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DOPAMINE D_2 RECEPTOR PHARMACOLOGY: *IN VITRO* ANALYSES AND *IN VIVO* PET IMAGING

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ABSTRACT

The goals of the present thesis were to study high affinity agonist binding at the D_2 dopamine receptor and to explore the role of agonist induced internalization of the receptor in positron emission tomography (PET) imaging studies. For this purpose, we combined *in vitro* studies of radioligand binding and receptor internalization with *in vivo* studies in rodents using PET.

A pharmacological characterization of the novel PET radioligand MNPA *in vitro* demonstrated that it is a potent and full agonist at the D_2 receptor. In membrane homogenates, MNPA bound to both a high and low affinity site of the D_2 receptor, of which the high affinity site was sensitive to guanosine triphosphate. In intact cells, however, MNPA bound to only one site of low affinity. As a typical agonist at G-protein coupled receptors, MNPA also induced receptor internalization, which was further augmented by arrestin proteins.

[^{11}C]MNPA was evaluated as an agonist radioligand and used to measure the baseline occupancy of the D_2 dopamine receptors by dopamine. *In vivo,* [^{11}C]MNPA was a D_2 selective radioligand. Comparison of [^{11}C]MNPA binding in dopamine depleted rats and control rats suggested that about 50% of the D_2 receptors are occupied by dopamine at baseline.

To study high affinity agonist binding *in vivo,* we compared the binding of [^{11}C]MNPA in control mice to that of dopamine-β-hydroxylase (DBH) knockout mice, which have been reported to exhibit an increased percentage of D_2 receptors in the high affinity state. The *in vivo* studies were accompanied by *in vitro* binding experiments in striatal membrane homogenates. We did not observe any differences in [^{11}C]MNPA BP_{ND} between DBH knockout and control mice. Nor did we find any differences in density or percentage of D_2 receptors in high affinity state with *in vitro* binding experiments. Combined, this study suggests that DBH knockout mice have normal densities of D_2 receptors in the high affinity state.

Agonist induced D_2 receptor internalization was evaluated using immunohistochemistry in striatal tissue slices from arrestin3 knockout and wild-type mice. Both dopamine and MNPA induced D_2 receptor internalization in wild-type tissue but not in knockout tissue. These results demonstrated that D_2 receptor internalization is mediated by arrestin3 and that the arrestin3 knockout mice can be used to study D_2 receptor internalization *in vivo.*

To determine whether the prolonged decrease of radioligand binding after amphetamine is caused by receptor internalization, we imaged wild-type and arrestin3 knockout mice, which are incapable of internalizing D_2 receptors. The mice were imaged with both the D_2 agonist [^{11}C]MNPA and the D_2 antagonist [^{18}F]fallypride. The effect of amphetamine on radioligand binding was examined at two time points; 30 min and 4 hours post-amphetamine. At 30 min, [^{11}C]MNPA showed greater displacement than [^{18}F]fallypride, but each radioligand gave similar displacement in knockout and wild-type mice. At 4 hours, the binding of both radioligands returned to baseline in knockout mice but remained decreased in wild-type mice. Our results suggest that the prolonged decrease of radioligand binding after amphetamine is primarily due to D_2 receptor internalization rather than dopamine displacement.

In conclusion, the present thesis demonstrates that MNPA is a potent and full agonist at the D_2 dopamine receptor and binds *in vitro* to both a high and low affinity state of the D_2 receptor. We also demonstrated that arrestin3 mediates internalization of the D_2 dopamine receptor and that the prolonged *in vivo* decrease of radioligand binding after amphetamine is likely due to receptor internalization rather than dopamine displacement.

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LIST OF ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
Arr3	Arrestin3
BP	Binding Potential
cAMP	Cyclic adenosine monophosphate
DAT	Dopamine transporter
DBH	Dopamine-β-hydroxylase
DMEM	Dulbecco's Modified Eagle Medium
EBSS	Earle's Balanced Salt Solution
ERK	Extracellular signal-regulated kinase
GDP	Guanosine 5'-diphosphate
GPCR	G-protein coupled receptor
Gpp(NH)p	Guanosine-5'-[β,γ-imido]triphosphate
GRK	G-protein coupled receptor kinase
GTP	Guanosine 5'-triphosphate
HEK cells	Human Embryonic Kidney cells
MNPA	(*R*)-2-CH_3O-*N*-*n*-propylnorapomophine
MRTM2	Multilinear Reference Tissue Model 2
NPA	*N*-*n*-propylnorapomorphine
PET	Positron Emission Tomography
PHNO	(+)4-propyl-9-hydroxynaphthoxazine
PKC	Protein Kinase C
SPECT	Single Photon Emission Computed Tomography

1. INTRODUCTION

1.1. G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors (GPCRs) constitute one of the largest super families of receptors. More than 800 members have been identified in the human genome and over 90% of the non-sensory GPCRs are expressed in the human brain (Vassilatis et al., 2003). Rhodopsin was the first member of the GPCR family to be characterized with a complete crystal structure (Palczewski et al., 2000). Based on the crystal structure and sequence homology of Rhodopsin, all GPCRs are predicted to contain seven transmembrane spanning domains with the amino-terminal facing towards the extracellular side and the carboxy-terminal facing the intracellular side. Various different classification techniques, such as pharmacological profiles and sequence homology, have been used to classify the GPCRs, although still discussed GPCRs are commonly divided into 4 to 6 different families, such as Rhodopsin like, Secretin like and Frizzled/smoothened receptors. Within the different families the receptors can be further divided into more specific sub-families such as the dopamine receptors and serotonin receptors (Davies et al., 2007; Fredriksson et al., 2003; Gao and Wang, 2006). The signaling pathways of GPCRs are broad and include among other neurotransmitters, light, odorants and hormones. Combined, the diversity and abundance of signaling pathways and receptors make the GPCRs one of the most attractive biological targets for the pharmaceutical industry (Kroeze et al., 2003; Vassilatis et al., 2003).

1.1.1. Signal transduction through G-proteins

The signal transduction of GPCRs is mediated through activation of heterotrimeric G-proteins. Heterotrimeric G-proteins and their role in signal transduction were first discovered and described by Alfred G. Gilman and Martin Rodbell, who in 1994 received the Nobel Prize in Physiology or Medicine for their discovery (Gilman, 1995; Rodbell, 1995). The G-protein consists of an α, β and γ-subunit of which the α-subunit contains a guanine nucleotide binding site, hence the name G-protein. Based on sequence homology and the signaling pathways of the α-subunit the G-proteins are usually separated into four classes: G_s, $G_{i/o}$, G_q and $G_{12/13}$. The G_s class stimulates and the $G_{i/o}$ class inhibits adenylate cyclase and cAMP formation. The G_q class stimulates phospholipase C and the last class $G_{12/13}$ regulates extracellular signal-regulated kinase (ERK) and c-jun kinase. The $\beta\gamma$-subunits, also called the $\beta\gamma$-complex, are not as well

described as the α-subunit but they have been linked to regulation of ion channels and intracellular kinases and introduces yet another level of complexity to the signal transduction through G-proteins (Neer, 1994; Simon et al., 1991).

The mechanism of G-protein activation is best described by the guanine nucleotide exchange cycle (Fig. 1). Briefly, G-protein activation is initiated when an agonist binds to the receptor. Upon agonist binding the receptor undergoes a conformational change that activates the G-protein by catalyzing the exchange of guanine nucleotide diphosphate (GDP) with guanine nucleotide triphosphate (GTP) at the α-subunit. After the guanine nucleotide exchange, the α and the βγ-subunits dissociate from the receptor and separately modulate signal transduction by interacting with intracellular effectors such as adenylate cyclase. Signal transduction is terminated when the bound GTP on the α-subunit is hydrolyzed to GDP and the heterotrimeric G-protein is reunited. The heterotrimeric G-protein can then again couple to a receptor and engage in another round of the guanine nucleotide exchange cycle (Gether et al., 2002; Oldham and Hamm, 2008; Rodbell, 1997).

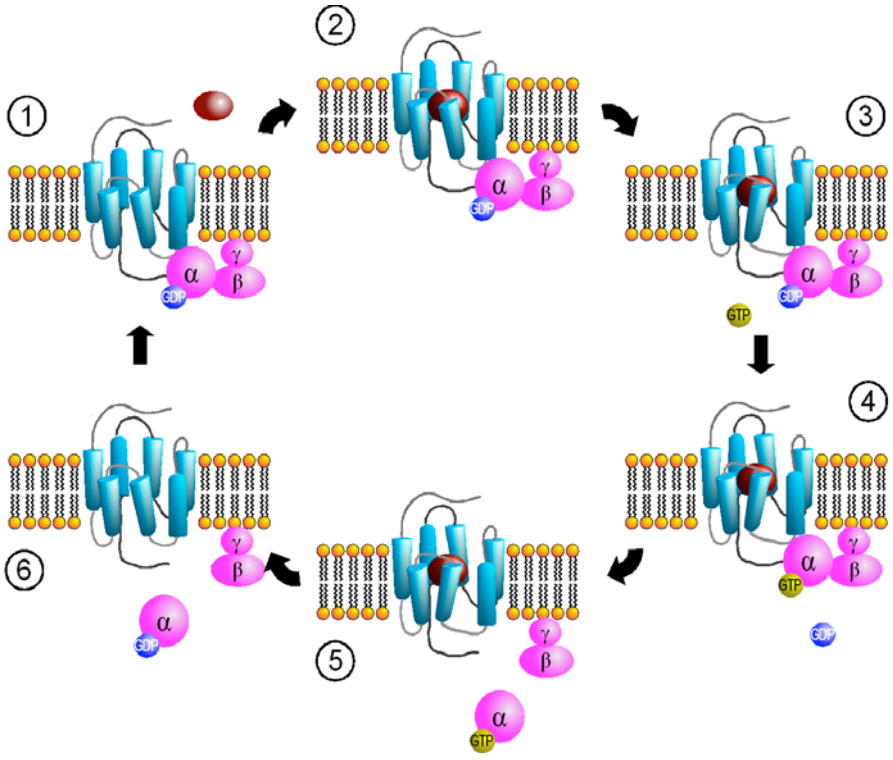

Fig. 1. The guanine nucleotide cycle describes the activation of a G-protein. **1-2**) The activation is initiated when an agonist binds to the GPCR. **3-4**) Agonist binding promotes a conformational change of the receptor, which activates the G-protein. The G-protein exchanges GDP with GTP at the α-subunit. **5-6**) The α-subunit and βγ-subunits dissociate from the receptor. G-protein signalling is terminated by hydrolysis of GTP to GDP at α-subunit and the heterotrimeric G-protein is reunited. Figure made by Sven Jähnichen, 2006.

