ^{125}I are frequently used for labelling ligands. They have slow radioactive decay because of their long half life (12.4 years), thus they can be stored and used for long periods (Jong *et al*., 2005). Moreover, they can be used to label ligands with very slight modifications on the structure (Flanagan, 2016; Jong *et al*., 2005).

The major advantages of radioligand binding assay include sensitivity, specificity and ease of use (Jong *et al.*, 2005). The major disadvantages of these assays are that radioactivity requires disposal of radioactive wastes and can also be a health hazard, and the need to separate free from bound ligand, which make these assays labour-intensive and relatively slow (Schnurr *et al.*, 2006). Scintillation proximity assay is an alternative method that doesn't require separation of bound and free radioligand. In scintillation proximity assay, the receptor is immobilized on a solid surface (bead) which contains a scintillant and the ligand is labelled with a radioactive isotope. In this method, only the radioligand bound to the scintillation beads can cause light emission from the bead, thus there is no need to separate the free and bound radioligand (Schnurr *et al.*, 2006). However, scintillation proximity assay is more time consuming than filtration based assay (Jong *et al*., 2005).

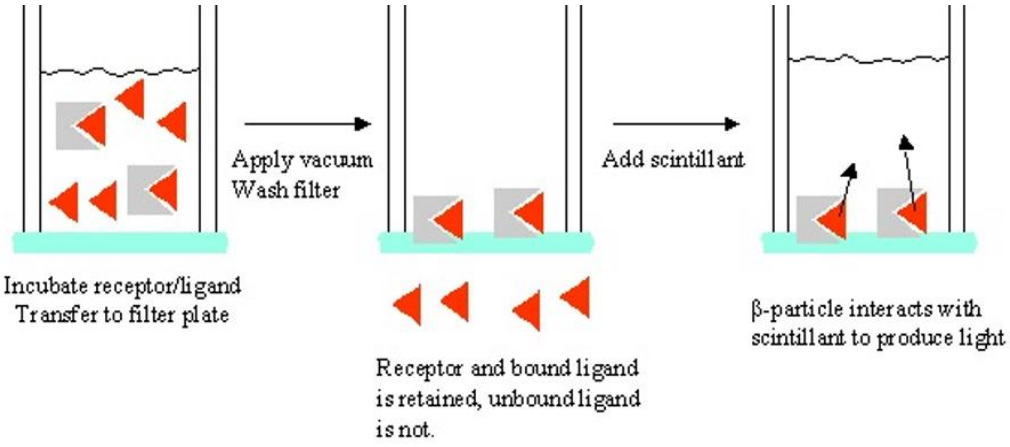

Figure 3. Procedure for filtration based radioligand binding assay. The receptor preparation and radioligand are incubated for a specific period of time at a specific temperature. The reaction is stopped by separating the receptor-bound radioligand from the free radioligand by vacuum filtration. Scintillant is then added to the filtermat containing the bound radioligand. The β particles emitted during radioactive decay interact with the scintillant to produce light which is detected by a luminescence counter (Auld *et al*., 2012)

1.3.3 Fluorescence anisotropy assay

Fluorescence anisotropy (FA) assay is a powerful method for studying ligand binding to receptors. FA assay is based on selective excitation of a subpopulation of fluorophores (a phenomenon known as photoselection) and monitoring the polarized emission (Shepherd and Fuentes, 2011). Plane polarized light from an appropriate polarizer is used to excite the sample and the fluorophore molecules having absorption transition dipole parallel to the

electric field vectors of the photons are more likely to get excited (Rinken *et al*., 2018). The excited molecules then emit polarized light with an electric field vector parallel to the emission transition dipole of the molecules (Rinken *et al*., 2018). There is partial depolarization of the emitted light if the molecules have rotational freedom (Rinken *et al*., 2018). The extent of depolarization can be characterized by measuring the change in fluorescence anisotropy r:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2 \cdot I_{\perp}} \quad \text{(a)}$$

where $I_{\parallel}$ is the fluorescence intensity when the excitation and emission polarization are parallel, $I_{\perp}$ is the fluorescence intensity when the excitation and emission polarization are perpendicular.

The total fluorescence intensity (TFI) is calculated as shown in (b):

$$TFI = I_{\parallel} + 2 \times I_{\perp} \quad \text{(b)}$$

Fluorescence anisotropy is related to the rotational correlation time τ_c (the average time taken by a molecule to rotate one radian) of the fluorescent particle and the fluorescence lifetime of the fluorophore τ by the Perrin's equation:

$$r = \frac{r_0}{\left(1 + \frac{\tau}{\tau_c}\right)} \quad \text{(c)}$$

where r_0 is the value of anisotropy at $t = 0$ after short pulse excitation (Shepherd and Fuentes, 2011).

For spherical molecules, the rotational correlation time is related to the molecular weight (M) of the molecule of interest according to the following equation:

$$\tau_c = \frac{\eta M \bar{v}_h}{RT} \quad \text{(d)}$$

where η is the viscosity of the solution, $\bar{v}_h$ is the specific volume of a hydrated molecule, R is the universal gas constant, and T is the absolute temperature (Shepherd and Fuentes, 2011).

These equations demonstrate that fluorescence anisotropy depends directly on the fluorophore's excited state lifetime τ and the rotational correlation time τ_c. When a fluorescent ligand binds to a GPCR, the size of the fluorescent particle increases, the

fluorescent particle rotates slower, thus generating high fluorescence anisotropy value (Figure 4).

The choice of fluorescent ligand is an important aspect of FA assay. A fluorescent ligand consists of a fluorophore and a pharmacophore. The attachment of the fluorophore to the pharmacophore should be rigid enough to prevent the fluorophore from rotating independently of the pharmacophore (Jameson and Ross, 2011). Additionally, the hydrophobicity of the fluorophore should be taken into account as it impacts the amount of nonspecific binding (Hughes *et al.*, 2014). Other important features that need to be considered include the fluorescence lifetime, excitation wavelength, quantum yield, extinction coefficient of the fluorophore (Lea and Simeonov, 2011). The fluorescence lifetime of the fluorophore (the time lapse between excitation of the fluorophore and fluorescence emission) should be close to the rotational correlation time of the molecule being studied to achieve a good fluorescence anisotropy signal to noise measurement (Gijsbers *et al*., 2016). The excitation wavelength of the fluorophore should be on the red side of the visible light spectrum to prevent autofluorescence (Jong *et al*., 2005). The quantum yield of the fluorophore (ratio of the number of emitted photons to the number of absorbed photons) and the molar extinction coefficient should also be as high as possible (Jong *et al*., 2005).

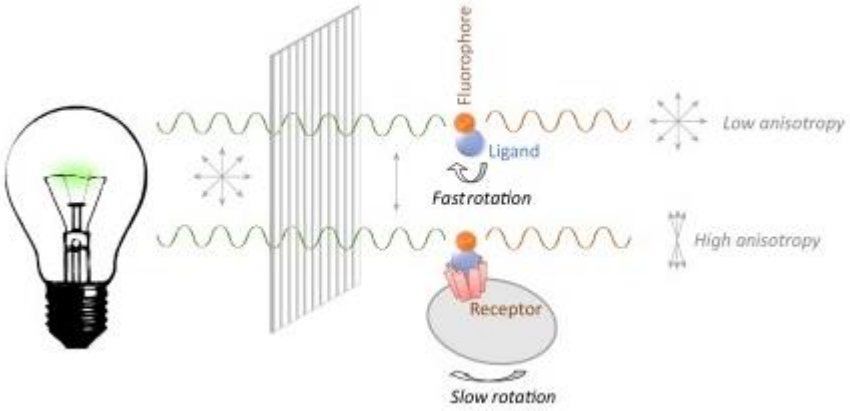

Figure 4. Principle of FA assay. Polarized light from a polarizer is used to excite a subpopulation of fluorophores. Binding of the fluorescent ligand to the receptor causes it to rotate slower, resulting in high fluorescence anisotropy, whereas the free fluorescent ligand in solution rotates fast and as a result the emitted light becomes highly depolarized, giving rise to low fluorescence anisotropy (Rinken *et al*., 2018).

1.4 Sf9/Baculovirus expression system

The source of receptors is a crucial aspect of receptor ligand binding assays. Due to the low natural abundance of GPCRs, heterologous expression of GPCRs is required (Massotte, 2003). GPCRs have been expressed in *E.coli*, yeast cells and mammalian cell lines (Massotte, 2003). The drawbacks of using bacteria for GPCR expression are the incapability of bacteria to perform necessary post-translational modifications important for protein function, lack of G proteins, and low expression levels (Massotte, 2003). Yeast cells are capable of performing post translational modifications similar to other more complex eukaryotic cells, but N-glycosylation of mammalian membrane proteins in yeast is not effective (Massotte, 2003). Mammalian cell lines such as CHO, HEK293, BHK-21 have been used for expressing GPCRs since they can perform the necessary post translational modifications, however, protein yields are low and scaling up is costly (Massotte, 2003; Saarenpää *et al*., 2015). Thus, a more effective system is required. Over-expression of GPCRs in insect cells has proven to be very efficient due to low maintenance requirements and the ability of insect cells to the required post translational modifications (Aloia *et al*., 2009).

Baculoviruses are enveloped, large double-stranded DNA viruses which mainly infect insects (Wang *et al*., 2016). The two insect cell lines widely used for GPCR expression are Sf21 and Sf9 derived from *Spodoptera frugiperda* ovarian tissue (Aloia *et al*., 2009). These cell lines enable replication of the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus which expresses the transgene encoding the GPCR during the viral infection and replication process (Aloia *et al*., 2009). In the Sf9/baculovirus expression system, the cDNA encoding the GPCR is first placed into a plasmid transfer under the polyhedrin promoter as the latter is a very strong promoter, and is not necessary for viral propagation in cell culture (Schneider and Seifert, 2010). Then, there is site specific transposition of the plasmid into a baculovirus shuttle vector (bacmid) (Luckow, 1993; Saarenpää *et al*., 2015). This bacmid is then propagated into *E.coli* bacteria, purified and transfected into Sf9 cells to produce recombinant baculoviruses (Saarenpää *et al*., 2015). During the insect cell infection cycle, budded baculoviruses (BBV) are formed as nucleocapsids which bud from the insect cell surface (Veiksina *et al*., 2014). Baculoviruses have a double lipid bilayer envelope derived from the Sf9 cell surface, containing membrane proteins from the host cell surface (Veiksina *et al*., 2014). The budded baculoviruses can be

separated from the Sf9 cells by centrifugation and used as a source of receptor for ligand binding assays (Veiksina *et al*., 2014).

Work involving baculoviruses are done on Biosafety Level 1, thus they are neither dangerous for the environment nor for humans (Veiksina *et al*., 2014).