1.1.2. High affinity agonist binding

High affinity agonist binding has been extensively studied *in vitro* using competition binding assays in membrane homogenates. These studies found that typical agonists at GPCRs bind to both a high and a low affinity site of the receptor. When GTP was added to the binding assay only low affinity binding was observed, demonstrating that the high affinity site is sensitive to GTP. In contrast to agonists, antagonists are not sensitive to GTP and bind with only one affinity to the receptors (Sibley et al., 1982; Wei and Sulakhe, 1979). Based on the sensitivity to GTP, high affinity agonist binding is thought to reflect a ternary complex consisting of the agonist, the receptor and a guanine nucleotide free G-protein. In the absence of GTP, the formation of the ternary complex is artificially stabilized in the binding assay, but in the presence of GTP, the complex is transient due to the GTP exchange at the G-protein, which promotes low affinity agonist binding (De Lean et al., 1980; Sibley et al., 1982).

Similar binding studies were also performed on intact live cells in order to study agonist binding on GPCRs under conditions that better reflect *in vivo* situations. Interestingly, high affinity agonist binding at the dopamine D_2 receptors was absent in intact cells (Sibley et al., 1983; Skinbjerg et al., 2009). However, studies of beta-adrenergic receptors demonstrated that high affinity agonist binding was detectable very briefly (≤1 min) in intact cells when binding assays were performed under non-equilibrium conditions. When the cells were exposed to agonists for longer periods only low affinity binding was observed. Several investigators proposed that endogenously produced GTP rapidly converts the receptors to low affinity upon agonist exposure and prevents stabilization of the ternary complex (Hoyer et al., 1984; Sibley et al., 1983; Toews et al., 1983). Combined, the studies in membrane homogenates and intact cells suggest that high affinity agonist binding (i.e. formation of the ternary complex) is transient due to the GTP exchange at the G-protein. While the high affinity state can be stabilized in a GTP free environment such as washed membrane homogenates, there is no support for such an accumulation or stabilization *in vivo* based on binding assays in intact cells.

1.1.3. Agonist induced receptor internalization

Receptor internalization is a mechanism to regulate the cell surface expression of receptors and is an important function to maintain homeostatic control in the cell. Therefore, a property of most agonists at GPCRs is to induce receptor internalization. Agonist induced receptor internalization primarily occurs through an arrestin-dynamin

dependent pathway involving clathrin coated pits. (Ferguson, 2001; Gainetdinov et al., 2004; Groer et al., 2007). The internalization process is initiated by receptor desensitization, which prevents further G-protein coupling and subsequent signaling (Fig. 2). Receptor desensitization involves phosphorylation of the intracellular domains of the receptor and binding of arrestin binding. Phosphorylation is mediated by G-protein coupled receptor kinases (GRKs) and/or second messenger activated protein kinases such as PKC. Previous studies suggested that receptor phosphorylation promoted arrestin binding, however, a recent study demonstrated that receptor phosphorylation is not necessary for arrestin binding or internalization (Ito et al., 1999; Marchese et al., 2008; Namkung et al., 2009). Instead, this study suggests that phosphorylation may play a role in regulation of post-endocytic trafficking (Namkung et al., 2009). Arrestin, on the other hand, plays an important role as a scaffolding protein in the internalization process. Arrestin targets the receptor for internalization by associating with adaptor proteins such as AP-2 and recruiting clathrin to form clathrin-coated pits. Dynamin pinches off these clathrin coated pits and thereby creates intracellular vesicles containing the receptor. Once internalized, the receptor is either targeted for degradation or recycled to the cell membrane (Pierce and Lefkowitz, 2001; Rappoport, 2008).

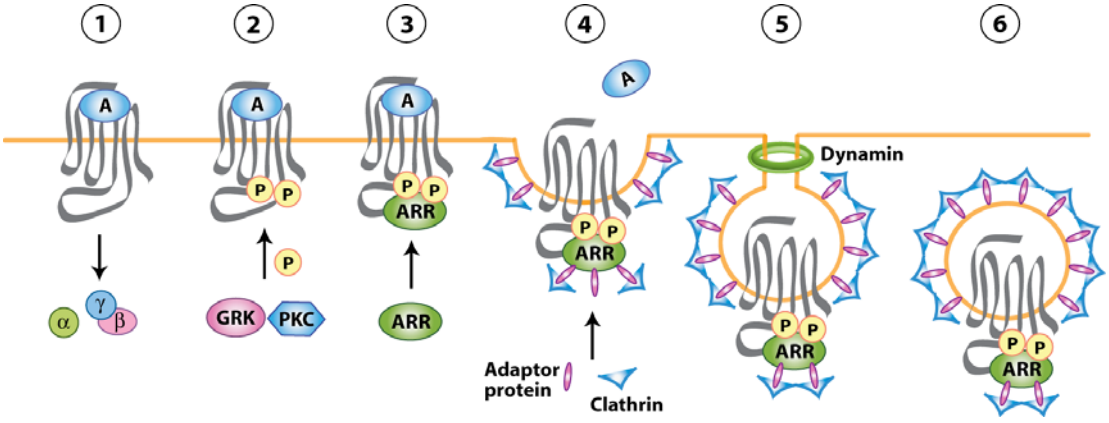

Fig. 2. Agonist induced internalization of GPCRs represented by the D_2 dopamine receptor. **1-2**) Following agonist binding (A) and G-protein activation, the second and third intracellular loops are phosphorylated (P) by G-protein coupled receptor kinase (GRK) and/or protein kinase C (PKC). **3-4**) Binding of the scaffolding protein, arrestin (ARR), leads to receptor desensitization and targets the receptor for internalization. Arrestin associates with an adaptor protein and recruits clathrin to form a clathrin-coated pit. **5-6**) The clathrin-coated pit is pinched off from the cell membrane by dynamin and the receptor is internalized. Figure modelled after Pierce and Lefkowitz (2001).

1.2. THE DOPAMINE SYSTEM

The dopamine system is one of the most widely studied neurotransmitter systems both *in vitro* and *in vivo.* All known dopamine receptors belong to the family of GPCRs and the dopamine system therefore serves as an excellent model for other GPCR systems.

In the brain the dopamine system can be divided into four main pathways; the tubero-hypophyseal system is involved in the regulation of hormone secretion and consists of neurons that project from the hypothalamus to the median eminence and pituitary gland. The mesolimbic and the mesocortical dopamine pathways are involved in cognition, emotion and reward mechanisms and play an important role in several psychiatric diseases such as drug addiction and schizophrenia. The mesolimbic and the mesocortical pathways both originate in the ventral tegmental area. While the mesolimbic pathway projects to the limbic structures such as nucleus accumbens, hippocampus and amygdala, the mesocortical pathway projects to the frontal cortex. The fourth dopaminergic pathway is the nigrostriatal pathway, which plays an important role in control of locomotion e.g. the loss of dopaminergic neurons in the nigrostriatal pathway is one of the most noticeable pathological features of Parkinson's disease. The nigrostriatal pathway projects from the substantia nigra to striatum and contains the highest density of dopaminergic neurons (Bedard et al., 1969; Bjorklund et al., 1970; Fuxe et al., 1974).

1.2.1. The dopamine receptors

To date five dopamine receptors have been cloned and characterized: D_1, D_2, D_3, D_4, and D_5 receptors. The dopamine receptors were first classified based on their pharmacological profiles and later sequence homology and are divided into two types, the D_1-like and the D_2-like receptors (Creese et al., 1983; Sibley and Monsma, 1992).

The D_1-like dopamine receptors include the D_1 and the D_5 receptor and mediate their signaling by activating G_s-proteins, which stimulate adenylate cyclase and increase cAMP formation (Monsma et al., 1990; Sunahara et al., 1991). The D_1 receptors are the most densely expressed of all dopamine receptors and are distributed throughout the forebrain with the highest density in striatum and substantia nigra.

The D_5 dopamine receptors are expressed at lower levels than the D_1 receptors and are mainly found in the striatum, the hippocampus and the thalamus (Boyson et al., 1986; Choi et al., 1995; Khan et al., 2000). Studies using specific antibodies for each

subtype have found that the D_1 and D_5 receptors are located separately at the cellular and subcellular levels suggesting distinct roles for each receptor subtype (Bergson et al., 1995).

The D_2-like dopamine receptors include the D_2, D_3, and the D_4 receptor and mediate their signaling by activating $G_{i/o}$ proteins, which inhibit adenylate cyclase and decrease cAMP formation (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). Soon after the initial cloning of the D_2 dopamine receptor it became clear that the receptor existed both as a short and a long isoform, of which the long isoform is formed by alternative splicing of the same gene (Dal Toso et al., 1989; Monsma et al., 1989). Studies comparing mice deficient of only the long isoform (D_{2L}-/-) and mice deficient of both isoforms (D_2-/-) suggested distinct neuronal functions of the two isoforms. Whereas the D_2 short isoform appeared to function as an autoreceptor at presynaptic sites, the long isoform primarily regulated postsynaptic events (De Mei et al., 2009; Lindgren et al., 2003; Usiello et al., 2000). The D_2 dopamine receptors are distributed in all areas receiving dopaminergic input and are expressed at high density in the striatum, the nucleus accumbens and the pituitary gland. The D_2 receptor is by far the best characterized of all the dopamine receptors mainly due to a large number of available ligands and its prominent role in psychiatric disorders (Toda and Abi-Dargham, 2007; Vallone et al., 2000).

The D_3 dopamine receptors are expressed in a more restricted pattern mostly of limbic origin and are generally present at a lower density than D_2 receptors. However, certain areas such as nucleus accumbens and the ventral striatum have high levels of both D_2 and D_3 receptors, which complicates quantitative binding studies as most ligands at the D_2-like receptors have high affinity for both receptor subtypes (Bouthenet et al., 1991; Murray et al., 1994).

The D_4 dopamine receptors are expressed at very low density in limbic and cortical areas in the human brain. Due to the low density the D_4 receptors are difficult to quantify with methods other than mRNA expression. In the human population the D_4 receptors exist in several polymorphic forms characterized by a varying number of amino acid repeats in the third intracellular loop, however, the exact functional significance of these forms is not well understood (Matsumoto et al., 1996; Missale et al., 1998; Strange, 1994).

1.2.2. Involvement of the dopamine system in psychiatric disorders

Dysfunction of the dopamine system is a common pathological characteristic of a number of psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and schizophrenia. Although psychiatric disorders are known to be heritable they are not associated with one specific genotype but are instead polygenic. In addition to the polygenic nature, environmental risk factors such as substance abuse increase the risk of developing psychiatric disorders. Combined, these factors complicate not only the ability to predict the risk of development but also clinical diagnosis and treatment of the psychiatric disorders (Porteous, 2008; Wallis et al., 2008). Currently no efficient biomarkers are known for clinical diagnosis of psychiatric disorders. However, much research has focused on using molecular imaging techniques such as Positron Emission Tomography (PET) to study general characteristics and develop effective biomarkers for clinical diagnosis. The present chapter will focus on two common but very different psychiatric disorders, ADHD and schizophrenia, which are both associated with dysfunction of the dopamine system.

ADHD is commonly characterized by several behavioral abnormalities such as attention deficit, impulsivity and hyperactivity (Biederman et al., 2008; Garrett et al., 2008). The disorder mainly manifests in childhood, but often persists into adolescence, and causes severe problems with performance and behavior in affected children (Faraone et al., 2003; Levy, 2004). In most children, however, ADHD is effectively treated with the psychostimulant drug methylphenidate, which blocks the dopamine transporter (DAT) and increases the levels of extracellular dopamine. The effective treatment with methylphenidate suggests that ADHD may be associated with dysregulation of DAT. However, the scientific evidence for this hypothesis is difficult to explain since imaging studies of DAT reported contrasting results. While some studies reported an increased density of the DAT in the striatum of ADHD patients (Cheon et al., 2003; Larisch et al., 2006; Spencer et al., 2007), other studies reported either no difference or reduced density of DAT in ADHD patients compared to healthy controls (Hesse et al., 2009; Jucaite et al., 2005; van Dyck et al., 2002; Volkow et al., 2007). As DAT regulates the extra synaptic dopamine levels another explanation for the pathophysiology of ADHD could be alteration of dopamine synthesis and release. A few PET studies have explored this alternative hypothesis by measuring the dopamine synthesis by [^{18}F]DOPA or L-[^{11}C]DOPA utilization. However, similar to the studies of DAT, the results were contrasting. While two studies reported reduced dopamine synthesis in ADHD patients another study reported increased dopamine

synthesis in ADHD patients compared to healthy controls (Ernst et al., 1998; Ernst et al., 1999; Forssberg et al., 2006). Combined, these studies suggest that ADHD is associated with dysfunction of the dopamine system and possible involvement of DAT, however, the exact pathophysiology of the disease is complex and not yet fully understood.

Schizophrenia, like ADHD, is associated with dysfunction of the dopamine system. The disorder mainly manifests in early adulthood and is predicted to affect about 0.3% to 2% of the population (Tandon et al., 2008). Schizophrenia is commonly characterized by positive and negative symptoms accompanied by cognitive impairment such as impaired working memory and lack of attention. The positive symptoms are characterized by excess or distortion of normal functions and include hallucinations, delusions and thought disorder. The negative symptoms are characterized by suppression or absence of rational behavior or thinking. For example, depressed thoughts and decreased ability to express emotions or initiate plans. Pharmacological treatment of schizophrenia with antipsychotic drugs effectively reduces the positive symptoms and number of illness episodes. All known antipsychotic drugs either block or partially inhibit the D_2 receptors, which are mainly located in the subcortical areas of the brain. Based on these observations the dopamine hypothesis of schizophrenia proposes that the positive symptoms are associated with elevated subcortical dopamine transmission and consequently, hyperstimulation of the D_2 receptors. The dopamine hypothesis has received solid support from both imaging and post mortem studies and is one of the most established hypotheses for the pathophysiology of schizophrenia (for review see Guillin et al., 2007). For example, several imaging studies reported that administration of psychostimulants increased the severity of the positive symptoms and induced a higher striatal dopamine release in schizophrenic patients than in healthy controls (Abi-Dargham, 2004; Breier et al., 1997; Laruelle et al., 1999). Another study reported higher baseline occupancy of the D_2 receptors by endogenous dopamine in schizophrenic patients than healthy controls (Abi-Dargham et al., 2000). In addition, numerous post mortem studies found increased density of striatal D_2 receptors in schizophrenic patients (Guillin et al., 2007). Combined, these studies suggest that the positive symptoms in schizophrenia are associated with elevated subcortical dopamine transmission and that pharmacological inhibition of D_2 receptor signaling regulates and stabilizes the dopamine transmission in these regions.

In contrast to the positive symptoms, the current forms of antipsychotic drugs do not effectively treat the negative and cognitive symptoms and the underlying biochemistry of these symptoms is, so far, relatively sparsely characterized. The dopamine hypothesis of schizophrenia suggests that the negative and cognitive symptoms are associated with decreased dopamine release in the cortical areas resulting in hypostimulation of the D_1 receptors (Abi-Dargham and Moore, 2003; Toda and Abi-Dargham, 2007). A few PET studies have focused on the involvement of D_1 receptors in the prefrontal cortex and the cognitive and negative symptoms of schizophrenia but the results have been inconclusive. One study found that schizophrenic patients had an increased density of D_1 receptors in the dorsolateral prefrontal cortex, an important area for working memory, but no change in other areas of the prefrontal cortex. The increased density of D_1 receptors in the dorsolateral prefrontal cortex correlated with the degree of working memory impairment in the schizophrenic patients (Abi-Dargham et al., 2002). However, another PET study reported a decreased density of D_1 receptors in the prefrontal cortex of schizophrenic patients (Okubo et al., 1997). These studies indicate that prefrontal D_1 dopamine receptors may be involved with the cognitive and negative symptoms of schizophrenia, but the exact regulation and expression of D_1 receptors in the prefrontal cortex is not fully known.

In summary, multiple studies and the effectiveness of pharmacological treatment demonstrates that the dopamine system is directly involved in the pathophysiology of psychiatric disorders. Although the knowledge of these disorders is steadily progressing, there are still many unanswered questions, which urge further research of the involvement of the dopamine system in psychiatric disorders.

1.3. MOLECULAR IMAGING OF THE DOPAMINE SYSTEM

Molecular imaging techniques like positron emission tomography (PET) and single photon emission computed tomography (SPECT) provide a non-invasive way to study the living brain and are highly desirable methods for clinical studies of neurological disorders. The present chapter will focus on molecular imaging of the dopamine system and the D_2 dopamine receptors. The chapter will also cover some of the aspects that would need to be addressed in order to further develop the understanding of *in vivo* molecular imaging. All current radioligands for the D_2 receptor also have relatively high affinity for the D_3 receptor, but for simplicity this chapter will refer to the D_2 receptor.

The dopamine system can be imaged by using specific radioligands for the D_2 receptor either under baseline conditions or in combination with pharmacological treatment. Under baseline conditions, a proportion of the receptors are occupied by dopamine and the radioligand measures those receptors that are available for binding i.e. the receptors that are not occupied by dopamine. By pharmacologically manipulating the dopamine release the radioligand binding will reflect the change of dopamine occupancy at the receptors. The most common ways to manipulate the dopamine system are dopamine depletion or stimulation, but administration of synthetic ligands for the dopamine receptors is also frequently used.

Dopamine depletion allows the complete pool of D_2 dopamine receptors to be available for radioligand binding. Dopamine depletion can be achieved by administration of the drugs like α-methyl-para-tyrosine and reserpine. The agent α-methyl-para-tyrosine inhibits tyrosine hydroxylase and thereby blocks dopamine synthesis. The agent reserpine blocks the transport of dopamine from the cytoplasm into vesicles and prevents subsequent release of dopamine and signal transmission. Only partial dopamine depletion can be achieved in humans and non-human primates due to the serious side effects of the depleting agents. However, complete dopamine depletion can be achieved in rodent models making them advantageous for this type of study (Engelman et al., 1968; Pfeifer et al., 1976; Seneca et al., 2008). Combined with baseline studies, dopamine depletion studies provide useful information about the proportion of receptors that are occupied by dopamine under baseline conditions and can be used as a measure of baseline dopaminergic neurotransmission (Abi-Dargham et al., 2000; Laruelle et al., 1997a; Verhoeff et al., 2001).

Pharmacological stimulation of dopamine release can be achieved by administration of psychostimulants such as amphetamine and cocaine. Psychostimulants act on the dopamine transporter (DAT) by blocking or reversing the reuptake of dopamine from the synaptic cleft leading to increased levels of extracellular dopamine. In the synaptic cleft, dopamine competes for the binding site at the D_2 dopamine receptor and displaces radioligand binding similar to *in vitro* competition assays. The extent of radioligand displacement by endogenous dopamine can be used as a measure of synaptic dopamine release (Dewey et al., 1991; Innis et al., 1992; Slifstein et al., 2004). Another approach to create an *in vivo* competition experiment is by administration of an unlabeled ligand selective for the D_2 receptor (or other dopamine receptor subtypes) together with the radioligand. This approach can be used to verify receptor specificity of the radioligand or to measure receptor occupancy by

the exogenous ligand e.g. a therapeutic drug (Finnema et al., 2009; Finnema et al., 2005; Kegeles et al., 2008).

In summary, PET imaging provides a method to explore molecular neurobiology *in vivo* by mimicking the methodology from *in vitro* binding experiments. Previous PET imaging studies have been limited to the use of antagonist radioligands, however, recent development of agonist radioligands has extended the ways one can explore molecular imaging but has also challenged the classical interpretation of the *in vivo* results (Laruelle, 2000; Seneca et al., 2006).