2 THE AIMS OF THE THESIS

D_3R receptor has been studied extensively using radioligand binding methods. However, there are very few fluorescent ligands that are available for studying D_3R. A2-TAMRA is a novel fluorescent ligand with a pharmacophore that has subnanomolar affinity for D_3R. Since there hasn't been previous success using other fluorescent ligands for characterizing D_3R in FA assay, we decided to use it for FA assay. The radioligand [^{3}H]-methylspiperone has been previously used for studying ligand binding to D_3R. So, we studied [^{3}H]-methylspiperone binding to D_3R in parallel. Previous studies have shown that BBV is a good source of receptors for radioligand binding assay (Allikalt and Rinken, 2017). So, we used BBV as a receptor source in both assays. Since D_1R has also been studied previously in FA assay using BBV and another fluorescent ligand (Allikalt *et al*., 2018), we wanted to compare how selective A2-TAMRA is for D_3R compared to D_1R.

Thus, we performed the following experiments:

- Competition binding experiments between D_3R ligands and A2-TAMRA in FA assay for determination of affinities of known D_3R ligands
- Saturation binding experiment with D_1R in FA assay to compare selectivity of A2-TAMRA for D_3R compared to D_1R
- Saturation binding experiments for determination of concentration of available binding sites of the receptor (B_{max}) and the equilibrium dissociation constant (K_d) of the receptor-[^{3}H]-methylspiperone complex
- Competition binding experiments for determination of affinities of known D_3R ligands in radioligand binding assay

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 MATERIALS

Reagents and receptor source

$MgCl_2$, KCl, $CaCl_2$, NaCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), HCl, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) were from Applichem. Pluronic acid F-127 was from Sigma Aldrich and Na- HEPES was from Amresco. Complete EDTA free protease inhibitor cocktail (PIC) was from Roche Diagnostics.

D_3R ligands Quinpirole, 7-hydroxypipat maleate, 7-hydroxyDPAT hydrobromide, Sulpiride, Spiperone were from TOCRIS and Dopamine, Apomorphine, Butaclamol, Raclopride, Terguride were from Sigma Aldrich. Stock solutions of these ligands were prepared in DMSO (Applichem) or milli Q water.

[^{3}H]-methylspiperone (83.8 C_i/mmol) was from Perkin Elmer.

A2-TAMRA, the fluorescent ligand used in FA assay was synthesized by Dr Erki Enkvist and Hanna Riia Allas of University of Tartu based on FAUC 346 pharmacophore. Saturation binding experiments performed by Maris Johanna Tahk have shown that A2-TAMRA has a dissociation constant of 0.13 ± 0.03 nM. Kinetic studies have provided an average k_{off} of (6 ± 3)× 10^{-4} s^{-1} and an average k_{on} of (4 ± 4)× 10^{-3} $nM^{-1}s^{-1}$. These rate constants were obtained with SB toolbox 2 (Schmidt and Jirstrand, 2006), modified by Tõnis Laasfeld (unpublished data).

The source of receptor for this work was budded baculovirus particles (BBV) and BBV preparations done previously by Maris Johanna Tahk and Anni Allikalt were used in FA and radioligand binding assays. Description of budded baculovirus preparation is in (Allikalt and Rinken, 2017).

3.1.2 Fluorescence anisotropy assay

FA assay buffer consisted of 1 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 135 mM NaCl, 0.1% pluronic acid F-127, Complete EDTA-free PIC (used according to manufacturer's protocol), 11 mM Na-HEPES (pH 7.4) and Milli-Q water.

Stocks of the D_3R ligands and fluorescent ligand were stored in DMSO at -20 °C and diluted with assay buffer on the day of experiment.

All competition binding experiments were done on black 96-well half Area, flat bottom Polystyrene NBS™ plates (Corning), with a final reaction volume of 100 µL in each well. Measurements were done in duplicates and at least three independent experiments were performed for each D_3R ligand. Fixed concentrations of A2-TAMRA (1 nM) and BBV (volume of receptor stock in well = 5 µL) were incubated with serial dilutions of D_3R ligands. All experiments had blank wells as well as positive control wells. Blank wells contained only assay buffer and same amount of BBV as in other wells. Positive control wells had only assay buffer, BBV, and fluorescent ligand. FA plates were measured for three hours using Synergy NEO plate reader (BioTek) at 27 °C, with polarizing excitation filter of 530 nm (bandwidth 30 nm) and dual emission of 590 nm (bandwidth 35 nm).

For saturation binding experiment with D_1R, serial dilutions of BBV were pipetted to two fixed concentrations of the fluorescent ligand (0.5 nM, and 3 nM). Nonspecific binding determined with 5 µM butaclamol and 30 µM butaclamol respectively. Total binding was determined in the absence of butaclamol. Blank wells having just assay buffer (50 µL) and BBV (50 µL) were included in the experiment. The plate was read for two hours in Synergy NEO plate reader (BioTek) at 27 °C, with polarizing excitation filter of 530 nm (bandwidth 30 nm) and dual emission of 590 nm (bandwidth 35 nm).

3.1.3 Radioligand binding assay

All radioligand binding experiments were performed on round bottom 96-well plates (Greiner) and the final reaction volume in each well was 250 µL. The assay buffer consisted of 50 mM Tris-HCl, 120 mM NaCl, 5 mM $MgCl_2$,5 mM KCl, 1 mM EDTA. Before the experiment, 1 mM DTT was added to the falcon tube in which assay buffer was prepared. For determination of K_d and B_{max}, fixed amount of BBV (Volume of receptor stock in well = 1.88 µL) was added to two times serial dilutions of [^{3}H]-methylspiperone (starting concentration in well = 10 nM). Nonspecific binding was determined in the presence of 1 µM butaclamol and total binding was determined in the absence of butaclamol.

For competition binding experiments, fixed amounts of BBV (volume of receptor stock in well = 0.94 µL) and [^{3}H]-methylspiperone (concentration in well = 2.5 nM) were added to serial dilutions of D_3R ligands. Measurements were done in triplicates and for some ligands we performed at least 3 independent experiments while for others we couldn't perform three independent experiments, since we were running out of radioligand stock solution.

For all radioligand binding experiments, after the samples were added to the plate, the reaction medium was incubated for one hour at 25 °C on a shaker at 400 rpm. After that, the bound radioligand was separated from the free radioligand by filtration through thick glass fiber filtermats (Perkin Elmer) in FilterMate Harvester (Perkin Elmer). The filtermats were then washed five times with ice cold washing buffer (consisting of 20 mM potassium phosphate, 100 mM NaCl, pH =7.4). After that, the filtermats were dried in a microwave oven at 800W for two minutes. Solid scintillant MeltiLex^{TM} B/HS was then melted onto the filtermats using MeltiLex^{TM} Heatsealer. Radioactivity from the filters were measured with Wallac MicroBeta TriLux 1450 Luminescence Counter (Perkin Elmer). Total concentration of the radioligand was determined by pipetting 50 µL of the radioligand to vials containing 3 ml scintillation fluid (Perkin Elmer) and measuring the radioactivity count from Wallac MicroBeta TriLux 1450 Luminescence Counter (Perkin Elmer).

3.1.4 Data analysis

FA assay

Experimental data from FA was analysed with Aparecium 2.0.20 software (http://gpcr.ut.ee/aparecium.html) and data fitting was done on GraphPad Prism 5.04.

Parallel and perpendicular fluorescence intensities from blank wells were subtracted from all intensities from all the other points before FA and TFI calculation.

Data from saturation binding experiment was analyzed using a global model implemented in GraphPad Prism assuming a single binding site for the fluorescent ligand, the possibility of nonspecific binding, and a potential change in quantum yield upon binding (Veiksina *et al*., 2014).

Data from competition binding experiments was analysed with *Gen5* tools of Aparecium software. IC_{50} values were calculated using nonlinear fitting method from competition binding curves, generated by dose-response inhibition model in GraphPadPrism. pIC_{50} values from FA competition binding experiments was converted to pK_i values using Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Radioligand binding assay

The concentration of free radioligand in nanomolar was calculated using the formula: concentration of free radioligand (nM) = x counts per minute (cpm)/ (specific activity of radioligand cpm/femtomol x V_{final} µL), where x is the value obtained from counting of total

radioligand concentration in vials, and V_{final} is the final reaction volume in each well. Non-specific binding count was subtracted from total binding count to obtain count for specific binding. Specific binding count was converted to receptor concentration in nanomolar using the formula: C (receptor) = y cpm / (specific activity of radioligand CPM/femtomol x Volume of receptor stock in each well in μL), where y = count from specific binding.

Data from saturation binding experiments was analysed using one site-specific binding model in GraphPad Prism.

Data from competition binding experiments was analysed using one site-fit K_i model in GraphPad prism.

Data presented shows mean ± S.E.M.

3.2 RESULTS

3.2.1 Characterization of binding of A2-TAMRA to D_3R

We first characterized binding of A2-TAMRA to D_3R with the speed at which FA increases in our experiments. Binding of A2-TAMRA to D_3R caused an increase in FA for approximately 35 minutes in the case of total binding (Figure 5). We also got a very good signal window since the theoretical maximum value of FA is 0.4 (Lakowicz, 2006).

In addition to FA, TFI increased upon binding of A2-TAMRA to the receptor (Supplementary Figure 1). There was an average increase of 15 ± 3 % in TFI upon binding of A2-TAMRA to D_3R.

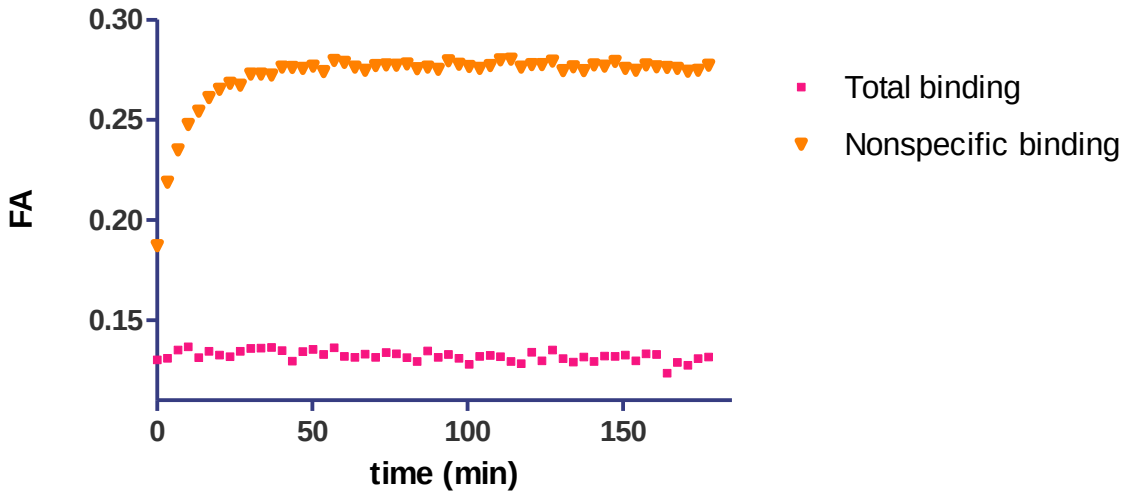

Figure 5. FA change in time caused by binding of A2-TAMRA to BBV displaying the D_3R. Nonspecific binding was determined with 316 µM 7-hydroxyDPAT hydrobromide. Total binding was determined in the absence of 7-hydroxyDPAT hydrobromide. FA took approximately 35 minutes to increase in the case of total binding. For nonspecific binding, FA increased very rapidly in the first few seconds, and there was no significant increase in FA throughout the reaction. Data shows representative of at least three independent experiments done in duplicate.