1.3.1. High affinity agonist binding to the D_2 dopamine receptor *in vivo*

Several imaging studies of the D_2 dopamine receptor have shown that antagonist radioligands are sensitive to both dopamine depletion and augmentation (Dewey et al., 1993; Ginovart et al., 1997; Innis et al., 1992; Laruelle et al., 1997a). PET studies combined with microdialysis demonstrated a dose dependent relationship between amphetamine challenge and dopamine release that correlated with radioligand displacement (Breier et al., 1997; Endres et al., 1997; Tsukada et al., 1999). These findings led to the proposal of the classical occupancy model (Fig. 3), which predicts that an increase of extracellular dopamine causes a decrease of radioligand binding due to competition from dopamine (i.e. dopamine displacement). Conversely, decreased extracellular dopamine levels will increase radioligand binding due to the lack of competition from dopamine (Laruelle, 2000).

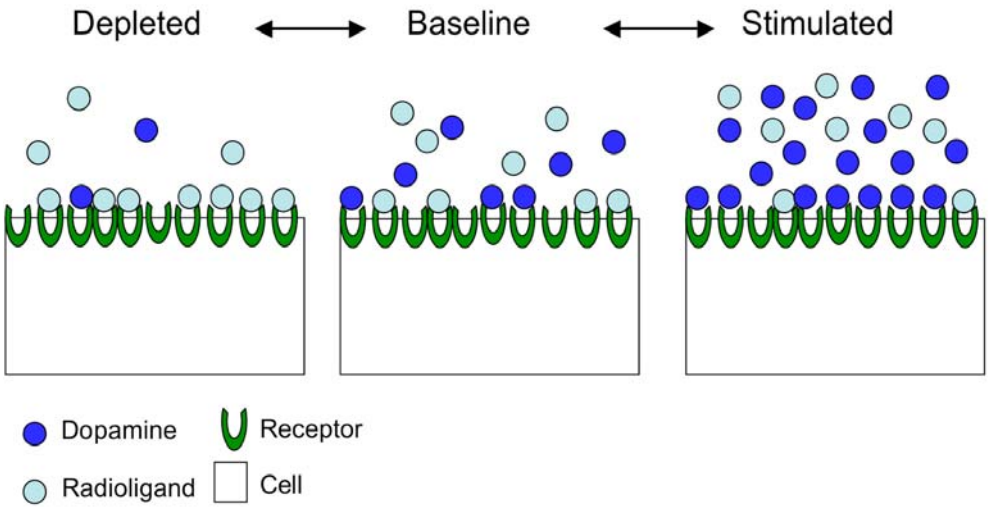

Fig. 3. The classical occupancy model for molecular imaging of neuroreceptors *in vivo*. Under baseline condition a proportion of the receptors are occupied by endogenous dopamine and competes with the radioligand for the binding site (middle panel). Stimulation of dopamine release increases dopamine competition and leads to decreased radioligand binding (right panel). Conversely, dopamine depletion decreases dopamine competition and leads to increased radioligand binding (left panel). Revised figure modelled after and proposed by Laruelle (2000).

Although a linear correlation was found between the amount of radioligand displacement and the dose of amphetamine challenge, the displacement was only modest compared to the increase of extracellular dopamine measured by microdialysis. For example, in the study of Tsukada et al. (1999) a ~1300% increase of extracellular dopamine only caused a ~35% displacement of radioligand binding and similar modest displacement was reported from other studies suggesting incomplete or weak competition by dopamine (Breier et al., 1997; Laruelle et al., 1997b; Tsukada et al., 1999). The incomplete radioligand displacement by dopamine was referred to as the ceiling effect and could not be fully explained by the classical occupancy model. In addition, administration of the D_2 antagonist raclopride completely displaced radioligand binding, suggesting that antagonists can displace the radioligand at a proportion of the receptors that are inaccessible for agonists (i.e. dopamine) binding (Laruelle, 2000; Laruelle et al., 1997b). The discrepancy between radioligand displacement by antagonists and agonists led to a revision of the classical occupancy model, which incorporated high affinity agonist binding into the model (Fig. 4). As mentioned previously, *in vitro* binding experiments demonstrated that agonists bind to both a high and a low affinity site of the D_2 receptor, whereas antagonists bind with equal affinity to all receptors in membrane homogenates (Sibley et al., 1982; Zahniser and Molinoff, 1978). Based on these *in vitro* binding studies, the revised occupancy model hypothesizes that dopamine preferentially binds to and competes at a proportion of D_2 dopamine receptors in a high affinity state for agonist binding. In contrast, antagonists bind and compete with equal affinity to the complete pool of D_2 receptors, which would explain the modest displacement of antagonist radioligands by endogenous dopamine but complete displacement with raclopride (Laruelle, 2000).

Assuming that a proportion of the D_2 receptors are in high affinity state for agonist binding the occupancy model also predicts that agonist radioligands would be more sensitive to dopamine displacement than antagonist radioligands. This prediction was first supported by an *ex vivo* experiment demonstrating that the D_2 receptor agonist, N-[^{3}H]propyl-norapomorphine (NPA), was displaced to a greater extent than the antagonist [^{11}C]raclopride after amphetamine challenge (Cumming et al., 2002). This study was later reproduced *in vivo* with three newly developed agonist radioligands for PET imaging, all of which consistently reported about two-fold greater displacement of the agonist radioligand than the antagonist [^{11}C]raclopride after amphetamine challenge (Ginovart et al., 2006; Narendran et al., 2004; Seneca et al., 2006)

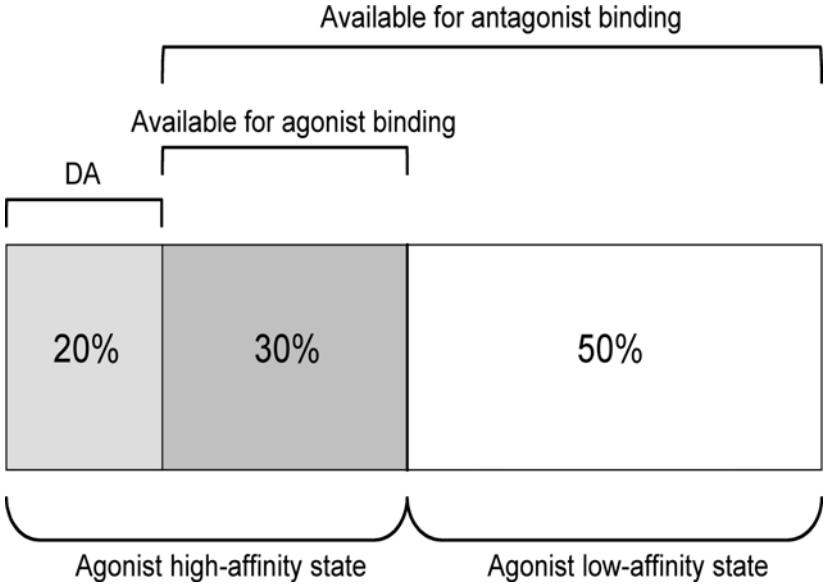

Fig. 4. The proposed occupancy model for agonists and antagonists binding to the D_2 dopamine receptor *in vivo*. The model assumes that 50% of the receptors are inducible into the high affinity state by agonists binding, and that 20% of the receptors are occupied by dopamine at baseline. The model proposes that dopamine and other agonists bind to the proportion of receptors in high affinity state, whereas antagonists bind with equal affinity to all receptors. Thus, amphetamine induced dopamine release would only displace antagonist radioligand binding at the fraction of receptors in the high affinity state. Figure modelled after and proposed by Laruelle (2000).

Although rather convincing data suggests that the D_2 receptors exist in a high and low affinity state for agonist binding in vivo an emerging amount of discrepant results have recently questioned this hypothesis. For example, in contrast to previous imaging studies that used amphetamine challenge Finnema and colleagues (2009) used an exogenously administered D_2 agonist, apomorphine, to establish an *in vivo* competition model. This study demonstrated that apomorphine dose dependently displaced both the agonist radioligand [^{11}C]-(*R*)-2-CH_3O-*N*-*n*-propylnorapomophine (MNPA) and the antagonist [^{11}C]raclopride. Most importantly, the displacement was monophasic and the ID_{50} and K_i values were indistinguishable between the two radioligands, thus providing no support for two affinity states of the D_2 dopamine receptor *in vivo* (Finnema et al., 2009). Similar results were reported from an *ex vivo* study, which used exogenously administered D_2 agonist, partial agonist, antagonist and amphetamine challenge to displace binding of the agonist [^{11}C](+)4-propyl-9-hydroxynaphthoxazine (PHNO) and the antagonist [^{3}H]raclopride (McCormick et al., 2008). The same group also studied [^{11}C]PHNO and [^{3}H]raclopride binding in three rat models known to

display an increased percentage of D_2 receptors in the high affinity state *in vitro.* If agonist radioligands bind only to receptors in the high affinity state, then [^{11}C]PHNO binding is expected to be greater in the high affinity rat model than control rats. However, [^{11}C]PHNO and [^{3}H]raclopride binding was indistinguishable from control rats suggesting that D_2 receptors do not exist in a high and low affinity state for agonist binding *in vivo* (McCormick et al., 2009).

A few PET studies have also examined high affinity agonist binding using a saturation binding approach to determine the B_{max} of agonist versus antagonist radioligands as a measure of the D_2 receptor density. If only a proportion of the D_2 receptors are in the high affinity state, then the B_{max} of agonist radioligands is expected to be lower than the B_{max} of antagonist radioligands. Consistent with the occupancy model, one study reported that B_{max} of the agonist [^{11}C]NPA was lower than that of the antagonist [^{11}C]raclopride, however, another study reported no differences in the B_{max} of the agonist [^{11}C]PHNO and [^{11}C]raclopride (Ginovart et al., 2006; Narendran et al., 2005). The different types of agonist radioligands may explain these discrepant results. However, both NPA and PHNO are well characterized agonists and bind to both a high and low affinity site in vitro (Sibley et al., 1982; Vasdev et al., 2007).