3.2.2 Determination of equilibrium dissociation constant (K_d) and concentration of available binding sites of the receptor (B_{max}) with [^{3}H]-methylspiperone

Saturation binding experiments were performed for determination of the K_d of [^{3}H]-methylspiperone-receptor complex and concentration of available binding sites of the

receptor (B_{max}) for [^{3}H]-methylspiperone (Figure 6). The K_d value is also required for calculations of K_i of the unlabelled D_3R ligands. Previous saturation binding experiments done with [^{3}H]-methylspiperone in CHO cell lines expressing D_3R have provided an average K_d of 0.6 ± 0.2 nM (Schmieg *et al*., 2016). Therefore we wanted to measure the K_d in BBV expressing D_3R. Three independent experiments were performed; the average K_d was 0.7 ± 0.2 nM, and the average B_{max} was 35 ± 4 nM.

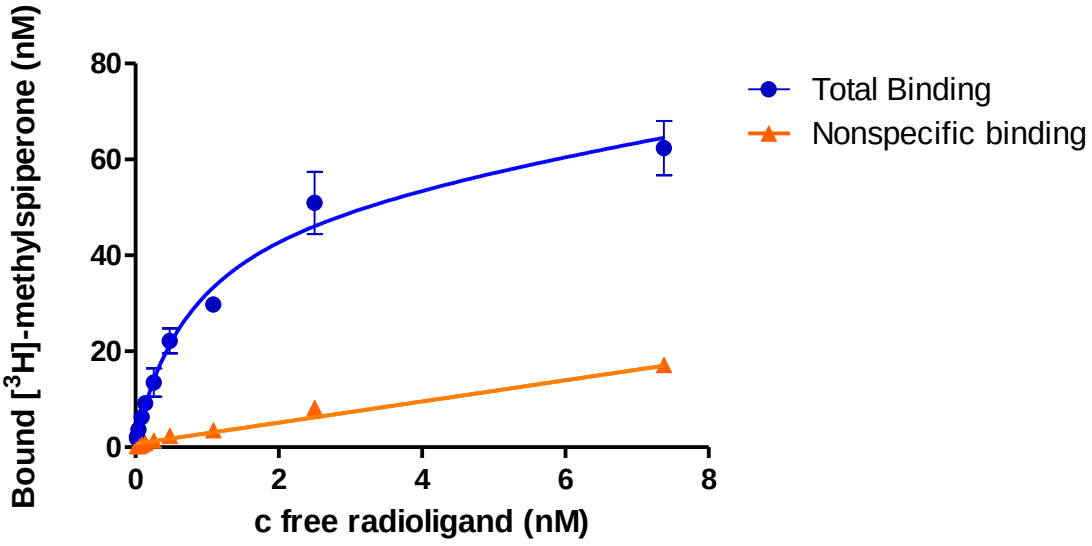

Figure 6. Binding of [^{3}H]-methylspiperone to D_3R on BBV. Serial dilutions of [^{3}H]-methylspiperone were incubated with BBV (volume of receptor stock in well = 1.88 µL) for one hour; nonspecific binding was determined with 1 µM butaclamol and total binding was determined in the absence of butaclamol. Data shows mean ± S.E.M from a representative experiment of three independent experiments performed in triplicates.

3.2.3 Comparison of selectivity of A2-TAMRA for D_3R compared to D_1R

D_1R on BBV has been previously studied in FA assay using another fluorescent ligand (Allikalt *et al*., 2018). Therefore, saturation binding experiment was performed with D_1R to determine selectivity of A2-TAMRA for D_3R compared to D_1R. The pharmacophore of A2-TAMRA is known to have a K_i value of 0.23 ± 0.02 nM with D_3R and a K_i value of 670 ± 15 nM with D_1R (Bettinetti *et al*., 2002).

For radioligand saturation binding experiments, we were able to use increasing concentrations of the radioligand. However, this approach doesn't work with FA saturation binding experiments. FA is a ratiometric assay, which means that it depends on the ratio of

both free and bound fluorescent ligand to get a good signal (Rinken *et al.*, 2018). Using excess of fluorescent ligand will increase the amount of unbound ligand, giving rise to a smaller change in FA. Thus, to measure K_d of a labelled ligand using FA, a different approach has to be used. This can be achieved by using two fixed concentrations of labelled ligand with serial dilutions of the receptor to provide increasing receptor concentration (Veiksina *et al.*, 2014). This approach was previously used by Maris Johanna Tahk to determine K_d of A2-TAMRA with D_3R (Figure 7A). The same approach was used to perform saturation binding experiment with D_1R. Increasing the receptor concentration provided increase in FA with both concentrations of A2-TAMRA. However, even at higher concentration of A2-TAMRA, there was a very small difference between nonspecific and total binding (Figure 7B).

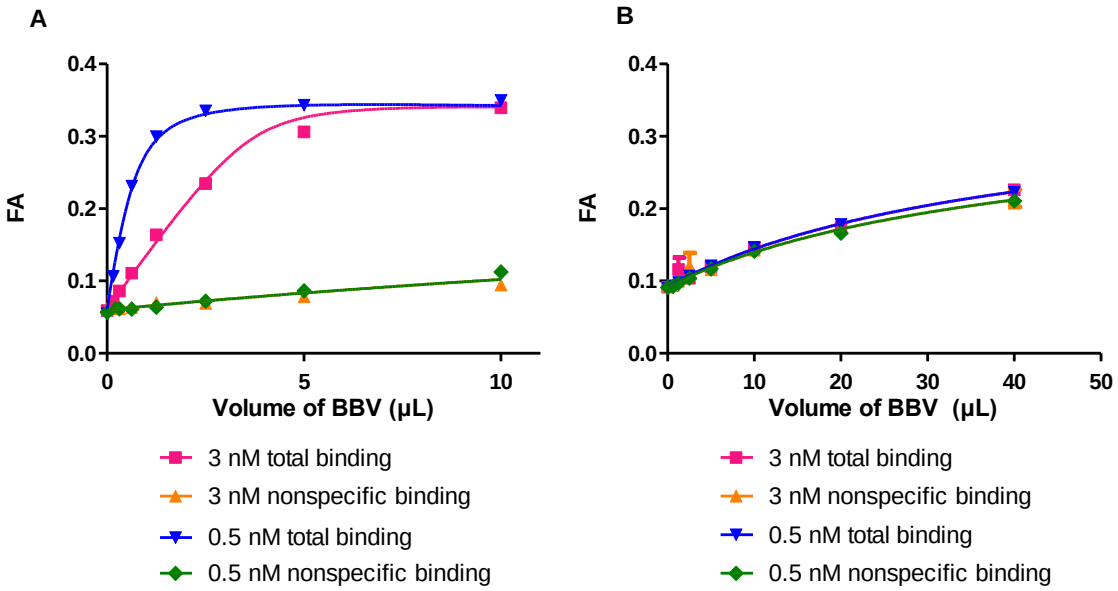

Figure 7. Binding of A2-TAMRA to D_1R and D_3R on BBV. (A) Binding of A2-TAMRA to D_3R. Serial dilutions of BBV were incubated with two fixed concentrations of A2-TAMRA (0.5 nM and 3 nM). Nonspecific binding was determined in the presence of 3 µM butaclamol and total binding was determined in the absence of butaclamol. Data shows representative experiment of four independent experiments done in duplicates. (B) Binding of A2-TAMRA to D_1R. Serial dilutions of BBV were incubated with two fixed concentrations of A2-TAMRA (0.5 nM and 3 nM). Nonspecific binding was determined with 5 µM and 30 µM butaclamol respectively. Total binding was determined in the absence of butaclamol.

3.2.4 Competition binding between D_3R ligands and A2-TAMRA and [^{3}H]-methylspiperone

Membrane preparations have been used previously as a source of receptor in radioligand competition binding experiments, but it has been shown previously that BBV preparations are also good receptor sources for radioligand binding assay (Allikalt and Rinken, 2017). Therefore, we used BBV as receptor source in both radioligand and FA competition binding experiments to determine the affinities of previously well characterized D_3R ligands (Figure 8).

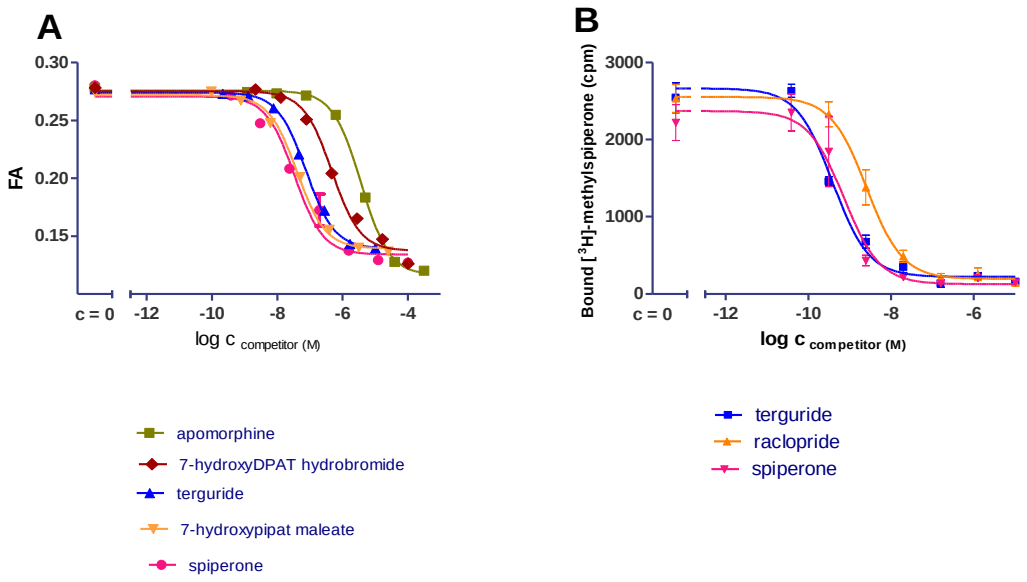

Figure 8. Competition binding between the D_3R ligands and A2-TAMRA or [^{3}H]-methylspiperone. (A) Competition binding between D_3R ligands and A2-TAMRA. Fixed concentrations of BBV (volume of receptor stock in well = 5 µL) and A2-TAMRA (concentration in well = 1 nM) were incubated with serial dilutions of the competitor ligands for three hours. Data shows representative experiment of at least three independent experiments done in duplicates. (B) Competition binding between D_3R ligands and [^{3}H]-methylspiperone. Fixed concentrations of BBV (volume of receptor stock in well = 0.94 µL) and [^{3}H]- methylspiperone (concentration in well = 2.5 nM) were incubated with serial dilutions of the competitors for one hour. Data shows mean ± S.E.M from a representative experiment of two to three independent experiments done in triplicates.

The incubation time of three hours was chosen for FA since A2-TAMRA hasn't been used with D_3R in competition binding experiments before, so we wanted to be sure that the incubation time is enough for equilibrium to be reached. FA assay enables monitoring of the change in IC_{50} values in real time. This enables us to know at what time the IC_{50} values are

stable. For the D_3R ligands ligands quinpirole, 7-hydroxypipat maleate, and raclopride, we noticed an intial drop in log IC_{50} in the beginning (Figure 9).

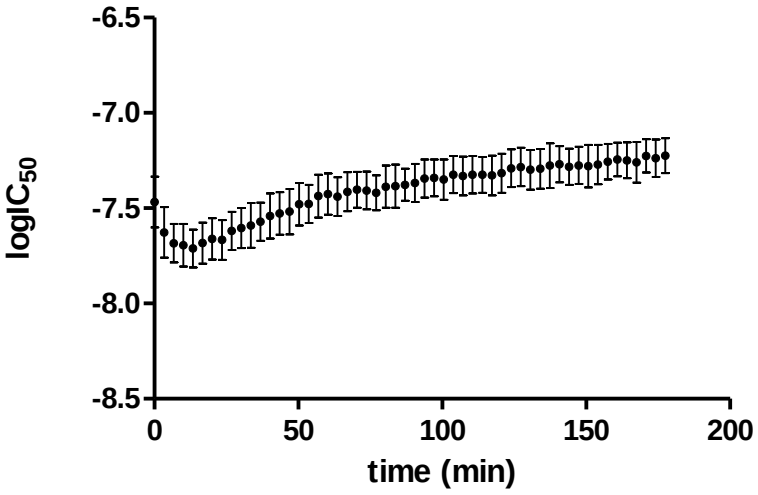

Figure 9. **Change in IC_{50} with time during displacement of A2-TAMRA by 7-hydroxypipat maleate.** $LogIC_{50}$ was obtained from competition binding experiments where D_3R ligands were competing with A2-TAMRA (concentration in well = 1 nM) for the receptor binding site. Data shows mean ± S.E.M.

For radioligand binding assay, the incubation time of one hour was chosen since this incubation time has been previously reported with [^{3}H]-methylspiperone (Schmieg *et al.*, 2016).

3.2.5 Correlation of affinities from FA and radioligand binding assay

To compare how consistent the two methods are with each other, the measured affinities of D_3R ligands from both methods were compared with each other. Since IC_{50} values depend on labelled ligand concentration, the IC_{50} values from FA assay were first converted to pK_i values using Cheng-Prusoff equation (Supplementary table 1) (Cheng and Prusoff, 1973). A very good correlation with very narrow confidence intervals (Figure 10) was obtained (unadjusted R^2 of the correlation = 0.94) between both methods. Also, the uncertainty was smaller in the case of FA.

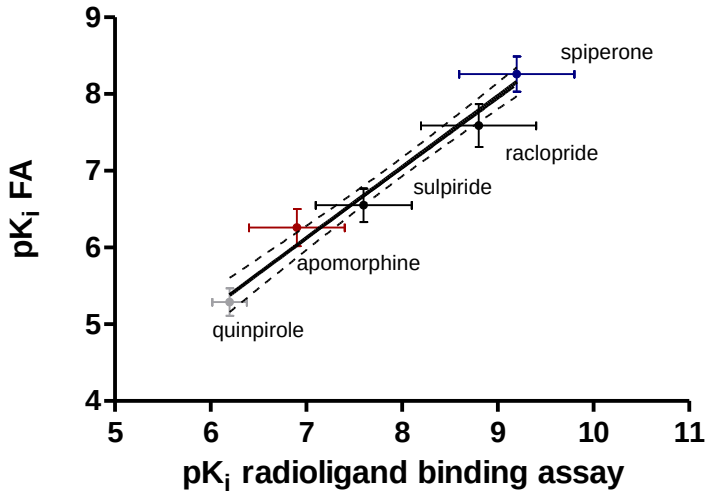

Figure 10. Correlation between pK_i obtained from FA assay and radioligand binding assay. Solid line shows linear regression of pK_i values measured from FA and radioligand binding assay. Dashed lines show 95% confidence intervals. Vertical error bars show uncertainty limits for FA assay, and horizontal error bars show uncertainty limits for radioligand binding assay.

3.3 DISCUSSION

Previous studies have shown that A2-TAMRA has a small K_d (materials section), implying high affinity for D_3R. This is demonstrated by the speed of FA increase in the case of total binding of A2-TAMRA to D_3R. Thus, A2-TAMRA is a suitable fluorescent ligand for screening known D_3R ligands in FA assay. We also saw that, compared to D_1R, A2-TAMRA is a D_3R selective ligand.

The pK_i values from FA and radioligand binding assay were compared with previously determined pK_i values (Burris *et al.*, 1995; Chumpradit *et al.*, 1994; Sautel *et al.*, 1995; Freedman *et al.*, 1994; MacKenzie *et al.*, 1994; Mierau *et al.*, 1995; Millan *et al.*, 1995, 2002; Sokoloff *et al.*, 1990; Pugsley *et al.*, 1995; Sokoloff *et al.*, 1992; Tang *et al.*, 1994; Van Tol *et al.*, 1991) to see how consistent our results are with previously determined pK_i values. pK_i values from literature were determined in radioligand competition binding experiments and each reference used a different radioligand. Also, in literature, cell lines such as CHO, and HEK293 were used. These have mammalian G proteins, which can couple to the receptor. The difference between literature affinities and affinities from both radioligand binding assay and FA assay was bigger in the case of the full agonists quinpirole and dopamine. There was just one reference for the full agonist 7-hydroxypipat maleate in literature and the FA pK_i was higher than that value. But the antagonists and partial agonists had a good correlation with literature data in the case of both radioligand binding and FA assay (unadjusted R^2 of the correlation = 0.88) (supplementary figures 2,3B). Agonists display higher affinity for the receptor when G proteins are coupled to the receptor (De Lean *et al.*, 1980; Gether, 2000). For this thesis, we used BBV as receptor source and these don't have G proteins, which may be the reason why the full agonists didn't have a good correlation with literature data. We nonetheless got a very good correlation between both methods and we saw that there is higher precision in the case of FA assay.

FA assay has several advantages over radioligand binding assay. FA is a fast and homogeneous assay while radioligand binding assay is time consuming and it requires the wash and filtration step. Since FA is a real time assay, it enables measurement of association and dissociation rate constants of the fluorescent ligand. Measurement of association and dissociation rate constants can be very challenging with the filtration based radioligand binding assay. From our experiments, we could also observe an intial drop in $logIC_{50}$ for some of the competitors. This could be a hint that the competitor ligand has slower binding kinetics compared to the fluorescent ligand. The binding kinetics of the competitors can also

be accurately measured using kinetic modelling, but this is subject for further studies. Additionally, FA is a high throughput assay, FA assay has been done in 384 well-plate format (Allen *et al*., 2000) as well as 1536 well-plate format (Harris *et al*., 2003).

Since we were able to successfully use A2-TAMRA for screening the D_3R ligands in FA assay, we can now consider other studies of D_3R using A2-TAMRA. Studies of receptors using fluorescent ligand can be used to either directly measure fluorescence or indirectly measure resonance energy transfer (RET) (Soave *et al*., 2020). For Nano-bioluminescence resonance energy transfer (NanoBRET) binding assay, the Nanoluc, which is a genetically modified luciferase, is fused to the GPCR of interest (Grätz *et. al*., 2020). It requires a fluorescent ligand whose excitation spectrum should overlap with the bioluminescence spectrum of NanoLuc, as BRET acceptor (Grätz *et. al*., 2020). After binding of a fluorescent ligand to the luciferase-tagged GPCR, the fluorescent ligand emits light due to bioluminescence resonance energy transfer (Grätz *et al*., 2020). The use of the fluorophore TAMRA has been previously shown to be good with BRET-based assays (Hoare *et al*., 2019; Sakyiamah *et al*., 2019; Stoddart *et al.,* 2015). In this work, we have shown that A2-TAMRA has very high affinity for D_3R and since it has slow dissociation kinetics, it can be used to study D_3R-ligand binding interactions in NanoBRET assay. NanoBRET assay can be used to study using receptor-ligand binding interactions in live cells as well and there is also less autofluorescence with NanoBRET assay (Brown *et al*., 2015; Dale *et al*., 2019).

Confocal microscopic studies of specific fluorescent ligand binding may be used for analysis of the distribution of receptors in tissue sections (Beaudet *et al*., 1998). For studies using tissue sections, it is important to choose a fluorophore emitting in the red or far red region to avoid cellular autofluorescence (Stoddart *et al*., 2016). TAMRA is an orange dye, with excitation peak at 552 nm, and emission peak at 578 nm (Blommel *et al.,* 2004*)*. Fluorophores that emit in the far red area include cyanine 5 (Patsenker *et al.,* 2011). However, it has a small fluorescence lifetime (Patsenker *et al.*, 2011). Thus, it is not suitable even for FA assay. For fluorescent ligand binding to receptors in tissue sections, a fluorescent ligand with a small dissociation rate constant is preferred, so that it still remains bound to the receptor after the wash steps involved in fluorohistochemical studies. So, swapping TAMRA fluorophore with Cy5 would not be suitable for tissue sections. However, there are different reagents and treatments, such as sodium borohydride and Sudan Black B that can be used to reduce autofluorescence from tissues (Baschong *et al*., 2001).

Fluorescent ligands have been used in Total Internal Reflection Fluorescence (TIRF) microscopy-based assays to study receptor dimerization. TIRF microscopy is a wide field imaging technique involving excitation of fluorescent molecules within a thin section of a sample (Stoddart *et al*., 2015). Due to its sensitivity and restricted illumination, TIRF is excellent for single molecule imaging and for identifying individual receptors and receptor complexes in a timeframe of seconds to gain insight about their mobility and clustering (Hern *et al*., 2010). D_3R forms dimers with D_1R, and D_2R and other GPCRs as well (Prieto, 2017). The results of the works for this thesis confirm that A2-TAMRA binds well to D_3R in BBV and studies have shown that BBV works in FA as well as in TIRF assay (Laasfeld *et al*., 2021). A2-TAMRA's high affinity for D_3R makes it a suitable candidate to be used in TIRF microscopy for single molecule imaging.

In conclusion, we can say that the results of the FA assay show that it can be adapted to high-thoughput format and we have validated the FA assay with a radioligand binding assay. The properties of A2-TAMRA opens up possibilities for studying D_3R using other fluorescence-based spectroscopy and microscopy methods.

SUMMARY

Dopamine receptors, which consist of five different subtypes (Dopamine D_{1-5} subtypes) play important roles in the central nervous system as well as in the periphery. Due to its limited distribution, Dopamine D_3 receptor is involved in emotional and cognitive functions. Abnormalities in D_3 receptor signalling are linked to several neuropsychiatric disorders. Thus, there have been several efforts to develop several ligands targeting Dopamine D_3 receptor. Development of a ligand suitable for therapeutic use involves several studies including studies of kinetic properties, measurement of the affinity of the ligand for the receptor and structural studies.