In summary, several PET studies have provided support for the hypothesis of two affinity states of the D_2 dopamine receptors *in vivo* but recent studies suggest that only one affinity state exists *in vivo.* Whether the latter would correspond to the high or the low affinity state is unknown. Important to consider is that the hypothesis of two affinity states of the D_2 dopamine receptors *in vivo* was based on binding studies carried out on membrane homogenates, whereas no notice was given to the binding studies in intact cells, which better reflects *in vivo* conditions. In contrast to membrane homogenates, agonist bound with only one affinity, corresponding to the low affinity site, in intact cells (Sibley et al., 1983). Thus, the existence of a high and low affinity site for agonist binding at the D_2 dopamine receptor *in vivo* is still elusive and more research is needed in order to fully explain the pharmacology of *in vivo* agonist and antagonist binding at the D_2 dopamine receptors.

1.3.2. Agonist induced internalization of the D_2 dopamine receptor *in vivo*

Another aspect of *in vivo* molecular imaging is the role of agonist induced internalization of the D_2 dopamine receptors. Although agonist induced internalization of D_2 dopamine receptors has been extensively studied *in vitro,* its effect on *in vivo* receptor binding has largely been unexplored. As mentioned earlier, many PET imaging studies use amphetamine or other psychostimulants to increase extracellular dopamine levels. Microdialysis studies demonstrated that amphetamine induces a massive increase of extracellular dopamine that peaks about 20 min after administration and returns to baseline over the next one to two hours (Laruelle et al., 1997b; Sharp et al., 1987; Tsukada et al., 1999). The massive increase of extracellular dopamine most likely induces internalization of the D_2 receptors, which may affect the radioligand binding, as the receptors may be inaccessible for the radioligand. PET studies using constant infusion of radioligand combined with amphetamine challenge were the first to suggest that receptor internalization may affect radioligand binding *in vivo.* Under steady state conditions, radioligand binding is supposed to reflect the fluctuations of extracellular dopamine induced by amphetamine. That is, radioligand binding is expected to rapidly decrease and then return to the initial level over the next couple of hours as extracellular dopamine returns to baseline levels. However, radioligand binding remained decreased for several hours after amphetamine administration and did not correlate well with the time course of dopamine release measured with microdialysis (Laruelle, 2000; Laruelle et al., 1997b). Moreover, recent studies reported that radioligand binding remains decreased for up to 24 hours after amphetamine challenge, strongly suggesting that the effect on radioligand binding is more than just direct dopamine displacement (Cardenas et al., 2004; Narendran et al., 2007). The prolonged decrease of radioligand binding observed after amphetamine challenge led to the proposal of an internalization model (Fig. 5), wherein radioligand binding is not only displaced by endogenous dopamine but also affected as a consequence of receptor internalization and up-regulation (Laruelle, 2000).

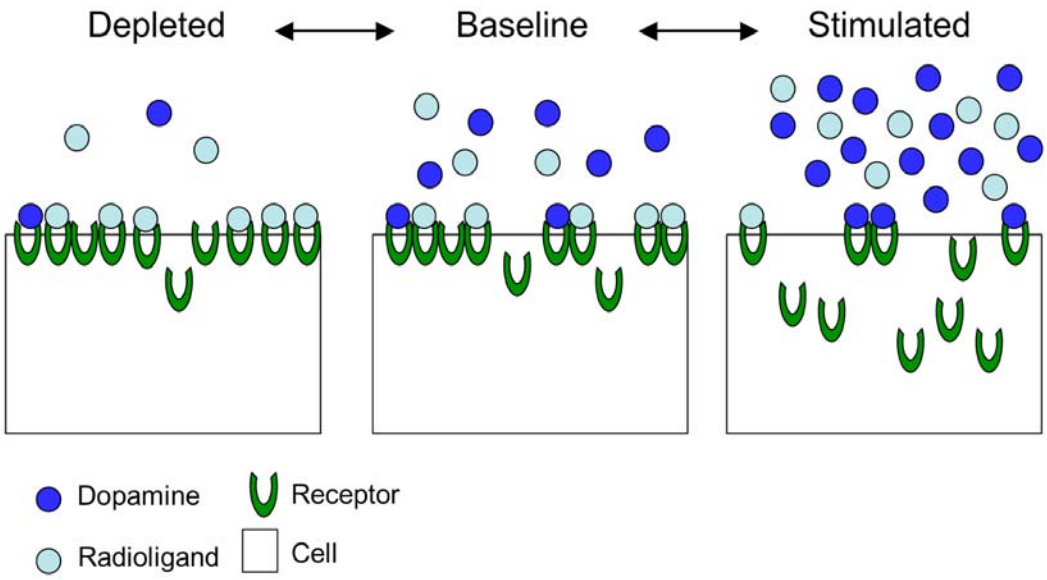

Fig. 5. The internalization model. In contrast to the classical occupancy model, this model includes both receptor trafficking and dopamine displacement as factors that can affect radioligand binding *in vivo*. The model proposes that stimulation of dopamine release will induce receptor internalization, which in combination with dopamine displacement contributes to decreased radioligand binding (right panel). Conversely, under dopamine depletion, receptor up-regulation at the cell membrane will contribute to increased radioligand binding (left panel). Revised figure modelled after and proposed by Laruelle (2000).

The internalization model adds another level of complexity to the interpretation of *in vivo* radioligand binding and raises the important question of whether radioligands can bind to internalized receptors. In order to cross the blood brain barrier, which is similar to a cell membrane, all radioligands are lipophilic molecules. Due to the lipophilic nature, the radioligands are expected to be able to enter the intracellular environment. The question is to what extent and whether they also bind to the internalized receptors. For example, the benzamide ligand raclopride has uniformly showed a decrease in radioligand binding after dopamine stimulation, whereas butyrophenone ligands, such as methylspiperone, showed either little to no change or even increased binding after dopamine stimulation (Hartvig et al., 1997; Kobayashi et al., 1995; Laruelle, 2000). Similar results have been reported from several *ex vivo* studies of benzamide and butyrophenone radioligands (Bischoff and Gunst, 1997; Sun et al., 2003; Young et al., 1991). One explanation for these discrepant results is the difference in lipophility (usually measured as the octanol/water partition coefficient, LogP) of the two radioligands. The benzamide, raclopride is less lipophilic (LogP ~

1.33) than the butyrophenones, methylspiperone and spiperone (LogP ~ 3.32 and 3.65 respectively). Therefore, the butyrophenone ligands would more readily cross the cell membrane and bind to internalized receptors than the less lipophilic raclopride (Laruelle, 2000). Another explanation for the discrepant results is vesicular trapping of the radioligand due to pH changes in the intracellular environment. Studies of endocytosis demonstrated that the internalization process involves a rapid acidification of the endocytic vesicles, which can trap ligands of weak bases in the vesicles due to acidotropic uptake (Maloteaux et al., 1983; Maxfield, 1982; Yamashiro et al., 1983). Notably, both spiperone and raclopride are weak bases and are thus sensitive to acidotropic uptake (i.e. vesicular accumulation). Using a pH sensitive dye method, a previous study reported the threshold for vesicular accumulation of spiperone to be 1 μM, whereas the threshold for raclopride was 100 μM, suggesting that spiperone enters the intracellular compartments more readily than raclopride (Rayport and Sulzer, 1995). A recent study used saturation binding experiments in intact cells to measure the affinity for membrane bound versus internalized receptors of the most common D_2 antagonist PET radioligands. All radioligands, including raclopride and methylspiperone, bound with relative high affinity ($K_i \leq 1$ nM) to internalized receptors, although the affinity for internalized receptors was about 2 fold lower than that of membrane bound receptors. This study suggests that all the radioligands bind to internalized receptors but the binding may be affected to some degree due to the 2 fold decrease in affinity (Laruelle et al., 2008).

In summary, a number of studies indicate that dopamine stimulation induces internalization of the D_2 receptors and that internalization may affect radioligand binding. However, to what extent radioligands cross the cell membrane and bind to internalized receptors is unclear. For example, the ceiling effect from dopamine displacement studies, mentioned in the previous chapter, was explained by dopamine competition at high affinity configured receptors. If the radioligand binds to internalized receptors, another explanation for the ceiling effect could be that receptor internalization partly prevents dopamine displacement, as dopamine does not cross the cell membrane. On the other hand, the prolonged decrease of radioligand binding after amphetamine challenge is difficult to explain if the radioligand binds to internalized receptors, although some reduction is expected due to the lower affinity for the relocated receptors.

In conclusion, PET imaging provides a useful and non-invasive way to explore molecular neurobiology *in vivo*, but there are still many questions that need to be

addressed to fully understand the pharmacology of radioligand binding at the D_2 receptors. The present thesis focused on two of these questions, namely high affinity agonist binding and the role of receptor internalization. The following papers represent the experimental work that was performed in order to elucidate these two important questions.

2. AIMS OF THE PROJECT

The aims of the present thesis were to elucidate some of the paradoxes connected to molecular imaging of the D_2 dopamine receptor, namely high affinity agonist binding and the role of agonist induced receptor internalization. For this purpose pharmacological studies in cell culture and brain tissue were combined with PET imaging.

The two primary aims of the project were as follows:

1. To study high affinity agonist binding to the D_2 dopamine receptor *in vivo.*

Paper I. PET study using [^{11}C]MNPA and dopamine depletion. The purpose of the study was to estimate the baseline occupancy of the D_2 dopamine by dopamine and evaluate the novel agonist radioligand [^{11}C]MNPA.

Paper II. *In vitro* pharmacological characterization of MNPA, a novel PET radioligand. The purpose of the study was to verify the agonistic properties MNPA.

Paper III. *In vivo* PET imaging using the agonist [^{11}C]MNPA and *in vitro* evaluation of high affinity agonist binding in dopamine-β-hydroxylase knockout mice. The purpose of the study was to evaluate a mouse model for studying high affinity agonist binding *in vivo.*

2. To study the role of agonist induced internalization of the D_2 dopamine receptor in PET imaging studies.

Paper II. *In vitro* pharmacological characterization of MNPA, a novel PET radioligand. The purpose of the study was to verify the agonistic properties MNPA including agonist induced internalization.

Paper IV. Evaluation of agonist induced D_2 dopamine receptor internalization in striatal tissue slices from arrestin3 knockout mice. The purpose of the study was to evaluate a mouse model for studying agonist induced internalization *in vivo.*

Paper V. PET imaging study using the antagonist [^{18}F]fallypride and the agonist [^{11}C]MNPA and amphetamine challenge. The purpose of the study was to determine if the prolonged decrease of radioligand binding after amphetamine is caused by D_2 receptor internalization.