For a long time, Dopamine D_3 receptor ligands have been labelled with a radioisotope for measuring affinities of unlabelled ligands for D_3 receptor. These radioligands have also been used to study localisation of receptors in diseased tissues. However, studies of radioligand binding to receptors can be labour intensive and radioactivity experiments require disposal of radioactive waste and can represent a health hazard. Thus, fluorescent ligands have emerged as a more promising alternative for studying ligand binding to receptors. They have been used to study several other receptors, but there are limited fluorescent ligands available for studying dopamine D_3 receptor. A2-TAMRA is a novel fluorescent ligand which has a pharmacophore that has been shown to have good affinity for D_3 receptor.

Fluorescence anisotropy assay is a powerful technique for studying ligand binding to receptors. Since there hasn't been any successful fluorescent ligand for studying Dopamine D_3 receptor in fluorescence anisotropy assay previously, we decided to use A2-TAMRA in fluorescence anisotropy assay. We used it to screen known unlabelled D_3 receptor ligands in fluorescence anisotropy assay. We also screened those ligands in parallel in radioligand binding experiments with a radioligand ([^{3}H]-methylspiperone) that has been previously used to study D_3 receptor. We used budded baculoviruses as our receptor source in both assays since they have been previously shown to be a good receptor source for both fluorescence anisotropy and radioligand binding assay. We also investigated whether A2-TAMRA would work in fluorescence anisotropy assay with another dopamine receptor as well (Dopamine D_1 receptor). In the end, we compared the results of fluorescence anisotropy assay with the radioligand binding assay.

We saw that A2-TAMRA binds well to D_3 receptor displayed on budded baculoviruses and it prefers D_3 receptor over D_1 receptor. We also got a very good correlation between both methods. Fluorescence anisotropy assay can also be used to monitor ligand binding in real

time and kinetic properties can be accurately measured using kinetic modelling, but this remains subject for further study. The properties of A2-TAMRA make it a suitable candidate for further studies using nanobioluminescence resonance energy transfer, and total internal reflection fluorescence microscopy.

REFERENCES

Allen, M., Reeves, J., & Geoffrey, M. (2000). High Throughput Fluorescence Polarization: A homogeneous Alternative to Radioligand Binding for Cell Surface Receptors. *Journal of Biomolecular Screening*, *5*.

Allikalt, A., Kopanchuk, S., & Rinken, A. (2018). Implementation of fluorescence anisotropy-based assay for the characterization of ligand binding to dopamine D1 receptors. *European Journal of Pharmacology*, *839*(September), 40–46. https://doi.org/10.1016/j.ejphar.2018.09.008

Allikalt, A., Laasfeld, T., Ilisson, M., Kopanchuk, S., & Rinken, A. (2021). Quantitative analysis of fluorescent ligand binding to dopamine D3 receptors using live-cell microscopy. *FEBS Journal*, *288*(5), 1514–1532. https://doi.org/10.1111/febs.15519

Allikalt, A., Purkayastha, N., Flad, K., Schmidt, M. F., Tabor, A., Gmeiner, P., Hübner, H., & Weikert, D. (2020). Fluorescent ligands for dopamine -. *Scientific Reports*, 1–13. https://doi.org/10.1038/s41598-020-78827-9

Allikalt, A., & Rinken, A. (2017a). Budded baculovirus particles as a source of membrane proteins for radioligand binding assay: The case of dopamine D1 receptor. *Journal of Pharmacological and Toxicological Methods*, *86*(April), 81–86. https://doi.org/10.1016/j.vascn.2017.04.004

Allikalt, A., & Rinken, A. (2017b). Budded baculovirus particles as a source of membrane proteins for radioligand binding assay: The case of dopamine D1 receptor. *Journal of Pharmacological and Toxicological Methods*, *86*(November 2016), 81–86. https://doi.org/10.1016/j.vascn.2017.04.004

Aloia, A., V.Glatz, R., McMurchie, E. J., & Leifert, W. R. (2009). GPCR Expression using Baculovirus-Infected Sf9 cells. In *Methods in Molecular Biology* (Vol. 552, Issue 2, pp. 115–129). https://doi.org/10.1007/978-1-60327-317-6

Auld, D. S., Farmen, M. W., Kahl, S. D., Kriauciunas, A., McKnight, K. L., Montrose, C., & Weidner, J. R. (2012). Receptor Binding Assays for HTS and Drug Discovery. In *Assay Guidance Manual* (pp. 1–42). http://www.ncbi.nlm.nih.gov/pubmed/22553864

Baschong, W., Suetterlin, R., & Laeng, R. H. (2001). Control of Autofluorescence of Archival Formaldehyde-fixed , Paraffin-embedded Tissue in Confocal Laser Scanning Microscopy (CLSM). *The Journal of Histochemistry and Cytochemistry*, *49*(12), 1565–1571.

Beaudet, A., Nouel, D., Stroh, T., Vandenbulcke, F., Dal-Farra, C., & Vincent, J. P. (1998). Fluorescent ligands for studying neuropeptide receptors by confocal microscopy. *Brazilian Journal of Medical and Biological Research*, *31*(11), 1479–1489. https://doi.org/10.1590/S0100-879X1998001100017

Beaulieu, & Gainetdinov, R. R. (2011). The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacol Ogical Reviews*, *63*(1), 182–217. https://doi.org/10.1124/pr.110.002642.182

Beaulieu, J., Gainetdinov, R. R., & Caron, M. G. (2009). Akt / GSK3 Signaling in the Action of Psychotropic Drugs. *Annual Review of Pharmacology and Toxicology*, *49*, 327–349. https://doi.org/10.1146/annurev.pharmtox.011008.145634

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1985). Phosphorylation of the Mammalian β -

Adrenergic Receptor by Cyclic AMP-dependent Protein Kinase. *The Journal of Biological Chemistry*, *260*(11), 7094–7101. https://doi.org/10.1016/S0021-9258(18)88892-5

Berg, K. A., & Clarke, W. P. (2018). Making sense of pharmacology: Inverse agonism and functional selectivity. *International Journal of Neuropsychopharmacology*, *21*(10), 962–977. https://doi.org/10.1093/ijnp/pyy071

Bettinetti, L., Schlotter, K., & Gmeiner, P. (2002). Interactive SAR Studies : Rational Discovery of Super-Potent and Highly Selective Dopamine D3 Receptor Antagonists and Partial Agonists. *J Med Chem*, *45*, 4594–4597.

Blommel, P., Hanson, G. T., & Vogel, K. W. (2004). Multiplexing Fluorescence Polarization Assays to Increase Information Content Per Screen: Applications for Screening Steroid Hormone Receptors. *Journal of Biomolecular Screening*, 294–302. https://doi.org/10.1177/1087057104264420

Borroto-Escuela, D. O., & Fuxe, K. (2019). Oligomeric Receptor Complexes and Their Allosteric Receptor-Receptor Interactions in the Plasma Membrane Represent a New Biological Principle for Integration of Signals in the CNS. *Frontiers in Molecular Neuroscience*, *12*. https://doi.org/10.3389/fnmol.2019.00230

Brown, N. E., Blumer, J. B., & Hepler, J. R. (2015). Protein-protein interactions: Methods and applications: Second edition. *Methods Mol Biol*, *1278*, 1-457–465. https://doi.org/10.1007/978-1-4939-2425-7

Burris, K. D., Pacheco, M. A., Filtz, T. M., Kung, M. P., Kung, H. F., & Molinoff, P. B. (1995). Lack of discrimination by agonists for d2 and d3 dopamine receptors. *Neuropsychopharmacology*, *12*(4), 335–345. https://doi.org/10.1016/0893-133X(94)00099-L

Bylund, D. B., & Toews, M. L. (2011). Radioligand binding methods for membrane preparations and intact cells. *Methods in Molecular Biology*, *746*, 135–164. https://doi.org/10.1007/978-1-61779-126-0_8

Cheng, Y.-C., & Prusoff, W. H. (1973). Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochemical Pharmacology*, *22*(23), 3099–3108. https://doi.org/10.1016/0006-2952(73)90196-2

Chien, E. Y. T., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., Shi, L., Newman, A. H., Javitch, J. A., Cherezov, V., & Stevens, R. C. (2010). Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science*, *330*(6007), 1091–1095. https://doi.org/10.1126/science.1197410

Chumpradit, S., Kung, M. P., Vessotskie, J., Foulon, C., Mu, M., & Kung, H. F. (1994). Iodinated 2-Aminotetralins and 3-Amino-1-benzopyrans: Ligands for Dopamine D2 and D3 Receptors. *Journal of Medicinal Chemistry*, *37*(24), 4245–4250. https://doi.org/10.1021/jm00050a021

Dale, N. C., Johnstone, E. K. M., White, C. W., & Pfleger, K. D. G. (2019). NanoBRET: The bright future of proximity-based assays. *Frontiers in Bioengineering and Biotechnology*, *7*(MAR), 1–13. https://doi.org/10.3389/fbioe.2019.00056

De Lean, A., Stadel, J. M., & Lefkowitz, R. J. (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *Journal of Biological Chemistry*, *255*(15), 7108–7117. https://doi.org/10.1016/s0021-9258(20)79672-9

Di Chiara, G., & Bassareo, V. (2007). Reward system and addiction: what dopamine does and doesn't do. *Current Opinion in Pharmacology*, *7*(1), 69–76. https://doi.org/10.1016/j.coph.2006.11.003

Farooqui, T., & Farooqui, A. A. (2016). Perspective and Directions for Future Research on Trace Amines and Neurological Disorders. In *Trace Amines and Neurological Disorders: Potential Mechanisms and Risk Factors*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-803603-7.00025-2

Ferguson, S. S. G., Ill, W. E. D., Colapietro, A., Barak, L. S., Menard, L., & Caron, M. G. (1996). Role of P-Arrestin in Mediating Agonist-Promoted G Protein-Coupled Receptor Internalization. *Science*, *271*, 3–6.

Flanagan, C. A. (2016). GPCR-radioligand binding assays. *Biophysical Methods in Cell Biology*, *132*, 191–215. https://doi.org/10.1016/bs.mcb.2015.11.004

François Sautel, Nathalie Griffon, Daniel Lévesque, Catherine Pilon, Jean-Charles Schwartz, P. S. (1995). A functional test identifies dopamine agonists selective for D3 versus D2 receptors. *NeuroReport*, *6*(2), 329–332.

Freedman, S. B., Patel, S., Marwood, R., Emms, F., Seabrook, G. R., Knowles, M. R., & McAllister, G. (1994). Expression and pharmacological characterization of the human D3 dopamine receptor. *Journal of Pharmacology and Experimental Therapeutics*, *268*(1), 417–426.

Fu, J., Lee, T., & Qi, X. (2014). The identification of high-affinity G protein-coupled receptor ligands from large combinatorial libraries using multicolor quantum dot-labeled cell-based screening. *Future Med Chem*, *6*(7), 809–823. https://doi.org/10.4155/fmc.14.38.The

Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J., & Caron, M. G. (2004). Desensitization of G protein-coupled receptors and neuronal functions. *Annual Review of Neuroscience*, *27*, 107–144. https://doi.org/10.1146/annurev.neuro.27.070203.144206

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine Reviews*, *21*(1), 90–113. https://doi.org/10.1210/edrv.21.1.0390

Gijsbers, A., Nishigaki, T., & Sánchez-puig, N. (2016). Fluorescence Anisotropy as a Tool to Study Protein-protein Interactions. *Journal of Visualized Experiments*, *October*, 1–9. https://doi.org/10.3791/54640

Gingrich, J. A., & Caron, M. G. (1993). Recent advances in the molecular biology of dopamine receptors. *Annual Review of Neuroscience*, *16*, 299–231. https://doi.org/10.1146/annurev.ne.16.030193.001503

Grätz, L., Tropmann, K., Bresinsky, M., Müller, C., Bernhardt, G., & Pockes, S. (2020). NanoBRET binding assay for histamine H2 receptor ligands using live recombinant HEK293T cells. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-70332-3

Hall, H., Farde, L., & Sedvall, G. (1988). Neural Transmission Human dopamine receptor subtypesmin vitro binding analysis using. *Journal of Neural Transmission*, *73*, 7–21.

Harris, A., Cox, S., Burns, D., & Norey, C. (2003). Miniaturization of Fluorescence Polarization Receptor-Binding. *Journal of Biomolecular Screening*, *8*(4), 410–420. https://doi.org/10.1177/1087057103256319

Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *Federation of American Societies for Experimental Biology*, *4*(11), 2881–2889.

Hern, J. A., Baig, A. H., Mashanov, G. I., Birdsall, B., Corrie, J. E. T., & Lazareno, S. (2010). Formation and dissociation of M 1 muscarinic receptor dimers seen by total internal re flection fluorescence imaging of single molecules. *Proc Natl Acad Sci U S A.*, *107*(6), 1–6. https://doi.org/10.1073/pnas.0907915107

Hoare, B. L., Bruell, S., Sethi, A., Gooley, P. R., Lew, M. J., Hossain, M. A., Inoue, A., Scott, D. J., & Bathgate, R. A. D. (2019). Multi-Component Mechanism of H2 Relaxin Binding to RXFP1 through NanoBRET Kinetic Analysis. *CellPress*, *11*, 93–113. https://doi.org/10.1016/j.isci.2018.12.004

Hughes, L. D., Rawle, R. J., Boxer, S. G., Alexa, C., Alexa, C., Alexa, C., Alexa, C., Bodipy-tmr, C., Invitrogen, P., Nhs-ester, O. R. A., Nhs-ester, A., Se, A., Alexa, M., Alexa, M., & Se, A. (2014). *Choose Your Label Wisely : Water-Soluble Fluorophores Often Interact with Lipid Bilayers*. *9*(2). https://doi.org/10.1371/journal.pone.0087649

Hulme, E. C., & Trevethick, M. A. (2010). Ligand binding assays at equilibrium: Validation and interpretation. *British Journal of Pharmacology*, *161*(6), 1219–1237. https://doi.org/10.1111/j.1476-5381.2009.00604.x

Iversen, S. D., & Iversen, L. L. (2007). Dopamine: 50 years in perspective. *Trends in Neurosciences*, *30*(5), 188–193. https://doi.org/10.1016/j.tins.2007.03.002

Jackson, T. (1991). Structure and function of G protein coupled receptors. *Pharmacology and Therapeutics*, *50*(3), 425–442. https://doi.org/10.1016/0163-7258(91)90052-N

Jacob, C., & Bunnett, N. W. (2006). Transmembrane Signaling by G Protein-Coupled Receptors. In *Physiology of the Gastrointestinal Tract* (Fourth Edi, pp. 63–90). Elsevier Inc. https://doi.org/10.1016/B978-0-12-088394-3.50006-4

Jameson, D. M., & Ross, J. A. (2011). Fluorescence Polarization/Anisotropy in Diagnostics and Imaging. *Chem Rev*, *110*(5), 2685–2708. https://doi.org/10.1021/cr900267p.Fluorescence

Jong, L. A. A. De, Uges, D. R. A., Piet, J., & Bischoff, R. (2005). Receptor – ligand binding assays : Technologies and Applications. *Journal of Chromatography B*, *829*, 1–25. https://doi.org/10.1016/j.jchromb.2005.10.002

Joyce, J., & Millan, M. (2005). Dopamine D 3 receptor antagonists as therapeutic agents. *Drug Discovery Today*, *10*(13). http://www.sciencedirect.com/science/article/pii/S1359644605034914

Kasai, R. S., Ito, S. V, Awane, R. M., Fujiwara, T. K., & Kusumi, A. (2018). The Class-A GPCR Dopamine D2 Receptor Forms Transient Dimers Stabilized by Agonists : Detection by Single-Molecule Tracking. *Cell Biochemistry and Biophysics*, 29–37. https://doi.org/10.1007/s12013-017-0829-y

Kelly, E., Bailey, C. P., & Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. *British Journal of Pharmacology*, *153*, 379–388. https://doi.org/10.1038/sj.bjp.0707604

Kim, K., Valenzano, K. J., Robinson, S. R., Yao, W. D., Barak, L. S., & Caron, M. G. (2001). Differential Regulation of the Dopamine D 2 and D 3 Receptors by G Protein-coupled Receptor Kinases and -Arrestins *. *Journal of Biological Chemistry*, *276*(40), 37409–37414. https://doi.org/10.1074/jbc.M106728200

Kobilka, B. K. (2007). G protein coupled receptor structure and activation. *Biochimica et Biophysica Acta - Biomembranes*, *1768*(4), 794–807.

Koob, G. F., & Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology*, *35*(1), 217–238. https://doi.org/10.1038/npp.2009.110

Krupnick, J. G., & Benovic, J. L. (1998). THE ROLE OF RECEPTOR KINASES AND ARRESTINS IN G PROTEIN – COUPLED RECEPTOR REGULATION. *Annual Review of Pharmacology and Toxicology*, *38*(1), 289–319.

Laasfeld, T., Ehrminger, R., Tahk, M. J., Veiksina, S., Kõlvart, K. R., Min, M., Kopanchuk, S., & Rinken, A. (2021). Budded baculoviruses as a receptor display system to quantify ligand binding with TIRF microscopy. *Nanoscale*, *13*(4), 2436–2447. https://doi.org/10.1039/d0nr06737g

Lakowicz, J. R. (2006). Fluorescence Anisotropy. In *Principles of Fluorescence Spectroscopy* (pp. 353–382). https://doi.org/10.1007/978-0-387-46312-4_10

Le Foll, B., Wilson, A. A., Graff, A., Boileau, I., & Di Ciano, P. (2014). Recent methods for measuring dopamine D3 receptor occupancy in vivo: Importance for drug development. *Frontiers in Pharmacology*, *5 JUL*(July), 1–12. https://doi.org/10.3389/fphar.2014.00161

Lea, W. A., & Simeonov, A. (2011). Fluorescence polarization assays in small molecule screening. *Expert Opinion on Drug Discovery*, *6*(1), 17–32. https://doi.org/10.1517/17460441.2011.537322

Li, Z. S., Schmauss, C., Cuenca, A., Ratcliffe, E., & Gershon, M. D. (2006). Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: Analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. *Journal of Neuroscience*, *26*(10), 2798–2807. https://doi.org/10.1523/JNEUROSCI.4720-05.2006

Li, Z., Zhou, X., & Dai, Z. (2012). Classification of G proteins and prediction of GPCRs-G proteins coupling specificity using continuous wavelet transform and information theory. *Amino Acids*, *43*(2), 793–804. https://doi.org/10.1007/s00726-011-1133-6

Luckow, V. A. (1993). Baculovirus systems for the expression of human gene products. *Current Opinion in Biotechnology*, 564–572.

Luttrell, L M, Ferguson, S. S. ., Y.Daaka, Miller, W. ., S.Maudsley, Rocca, G. . Della, F.-T.Lin, H.Kawakatsu, K.Owada, D.K.Luttrell, Caron, M. ., & Lefkowitz, R. . (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science*, *283*, 655–661.

Luttrell, Louis M. (2006). Transmembrane Signaling by G Protein-Coupled Receptors. In *Methods in Molecular Biolody* (Vol. 332, pp. 3–49).

MacKenzie, R. G., VanLeeuwen, D., Pugsley, T. A., Shih, Y. H., Demattos, S., Tang, L., Todd, R. D., & O'Malley, K. L. (1994). Characterization of the human dopamine D3 receptor expressed in transfected cell lines. *European Journal of Pharmacology: Molecular Pharmacology*, *266*(1), 79–85. https://doi.org/10.1016/0922-4106(94)90212-7

Martel, J. C., & McArthur, S. G. (2020). Dopamine Receptor Subtypes , Physiology and Pharmacology : New Ligands and Concepts in Schizophrenia. *Frontiers in Neuroscience*, *11*, 1–17. https://doi.org/10.3389/fphar.2020.01003

Massotte, D. (2003). G protein-coupled receptor overexpression with the baculovirus-insect

cell system: A tool for structural and functional studies. *Biochimica et Biophysica Acta - Biomembranes*, *1610*(1), 77–89. https://doi.org/10.1016/S0005-2736(02)00720-4

McKinney, M., & Raddatz, R. (2006). Practical aspects of radioligand binding. *Current Protocols in Pharmacology / Editorial Board, S.J. Enna (Editor-in-Chief) ... [et Al.]*, *Chapter 1*(1), 1–42. https://doi.org/10.1002/0471141755.ph0103s33

Meiser, J., Weindl, D., & Hiller, K. (2013). Complexity of dopamine metabolism. *Cell Communication and Signaling*, *11*(1), 1–18. https://doi.org/10.1186/1478-811X-11-34

Mierau, J., Schneider, F. J., Ensinger, H. A., Chio, C. L., Lajiness, M. E., & Huff, R. M. (1995). Pramipexole binding and activation of cloned and expressed dopamine D2, D3 and D4 receptors. *European Journal of Pharmacology: Molecular Pharmacology*, *290*(1), 29–36. https://doi.org/10.1016/0922-4106(95)90013-6

Millan, M. J., Maiofiss, L., Cussac, D., Audinot, V., Boutin, J. A., & Newman-Tancredi, A. (2002). Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I. A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics*, *303*(2), 791–804. https://doi.org/10.1124/jpet.102.039867

Millan, M. J., Vian, J., Gobert, A., & Bervoets, K. (1995). D3 Receptor Activation in the Rat In Vivo and Their Modulation and Catalepsy. *The Journal of Pharmacology and Experimental Therapeutics*, *275*(2), 885–898.