3. MATERIALS AND METHODS

This section will provide a description of the various methods used in the studies as well as a description of the animal models. More detailed information regarding the individual experiments is provided in the papers.

3.1. *IN VITRO* STUDIES

3.1.1. Cell culture

Cell culture studies were carried out on human embryonic kidney 293T (HEK293T) cells cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum, 1mM sodium pyrovate, 10 μg/mL gentamicin, 50 U/mL penicillin and 50 μg/mL streptomycin. Cells were kept under standard condition at 37 °C in 5% CO_2. For experiments, the cells were transiently transfected using a calcium phosphate transfection kit.

3.1.2. cAMP assay

HEK293T cells expressing D_{2L} or D_3 dopamine receptors and adenylate cyclase 5 were seeded in poly (D)-lysine coated 24 well plates at a density of 1.5 x 10^5 cell/well one day before experiment. Forskolin-stimulated cAMP accumulation was assessed by 10 min incubation in assay buffer (DMEM, 20 mM Hepes, 3 μM forskolin, 30 μM RO, 10 μM propranolol, 0.2 mM sodium metabisulfite) containing various amounts of the agonists. Basal activity was measured by incubation without forskolin. Buffer was removed and cells were lysed with 200 μL/well 3% perchloric acid and placed on ice for 30 min. The cell lysate was neutralized with 80 μL/well $KHCO_3$ for 10 min followed by 10 min centrifugation at 1300 x g. After centrifugation 50 μl supernatant from each well was added to reaction tubes containing 50 μL [^{3}H]cAMP (3nM) and 250 μL protein kinase A and incubated on ice for 90 min. The assay was terminated by incubation with 250 μL charcoal solution for 10 min followed by 20 min centrifugation at 2000 x g. Radioactivity of the samples was measured by liquid scintillation spectroscopy and cAMP concentration was calculated from a standard curve using 0.1-27 pmol cAMP.

3.1.3. Radioligand binding assays

For membrane binding assays, HEK293T cells were transfected with D_{2L} or D_3 dopamine receptor and harvested the day after transfection. Membrane homogenates were prepared by 10 min incubation in lysis buffer (5 mM Tris-HCl, 5 mM $MgCl_2$) followed by disruption in a glass homogenizer. The membrane suspension was washed and centrifuged twice for 30 min at 34000 x g in binding buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$). Subsequently, the membranes were resuspended in binding buffer containing 0.2 mM sodium metabisulfite and used for binding assay. For competition assays the membranes were incubated for one hour in binding buffer containing 0.2 mM sodium metabisulfite, ~0.2 nM [^{3}H]methylspiperone and increasing concentrations of competing ligand (MNPA, NPA or dopamine) +/- 100 μM GTP. For saturation assays, the membranes were incubated for 15 min in binding buffer containing 0.2 mM sodium metabisulfite and increasing concentrations of [^{3}H] labeled ligand (methylspiperone, MNPA or NPA) in the presence or absence of +/- 100 μM Gpp(NH)p. For all membrane binding assays, the final assay volume was adjusted to 1 mL with binding buffer.

For intact cell binding assays HEK293T cells were transfected with D_{2L} dopamine receptor and/or arrestin2, arrestin3, empty vector, D_3 dopamine receptor and seeded onto poly (D)-lysine coated 24 well plates at a density of 2 x 10^5 cells/well. On the day of the experiment the plates were washed once with 37 °C Earle's Balanced Salt Solution (EBSS) and incubated for one hour in 500 μL EBSS containing ~2 nM [^{3}H]sulpiride, 0.2 mM sodium metabisulfite and increasing concentrations of competing ligand (MNPA, NPA or dopamine) for competition assays or increasing concentrations of [^{3}H]sulpiride (up to ~12 nM) for saturation assays. For internalization assays, plates were incubated for 30 min at 37 °C in DMEM containing 20 mM HEPES, 0.2 mM sodium metabisulfite and either 10 μM dopamine or 50 nM MNPA before binding assays. After incubation the plates were washed three times with 37 °C EBSS and cells were lysed in 500 μL Triton-X and transferred to scintillation vials.

All binding assays were performed at 37 °C, non-specific binding was determined in the presence of 5 μM (+)-butaclamol and samples were quantified by liquid scintillation spectroscopy.

3.1.4. Confocal microscopy

For confocal microscopy HEK293T cells were transfected with D_{2L}-YFP and arrestin2 or arrestin3 and seeded in 35 mm glass bottom culture dishes at a density of 2 x 10^5 cells per dish. The next day cells were washed once with 37 °C EDSS and covered with 1 mL 37 °C Opti-MEM media. Confocal microscopy was performed at room temperature using a confocal laser scanning microscope at 100X magnification. A time series of confocal images was acquired over a period of ~ 17 min with the first confocal image captured immediately prior to adding 1 mL Opti-MEM containing either 10 μM dopamine or 50 nM MNPA.

3.2. RODENT MODELS

3.2.1. Rats

Male Sprague Dawley rats weighing 375 ± 94 g were obtained from Taconic Farm, Germantown, NY. All rat and mouse procedures described in the material and methods section were performed in accordance with Guide for Care and Use of Laboratory Animals and approved by the National Institute of Mental health Animal Care and Use committee.

3.2.2. Dopamine-β-hydroxylase knockout mice a model for studying high affinity agonist binding *in vivo*

Dopamine-β-hydroxylase (DBH) knockout and heterozygous mice were kindly provided from Dr. David Weinshenker (Emory university, Atlanta, GA, USA). Mice were generated and characterized as described (Thomas et al., 1998; Thomas et al., 1995). Briefly, the strain was maintained on a mixed 129/SvEv and C57BL/6J background. Heterozygous females were bred with knockout males and pregnant females were given isoproterenol, phenylephrine and vitamin C during E9.5-E14.5 and thereafter dihydroxyphenylserine until birth to rescue DBH knockout animals from embryonic lethality. DBH heterozygous mice were used as control animals since they have normal catecholamine levels indistinguishable from wild-type animals (Thomas et al., 1998; Thomas et al., 1995). DBH knockout mice were identified by the delayed growth and ptosis phenotypes and the genotype was further confirmed by PCR. Mice 6-9 month weighing 31 ± 5 g were used for the experiments. A total of 20 mice (10 of each genotype) of mixed gender were used for PET imaging and dissected striatum

from 10 mice (5 of each genotype) were used for *in vitro* binding studies. All groups were matched for gender and age (Paper III).

3.2.3. Arrestin3 knockout mice a model for studying internalization *in vivo*

Arrestin3 (arr3) knockout and wild-type mice were a kind gift from Robert J. Lefkowitz, (Duke University, Durham, NC, USA). The mice were bred and characterized as described (Bjork et al., 2008; Bohn et al., 1999). Briefly, the strain was kept on a mixed SV129 and C57BL/6J background and homozygous knockout or wild-type mice were obtained from littermate breeding of heterozygous parents. No phenotypic abnormalities were observed in the arr3 knockout mice. For characterization of the arr3 knockout mice as a model for studying internalization *in vivo* (paper IV), brains were dissected from 3-6 month old male mice. To study the role of internalization on PET imaging *in vivo* (paper V) mice 2-6 months of age and weighing 24 ± 6 g of mixed gender were used. The mixed gender was chosen due to limited breeding. A total of 60 mice (30 of each genotype) were used for PET imaging. All groups were matched with respect to gender and age.

3.3. CHARACTERIZATION OF MOUSE MODELS

3.3.1. Immunohistochemistry

Brains from arr3 knockout and wild-type mice were removed and frozen on powdered dry ice. On experimental day the brains were cut in 10 μm coronal sections (1.34-0.02 mm from bregma), thaw mounted onto glass slides and allowed to air dry at room temperature. Sections were hydrated for 5 min in phosphate buffered saline (PBS, pH 7.2) followed by 30 min incubation in internalization cocktail; PBS containing 2% ascorbic acid and either 100 μM dopamine, 100 nM MNPA, 50 nM SKF-81297 or nothing for controls. For blocking experiments sections were pre-incubated in 100 nM eticlopride for 10 min followed by incubation in internalization cocktail containing both agonist and antagonist. Incubations were carried out in room temperature in a moist foil covered box. Sections were then fixed for 5 min in PBS containing 4% paraformaldehyde and rinsed for 5 min in PBS before incubation overnight at 4 °C in (1:200) primary anti-D_2 dopamine receptor antiserum as described (Ariano et al., 1993; McVittie et al., 1991). The next day, the sections were rinsed twice for 15 min in PBS and incubated for 1.5 hour at 4 °C in CY3 fluorescently labeled antiserum.

Subsequently sections were rinsed twice for 15 min in PBS and left to air dry in a light protected container before microscopy. Images of striatal tissue were captured on an Olympus BX41 epifluorescence microscope at 20X magnification. Fluorescence intensity was measured and quantified using Adobe Photoshop C2S histogram function as described (Ariano et al., 2005). Background was defined as the luminosity of the fiber bundles perforating the striatum.

3.3.2. Binding assay on brain homogenates

Brains from the knockout and control mice were removed and striatal tissue was dissected, frozen in liquid nitrogen and stored at -80 °C until use. The striatal tissue was homogenized using a polytron 5 sec at 4500 rpm and two times 5 sec at 20000 rpm followed by 20 strokes with a glass homogenizer. Binding studies were performed as described under cell culture studies with the addition of 50 nM ketanserin.

3.4. POSITRON EMISSION TOMOGRAPHY STUDIES

3.4.1. Radioligands

[^{11}C]MNPA was prepared by ^{11}C-methylation of the precursor (*R*)-2-hydroxy-10,11-acetonide-NPA using a two-step labeling method (Steiger et al., 2009). The chemical purity was >98% and the radiochemical purity was >95%. For rat experiments (Paper I), the specific activity at time of injection was 116 ± 21 GBq/μmol (n=16 syntheses). For mouse experiments (paper III and V respectively), the specific activity at time of injection was 82 ± 24 and 73 ± 23 GBq/μmol (*n* =8 and 17 syntheses).