Mishra, A., Singh, S., & Shukla, S. (2018). Physiological and Functional Basis of Dopamine Receptors and Their Role in Neurogenesis: Possible Implication for Parkinson's disease. *Journal of Experimental Neuroscience*, *12*. https://doi.org/10.1177/1179069518779829

Missale, C., Russel Nash, S., Robinson, S. W., Jaber, M., & Caron, M. G. (1998). Dopamine receptors: From structure to function. *Physiological Reviews*, *78*(1), 189–225. https://doi.org/10.1152/physrev.1998.78.1.189

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 681, Dopamine. Retrieved May 6, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Dopamine

Nelson, D. L., & Cox, M. M. (2017). Biosignaling. In *Lehninger Principles of Biochemistry* (7th ed., pp. 1129–1264).

Neubig, R. R., Spedding, M., Kenakin, T., & Christopoulos, A. (2003). International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification . XXXVIII . Update on Terms and Symbols in Quantitative Pharmacology. *Pharma*, *55*(4), 597–606. https://doi.org/10.1124/pr.55.4.4.and

Newton, P., Harrison, P., & Clulow, S. (2008). A Novel Method for Determination of the Affinity of Protein : Protein Interactions in Homogeneous Assays. *Journal of Biomolecular Screening*, *137*(7), 674–682. https://doi.org/10.1177/1087057108321086

Paton, W. D. M., & Rang, H. P. (1965). The Uptake of Atropine and Related Drugs by Intestinal Smooth Muscle of the Guinea-Pig in Relation to Acetylcholine Receptors. *Proc R Soc Lond B Biol Sci.*, *163*. https://doi.org/10.1098/rspb.1965.0058

Patsenker, L. D., Tatarets, A. L., Povrozin, Y. A., & Terpetschnig, E. A. (2011). Long-wavelength fluorescence lifetime labels. *Bioanal Rev*, *3*, 115–137. https://doi.org/10.1007/s12566-011-0025-2

Pierce, K. L., & Lefkowitz, R. J. (2001). β -ARRESTINS IN THE REGULATION OF G-PROTEIN-COUPLED RECEPTORS. *Nat Rev Neurosci*, *2*(10), 727–733.

Pierre, S., Bruno, G., Marie-Pascale, M., Marie-Louise, B., & Jean-Charles, S. (1990). Molecular Cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature*, *347*.

Pitcher, J. A., Freedman, N. J., & Lefkowitz, R. J. (1998). G PROTEIN – COUPLED RECEPTOR KINASES. *Annual Review of Biochemistry*, *67*, 653–692.

Prieto, G. A. (2017). Abnormalities of Dopamine D 3 Receptor Signaling in the Diseased Brain. *Journal of Central Nervous System Disease*, *9*, 1–8. https://doi.org/10.1177/1179573517726335

Pugsley, T. A., Davis, M. D., Akunne, H. C., Mackenzie, R. G., Shih, Y. H., Damsma, G., Wikstrom, H., Whetzel, S. Z., Georgic, L. M., Cooke, L. W., Demattos, S. B., Corbin, A. E., Glase, S. A., Wise, L. D., Dijkstra, D., & Heffner, T. G. (1995). Neurochemical and functional characterization of the preferentially selective dopamine D3 agonist PD 128907. *Journal of Pharmacology and Experimental Therapeutics*, *275*(3), 1355–1366.

Rajagopal, S., & Shenoy, S. K. (2018). GPCR desensitization: Acute and Prolonged Phases. *Cellular Signaling*, *41*, 9–16. https://doi.org/10.1016/j.cellsig.2017.01.024.GPCR

Rinken, A., Lavogina, D., & Kopanchuk, S. (2018). Assays with Detection of Fluorescence Anisotropy : Challenges and Possibilities for Characterizing Ligand Binding to GPCRs. *Trends in Pharmacological Sciences*, 1–13. https://doi.org/10.1016/j.tips.2017.10.004

Román, V., Gyertyán, I., Sághy, K., Kiss, B., & Szombathelyi, Z. (2013). Cariprazine (RGH-188), a D3-preferring dopamine D 3/D2 receptor partial agonist antipsychotic candidate demonstrates anti-abuse potential in rats. *Psychopharmacology*, *226*(2), 285–293. https://doi.org/10.1007/s00213-012-2906-7

Rondou, P., Haegeman, G., & Van Craenenbroeck, K. (2010). The dopamine D4 receptor: biochemical and signalling properties. *Cellular and Molecular Life Sciences : CMLS*, *67*(12), 1971–1986. https://doi.org/10.1007/s00018-010-0293-y

Rosenbaum, D., Rasmussen, S., & Brian, K. (2014). The structure and function of G protein coupled receptors. *Nature*, *459*(7245), 356–363. https://doi.org/10.1038/nature08144.The

Saarenpää, T., Jaakola, V., & Goldman, A. (2015). Baculovirus-Mediated Expression of GPCRs in Insect Cells. In *Methods in Enzymology* (pp. 185–218). https://doi.org/10.1016/bs.mie.2014.12.033

Sabirsh, A., Wetterholm, A., Bristulf, J., Leffler, H., Haeggström, J. Z., & Owman, C. (2005). Fluorescent leukotriene B 4 : potential applications. *Journal of Lipid Research*, *46*, 1339–1346. https://doi.org/10.1194/jlr.D500005-JLR200

Sakyiamah, M. M., Nomura, W., Kobayakawa, T., & Tamamura, H. (2019). Development of a NanoBRET-based sensitive screening method for CXCR4 ligands. *Bioconjugate Chemistry*, *30*(5), 1442–1450. https://doi.org/10.1021/acs.bioconjchem.9b00182

Schmidt, H., & Jirstrand, M. (2006). Systems Biology Toolbox for MATLAB : a computational platform for research in systems biology. *Bioinformatics*, *22*(4), 514–515. https://doi.org/10.1093/bioinformatics/bti799

Schmieg, N., Rocchi, C., Romeo, S., Maggio, R., Millan, M. J., & Mannoury La Cour, C. (2016). Dysbindin-1 modifies signaling and cellular localization of recombinant, human D3 and D2 receptors. *Journal of Neurochemistry*, *136*(5), 1037–1051.

https://doi.org/10.1111/jnc.13501

Schneider, E. H., & Seifert, R. (2010). Sf9 cells: A versatile model system to investigate the pharmacological properties of G protein-coupled receptors. *Pharmacology and Therapeutics*, *128*(3), 387–418. https://doi.org/10.1016/j.pharmthera.2010.07.005

Schnurr, B., Ahrens, T., & Regenass, U. (2006). Optical assays in drug discovery. In *Comprehensive Medicinal Chemistry II* (Vol. 3, pp. 577–598). https://doi.org/10.1016/b0-08-045044-x/00100-0

Shenoy, S. K., & Lefkowitz, R. J. (2003). Multifaceted roles of β -arrestins in the regulation of seven-membrane- spanning receptor trafficking and signalling. *Biochemical Journal*, *375*, 503–515.

Shenoy, S. K., & Lefkowitz, R. J. (2005). Seven-Transmembrane Receptor Signaling Through β -Arrestin. *Science Signalling*, *308*. https://doi.org/10.1126/stke.2005/308/cm10

Shepherd, T. R., & Fuentes, E. J. (2011). Structural and Thermodynamic Analysis of PDZ – Ligand Interactions. In *Methods in Enzymology* (Vol. 488, pp. 81–100). https://doi.org/10.1016/B978-0-12-381268-1.00004-5

Silverdale, M. A., Nicholson, S. L., Ravenscroft, P., Crossman, A. R., Millan, M. J., & Brotchie, J. M. (2004). Selective blockade of D 3 dopamine receptors enhances the anti-parkinsonian properties of ropinirole and levodopa in the MPTP-lesioned primate. *Experimental Neurobiology*, *188*, 128–138. https://doi.org/10.1016/j.expneurol.2004.03.022

Soave, M., Briddon, S. J., Hill, S. J., & Stoddart, L. A. (2020). Fluorescent ligands: Bringing light to emerging GPCR paradigms. *British Journal of Pharmacology*, *177*(5), 978–991. https://doi.org/10.1111/bph.14953

Sokoloff, P., Diaz, J., Foll, B., Guillin, O., Leriche, L., Bezard, E., & Gross, C. (2006). The Dopamine D3 Receptor: A Therapeutic Target for the Treatment of Neuropsychiatric Disorders. *CNS & Neurological Disorders - Drug Targets*, *5*(1), 25–43. https://doi.org/10.2174/187152706784111551

Sokoloff, Pierre, Andrieux, M., Besançon, R., Pilon, C., Martres, M. P., Giros, B., & Schwartz, J. C. (1992). Pharmacology of human dopamine D3 receptor expressed in a mammalian cell line: comparison with D2 receptor. *European Journal of Pharmacology: Molecular Pharmacology*, *225*(4), 331–337. https://doi.org/10.1016/0922-4106(92)90107-7

Sokoloff, Pierre, Giros, B., Martres, M. P., Bouthenet, M. L., & Schwartz, J. C. (1990). Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature*, *347*(6289), 146–151. https://doi.org/10.1038/347146a0

Stoddart, L. A., Johnstone, E. K. M., Wheal, A. J., Goulding, J., Robers, M. B., Machleidt, T., Wood, K. V, Hill, S. J., & Pfleger, K. D. G. (2015). Application of BRET to monitor ligand binding to GPCRs Europe PMC Funders Group. *Nat Methods*, *12*(7), 661–663. https://doi.org/10.1038/nmeth.3398.Application

Stoddart, L. A., Kilpatrick, L. E., Briddon, S. J., & Hill, S. J. (2015). Probing the pharmacology of G protein-coupled receptors with fluorescent ligands. *Neuropharmacology*, *98*, 48–57. https://doi.org/10.1016/j.neuropharm.2015.04.033

Sun, N., Zhang, X., Guo, S., Le, H. T., Zhang, X., & Kim, K. M. (2018). Molecular mechanisms involved in epidermal growth factor receptor-mediated inhibition of

dopamine D3 receptor signaling. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1865*(9), 1187–1200. https://doi.org/10.1016/j.bbamcr.2018.06.001

Svennilson, J., & Aperia, A. (2000). Dopamine in the developing kidney. *International Journal of Developmental Biology*, *43*(5), 441–443. https://doi.org/10.1387/ijdb.10535321

Tabor, A., Möller, D., Hübner, H., Kornhuber, J., & Gmeiner, P. (2017). Visualization of ligand-induced dopamine D2S and D2L receptor internalization by TIRF microscopy. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-11436-1

Tang, L., Todd, R. D., Heller, A., & O'Malley, K. L. (1994). Pharmacological and functional characterization of D2, D3 and D4 dopamine receptors in fibroblast and dopaminergic cell lines. *Journal of Pharmacology and Experimental Therapeutics*, *268*(1), 495–502.

Tuteja, N. (2009). Signaling through G protein coupled receptors. *Plant Signaling and Behavior*, *4*(10), 942–947. https://doi.org/10.4161/psb.4.10.9530

Vallone, D., Picetti, R., & Borrelli, E. (2000). Structure and function of dopamine receptors. *Neuroscience and Behavioural Reviews*, *24*(99), 125–132.

Van Tol, H. H. M., Bunzow, J. R., Guan, H. C., Sunahara, R. K., Seeman, P., Niznik, H. B., & Civelli, O. (1991). Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature*, *350*(6319), 610–614. https://doi.org/10.1038/350610a0

Veiksina, S., Kopanchuk, S., & Rinken, A. (2014). Biochimica et Biophysica Acta Budded baculoviruses as a tool for a homogeneous fl uorescence anisotropy-based assay of ligand binding to G protein-coupled receptors : The case of melanocortin 4 receptors. *BBA - Biomembranes*, *1838*(1), 372–381. https://doi.org/10.1016/j.bbamem.2013.09.015

Villar, V. A. M., Jones, J. E., Armando, I., Palmes-Saloma, C., Yu, P., Pascua, A. M., Keever, L., Arnaldo, F. B., Wang, Z., Luo, Y., Felder, R. A., & Jose, P. A. (2009). G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. *Journal of Biological Chemistry*, *284*(32), 21425–21434. https://doi.org/10.1074/jbc.M109.003665

Wang, Q., Bosch, B., Vlak, J. M., Oers, M. M. Van, Rottier, P. J., & Lent, J. W. M. Van. (2016). Budded baculovirus particle structure revisited. *Journal of Invertebrate Pathology*, *134*, 15–22. https://doi.org/10.1016/j.jip.2015.12.001

Witkovsky, P. (2004). Dopamine and retinal function. *Documenta Ophthalmologica*, *108*(1), 17–39. https://doi.org/10.1023/B:DOOP.0000019487.88486.0a

Xi, Z., & Gardner, E. L. (2007). Pharmacological Actions of NGB 2904 , a Selective Dopamine D 3 Receptor Antagonist , in Animal Models of Drug Addiction. *CNS Drug Reviews*, *13*(2), 240–259.

Yakimchuk 2011

Konstantin Yakimchuk

MATER METHODS 2011;1:199

Yuan, J., Chen, X., Brodbeck, R., Primus, R., Braun, J., Wasley, J. W. F., & Thurkauf, A. (1998). NGB 2904 AND NGB 2849 : TWO HIGHLY SELECTIVE DOPAMINE D3 RECEPTOR ANTAGONISTS . *Bioorganic and Medicinal Chemistry Letters*, *8*, 2715–2718.

Supplementary

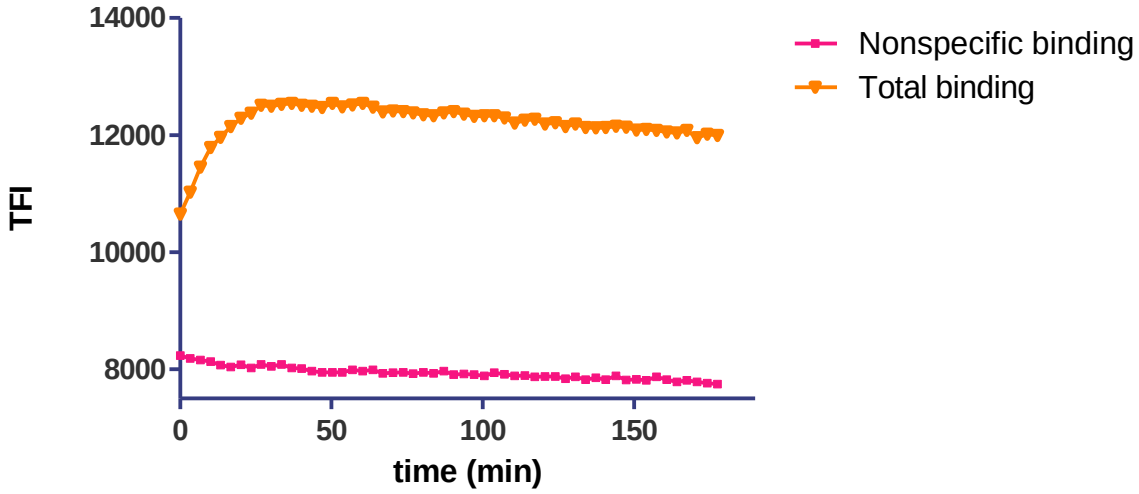

Figure 1. TFI change in time caused by binding of A2-TAMRA to D_3R displayed on BBV. Nonspecific binding was determined with 316 µM 7-hydroxyDPAT hydrobromide. Total binding was determined in the absence of 7-hydroxyDPAT hydrobromide. Data shown is from a representative experiment of at least three independent experiments done in duplicates.

Table 1. pK_i values of D_3R ligands determined in competition binding experiments between D_3R ligands and A2-TAMRA and [^{3}H]-methylspiperone. Serial dilutions of competitor ligands were incubated with fixed concentrations of BBV and A2-TAMRA for FA assay or [^{3}H]-methylspiperone for radioligand binding assay. Data shows mean ± S.E.M of at least three independent experiments done in duplicates (for FA experiments) or triplicates (for radioligand binding assay).

	pK_i		
	FA[a]	**Radioligand binding assay**	**Literature**
Antagonists			
Sulpiride	6.6 ± 0.1	7.6 ± 0.4	7.3 ± 0.5
Butaclamol	7.5 ± 0.2		8.5 ± 0.3
Spiperone	8.3 ± 0.1	9.2 ± 0.4	9.2 ± 0.1
Raclopride	7.6 ± 0.2	8.8 ± 0.5	8.9 ± 0.2
Agonists			
Quinpirole (full agonist)	5.3 ± 0.2	6.2 ± 0.2	8.1 ± 0.3
Dopamine (full agonist)	5.4 ± 0.1		7.7 ± 0.2
7-hydroxypipat maleate (full agonist)	8.2 ± 0.1		7.5 ± 2.1
7-hydroxy DPAT hydrobromide	7.2 ± 0.1		8.8 ± 0.2
Terguride (partial agonist	8.1 ± 0.1		9.0 ± 0.1
Apomorphine (partial agonist)	6.3 ± 0.2	6.9 ± 0.3	7.5 ± 0.2

FA[a] – pK_i values for FA experiments were calculated using Cheng-Prusoff equation (Cheng and Prusoff, 1973).

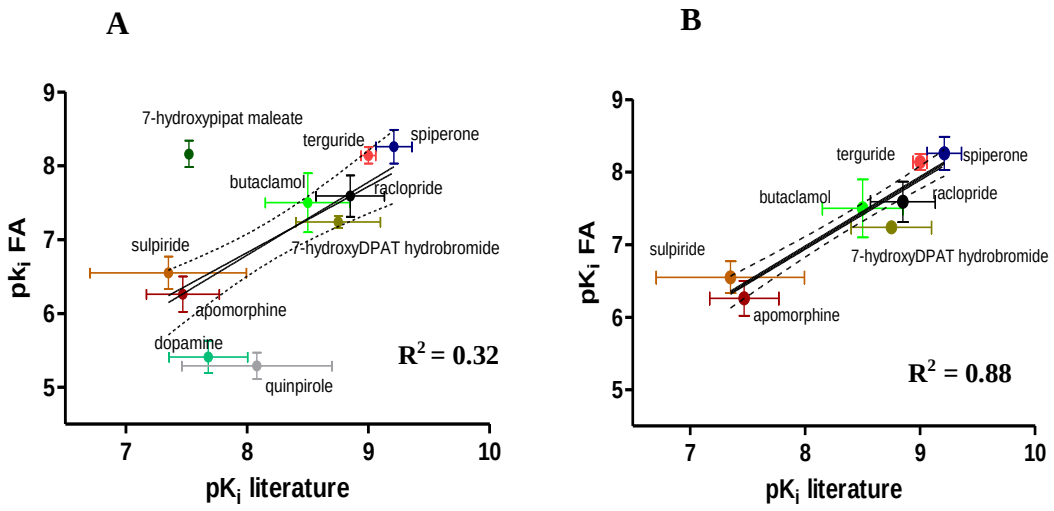

Figure 2. Correlation plots of pK_i from FA assay and pK_i from literature. (A) Correlation between FA-obtained pK_i and pK_i values from literature for the ten D_3R ligands. Solid line shows linear regression, dashed lines represent 95 % confidence intervals, vertical error bars show uncertainty limits for FA-obtained pK_i values and horizontal bars show uncertainty limits for literature pK_i values. (B) Correlation between FA-obtained pK_i values and literature pK_i values except for the full agonists quinpirole, 7-hydroxypipat maleate, and dopamine.

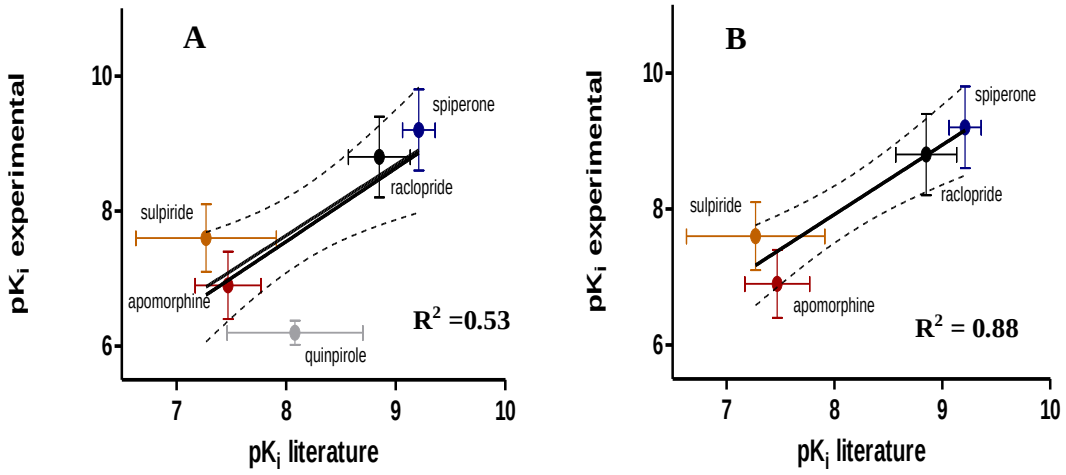

Figure 3. Correlations between pK_i values obtained from radioligand binding assay and pK_i values from literature. (A) Correlation between pK_i values obtained from radioligand binding assay and literature pK_i for the antagonists sulpiride, raclopride, spiperone, the partial agonist apomorphine, and the full agonist quinpirole. Solid line shows linear regression, dashed lines represent 95 % confidence intervals; vertical error bars show uncertainty limits for pK_i values determined in radioligand binding assay and horizontal error bars show uncertainty limits for pK_i values from literature. (B) Correlation between pK_i values from radioligand binding assay and literature pK_i except for the full agonist quinpirole.

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, Lakshmi Thoondee,

(*author's name*)

1. herewith grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

Characterization of ligand binding to Dopamine D_3 receptor using fluorescence anisotropy and radioligand binding,

(*title of thesis*)

supervised by Maris Johanna Tahk, Tõnis Laasfeld, and Prof. Ago Rinken.

(*supervisors' names*)

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.

3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.

4. I certify that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Lakshmi Thoondee

24/05/2021