[^{18}F]Fallypride was synthesized based on the literature method (Mukherjee et al., 1995), but using a microwave-accelerated Synthia radiosynthesis platform (Bjurling et al., 1995; Lazarova et al., 2007; Lu et al., 2009). The chemical purity was >99% and the radiochemical purity was >95%. The specific activity at time of injection was 151 ± 54 GBq/μmol (*n* = 22 syntheses).

3.4.2. PET cameras

For paper I and III, rodents were scanned on the Advanced Technology Laboratory Animal Scanner (ATLAS), with a reconstructed resolution of 1.6 mm full-width at half maximum (Seidel et al., 2003). For paper V, half of the mice were scanned on the ATLAS and the other half were scanned on a microPET Focus 120 scanner (Siemens Medical Solutions, Inc. Knoxville, TN, USA), which has a similar high resolution (Kim et al., 2007). For all studies, dynamic emission data were collected continuously for 90 min ([^{11}C]MNPA) or for 150 min ([^{18}F]fallypride) with increasing duration of time frames from 20 s to 20 min. Data were constructed with an OSEM algorithm without attenuation or scatter correction.

3.4.3. Experimental procedures

For all PET imaging studies, anesthesia was induced with 5% isoflurane and maintained with 1.5% isoflurane through a nose cone. Body temperature was maintained between 36.5 and 37°C with a heating pad or a heating lamp. Drugs for dopamine depletion or augmentation were administered i.p. Radioligands and displacement compounds were administered through penile vein catheter in rats and a tail vein catheter in mice.

Occupancy of $D_{2/3}$ receptors by endogenous dopamine

To study the occupancy of $D_{2/3}$ receptors by endogenous dopamine in rats, [^{11}C]MNPA was injected in through a penile vein catheter as a bolus (24 ± 4 MBq) followed by constant infusion (47 ± 5 MBq). Dopamine was depleted by administration of reserpine and α-methyl-paratyrosine. Reserpine (5 mg/kg, i.p.) was given 24 hours before the PET study and α-methyl-paratyrosine (20 mg/kg, i.p) administered 4 and 1 hours before the PET study. For radioligand displacement studies, raclopride (2mg/kg i.v.) and the specific D_3 DAR antagonist BP897 (0.25 and 0.5 mg/kg, i.v) were injected after 50 min during steady state conditions.

To measure radiometabolites rats were injected i.v with [^{11}C]MNPA (41.8 ± 4 MBq, injected mass 1.1 ± 0.3 nmol/kg). Blood samples were collected and the brains were removed 30 min after radioligand injection. Plasma samples, isolated from whole blood, and brains were analyzed for radiometabolites by high-performance liquid chromatography (HPLC) as described (Zoghbi et al., 2006).

Imaging high affinity agonist binding in knockout mice

To study high affinity agonist binding in vivo, DBH knockout and heterozygous mice were imaged at baseline with the agonist [^{11}C]MNPA. Radioligand was injected as a bolus through a tail vein catheter with a mean injected activity of 13 ± 6 MBq, which was accompanied by 0.17 ± 0.09 nmol of carrier.

Imaging agonist induced receptor internalization in arrestin3 knockout mice

To study the role of agonist induced internalization, mice were divided into three groups matched for sex and genotype and imaged with either [^{11}C]MNPA or [^{18}F]fallypride. After an initial baseline scan, the mice had a second scan, either with drug treatment or as retest baseline. One group received amphetamine (3 mg/kg, i.p.) 30 min before the scan; another group received the same dose of amphetamine 4 hours before the scan; and the last group was a retest and did not receive any treatment. The mean injected activity of [^{11}C]MNPA was 9 ± 1 MBq, which was accompanied by 0.14 ± 0.05 nmol of carrier. For [^{18}F]fallypride studies, the mean activity injected into mice was 5 ± 1 MBq, which was accompanied by 0.04 ± 0.02 nmol of carrier.

3.4.4. Image analysis

Images were analyzed with pixel-wise modeling software (PMOD Technologies, Zurich, Switzerland) using the two-parameter multilinear reference tissue model (MRTM2) (Ichise et al., 2003). For constant infusion studies an additional equilibrium model was used to analyze the data, in which the average radioactivity is measured from time 45 to 90 min when steady state conditions are established. Regions of interest were visually identified with guidance from a mouse or rat brain stereotactic atlas (Paxinos and Franklin, 2001). Regions were similar in size for the left and right striatum respectively. For rat studies the total striatal region (left and right) was 50 mm^3 and the cerebellum region 35 mm^3, for mouse studies the total striatal region was 14.6 mm^3 and the cerebellum region 12.8 and 25.6 mm^3 for [^{18}F]fallypride and [^{11}C]MNPA respectively. For both the kinetic and the equilibrium models the outcome measure was binding potential expressed relative to non-specific binding, BP_{ND}, defined as the ratio of striatum - cerebellum/cerebellum (Innis et al., 2007).

3.4.5. Statistical analysis

Data analysis and curve fitting were performed using Graphpad prism and PASW (SPSS) statistical software. Statistical methods included F-test, two-tailed independent *t*-test and repeated measures analysis of variance (rmANOVA). For all studies the level of significance were set at $P<0.05$.

4. RESULTS AND DISCUSSION

4.1. OCCUPANCY OF DOPAMINE $D_{2/3}$ RECEPTORS BY ENDOGENOUS DOPAMINE (PAPER I)

Occupancy of the D_2 receptors by endogenous dopamine has been measured in monkeys and humans with PET imaging using antagonist radioligands and dopamine depletion. However, the estimates have varied almost three fold. This variability may have been caused by incomplete dopamine depletion or the use of antagonist radioligands, which are less sensitive to fluctuations in endogenous dopamine than agonist radioligands. In this study we used the novel agonist radioligand [^{11}C]MNPA to estimate the dopamine occupancy of the D_2 receptors in rats. The occupancy by endogenous dopamine was estimated using the following equation: (depleted BP_{ND} – baseline BP_{ND})/ depleted BP_{ND}. Complete dopamine depletion was achieved by administration of both α-methyl-paratyrosine and reserpine. For PET imaging, [^{11}C]MNPA was injected as a bolus plus constant infusion to achieve steady state concentration in the body and equilibrium receptor binding in the brain. The *in vivo* selectivity of [^{11}C]MNPA for the D_2 and D_3 receptors was evaluated by radioligand displacement with the mixed D_2 and D_3 antagonist, raclopride, and the selective D_3 partial agonist, BP897. In addition to PET imaging, we also analyzed radiometabolites in the brain and plasma.

The baseline occupancy of D_2 receptors by endogenous dopamine was estimated to be ~ 53% (Fig. 6). Previous imaging studies have reported the baseline occupancy of D_2 receptors by endogenous dopamine in the range of 10% to 30% in human and monkeys, thus much lower than the present study (Abi-Dargham et al., 2000; Ginovart et al., 1997; Laruelle et al., 1997a; Verhoeff et al., 2002). This difference could be due to the use of antagonist radioligands in the previous studies. In the present study we used an agonist radioligand, which is more sensitive to endogenous dopamine (Seneca et al., 2006). Another contributing factor is the extent of

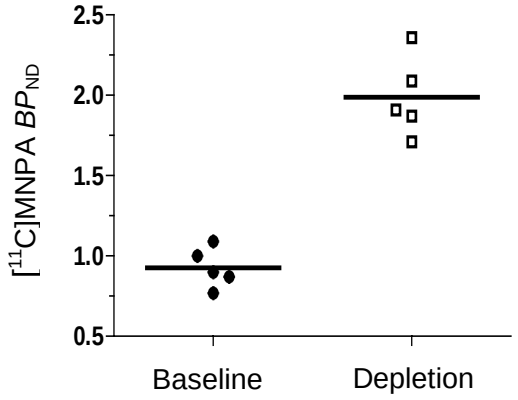

Fig. 6. [^{11}C]MNPA BP_{ND} at baseline (0.93 ± 0.12; mean ± SD) and after dopamine depletion (1.99 ± 0.25). Dopamine depletion significantly increased BP_{ND} (P<0.001, n = 5 rats in each group).

dopamine depletion that can be obtained the different species. The previous studies were performed in humans and monkeys and due to the severe side effects of the depleting drugs only partial dopamine depletion can be achieved these species. Our study was performed in rats, in which complete dopamine depletion can be achieved using the method previously described (Guo et al., 2003). Combined with imaging using an agonist radioligand, this study provides a more robust and accurate measurement of the baseline occupancy of D_2 receptors by dopamine.

The *in vivo* selectivity of [^{11}C]MNPA for the D_2 and D_3 dopamine receptor subtypes was evaluated by injection of either the selective D_3 partial agonist BP897 (0.5 mg/kg, i.v.) or the mixed D_2 and D_3 antagonist raclopride (2 mg/kg, i.v.). Whereas BP897 displaced less than 10% of the binding, raclopride displaced the radioligand binding by 83%, suggesting that [^{11}C]MNPA primarily binds to the D_2 dopamine receptor *in vivo.* To verify that dopamine depletion does not change the metabolism of [^{11}C]MNPA and to exclude the possibility of radiometabolites contributing to specific binding, we measured radiometabolites from the brain and plasma *ex-vivo*, 30 min after radioligand injection. Radiometabolites contributed to ~70% of the total activity in the plasma but only ~10% in the brain and were not affected by dopamine deletion, thus verifying specific [^{11}C]MNPA binding in brain and excluding pharmacologically effects of dopamine depletion on radiometabolism.

In summary, this study suggests that about half of the striatal D_2 receptors are occupied by dopamine in vivo. In addition, we demonstrated that [^{11}C]MNPA is a D_2 selective agonist *in vivo* and a suitable radioligand for future PET studies of the D_2 dopamine receptor.

4.2. MNPA INTERACTIONS WITH D_2 AND D_3 RECEPTORS (PAPER II)

PET radioligands derived from agonist ligands have recently been developed with the purpose of studying the D_2 receptors in the high affinity state *in vivo.* One such agonist radioligand is [^{11}C]MNPA, a methoxy derivative of the well known D_2 and D_3 agonist NPA (Finnema et al., 2007; Gao et al., 1990; Steiger et al., 2009). Despite its utility as a PET radioligand a pharmacological characterization of the agonistic properties of MNPA was not reported. To determine the agonistic properties of MNPA at the D_2 and D_3 dopamine receptors, we compared the pharmacological effects of MNPA to that of the reference compounds NPA and dopamine using recombinant D_2 and D_3 dopamine receptors expressed in HEK293T cells.

The functional agonistic properties were determined by inhibition of cAMP formation. We found that MNPA was a full agonist at both the D_2 and D_3 receptor and inhibited cAMP formation to the same extent as dopamine. Compared to dopamine, MNPA was 50 times more potent at the D_2 receptor but equally potent at the D_3 receptor.

Competition assays in membrane homogenates showed that MNPA bound as a typical agonist to both a high and low affinity site of the D_2 receptor (K_{iH} ~ 0.1 nM and K_{iL}~15 nM) of which the high affinity site was sensitive to the addition of GTP in the assay (Fig. 7). Similar binding at the D_2 receptor was observed for the reference compounds NPA and dopamine. Competition assays in membrane homogenates from cells expressing D_3 receptors also showed that MNPA bound to both a high and low affinity site (K_{iH} ~ 3 fM and K_{iL}~ 1 nM). Whereas a similar binding profile was observed with NPA, the endogenous agonist dopamine bound with only one affinity to the D_3 receptor. Moreover, none of the agonists were sensitive to GTP, suggesting that the high affinity binding site observed with MNPA and NPA was not connected to G-protein coupling. One question of interest to the utility of MNPA and NPA as PET radioligands though, is whether the high affinity binding site at the D_3 receptor is present *in vivo*. Since the affinity for the D_3 receptor was in the fM range, MNPA and NPA would potentially be D_3 selective agonists *in vivo*. However, PET studies in rats using the mixed D_2 and D_3 antagonist, raclopride and the selective D_3 partial agonist, BP897, suggest that MNPA preferentially binds to the D_2 receptor *in vivo* (paper I).

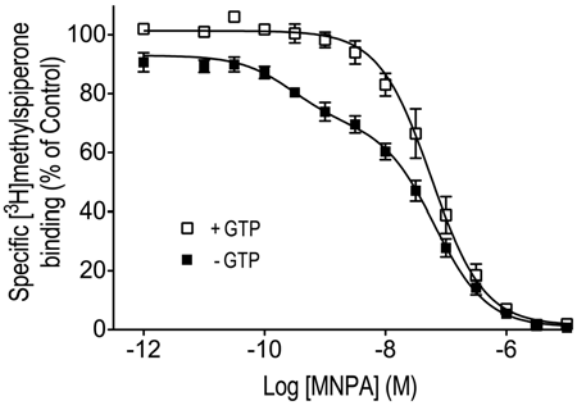

Fig. 7. Competition binding with [^{3}H]methyl-spiperone and MNPA in membrane homogenates from cells expressing D_2 dopamine receptors with (□) and without (■) GTP in the assay. The graphs represent means ± SEM from 5 experiments performed in triplicates.

As another approach to study high affinity agonist binding in membrane homogenates, we performed saturation binding using [^{3}H]-labeled MNPA and NPA. Similar to competition studies, saturation binding was sensitive to the GTP analogue Gpp(NH)p in homogenates from cells expressing the D_2 receptor but not the D_3 receptor. Previous studies reported both guanine nucleotide insensitivity and sensitivity for agonist binding at the D_3 receptor, suggesting that agonist binding at the D_3 receptor

is more complex than at the D_2 receptor (Chio et al., 1994; Freedman et al., 1994; Levesque et al., 1992; Vanhauwe et al., 1999).

In addition to membrane homogenates, we also performed competition binding on intact live cells, which better reflects *in vivo* conditions. In agreement with previous results (Sibley et al., 1983), all three agonists MNPA, NPA and dopamine bound with only one affinity to the D_2 receptor, which best corresponded to the low affinity site observed in membrane homogenates (Fig. 8). One major difference between binding assays performed in membrane homogenates versus intact cells is that the live cells produce endogenous GTP. Thus, the *in vitro* studies on intact cells do not support high affinity agonist binding *in vivo*, as suggested by the occupancy model for PET radioligands. However, the present binding assays were performed under equilibrium conditions. High affinity agonist binding has previously been detected very briefly (<1 min) in intact cells expressing beta-adrenergic receptors under non-equilibrium conditions, suggesting that high affinity agonist binding is detectable under these conditions. Notably, PET studies are often performed under non-equilibrium conditions using bolus injection of the radioligand. Due to the different conditions of the *in vivo* versus *in vitro* binding assays, no definite conclusion can be made to determine whether agonists bind to high affinity site *in vivo* and more research is needed to elucidate the existence of high affinity agonist binding *in vivo.*

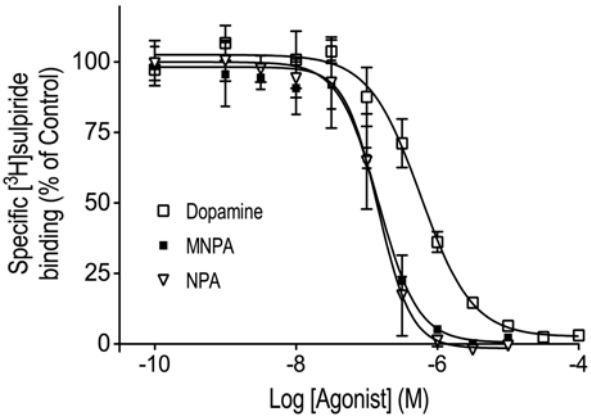

Fig. 8. Competition assay with [^{3}H]sulpiride and the agonists dopamine (□), MNPA (■) and NPA (▽) in intact cells expressing the D_2 dopamine receptor. The graphs represent means ± SEM from 5 experiments performed in triplicates.

Agonist induced internalization of the D_2 receptor was studied using two methods, loss of cell surface radioligand binding and confocal microscopy. The loss of cell surface binding was measured with the hydrophilic radioligand [^{3}H]sulpiride. Agonist pre-treatment reduced cell surface binding with ~10 and ~20% for dopamine and MNPA respectively in cells expressing only the D_2 receptor. The loss of cell surface binding increased by 2 to 3 fold when cells were co-transfected with either arrestin2 or arrestin3, suggesting an important role of arrestin protein in receptor internalization.

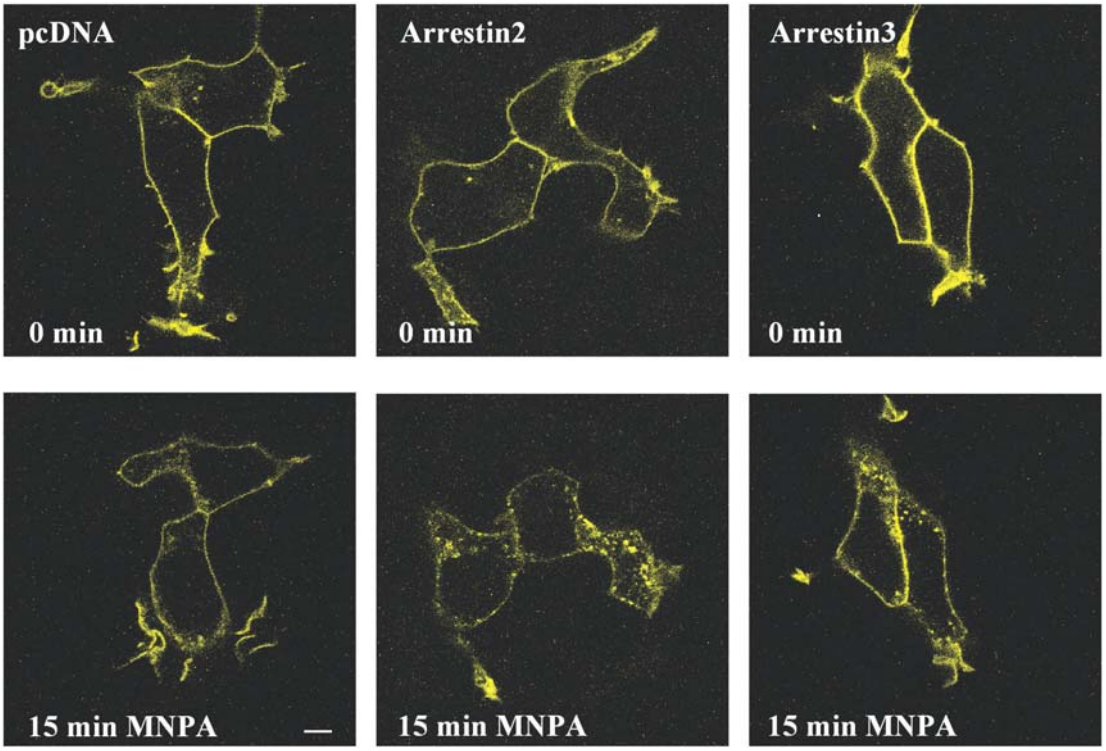

Fig. 9. Agonist induced internalization assed via confocal microscopy. HEK293T cells were transfected with D_2-YFP with either empty vector or constructs for arrestin2 or arrestin3. Confocal images were captured right before (0 min, upper panels) and 15 min after (lower panels) 50 nM MNPA were added. Bar 10 μm.

Confocal microscopy was used to visualize the agonist induced internalization using a D_2 receptor construct with yellow fluorescent protein (D_2-YFP). Similar to cell surface radioligand binding, co-transfection with arrestin markedly increased D_2 receptor internalization (Fig. 9). For both cell surface binding and confocal microscopy, MNPA appeared to induce greater receptor internalization than dopamine.

In summary, these results demonstrate that MNPA is a full agonist at both the D_2 and D_3 dopamine receptor. MNPA is 50 times more potent than dopamine at the D_2 receptor but has similar potency as dopamine at the D_3 receptor. In membrane homogenates, MNPA binds to both a high and low affinity site of the D_2 and the D_3 receptor, but only the high affinity site at the D_2 receptor is sensitive to GTP. In intact cells, high affinity binding was not observed. Similar to most agonists at G-protein coupled receptors, MNPA induced D_2 receptor internalization, which was augmented by co-transfection of arrestin proteins.

4.3. DBH KNOCKOUT MICE HAVE NORMAL DENSITIES OF D_2 DOPAMINE RECEPTORS IN THE HIGH AFFINITY STATE (PAPER III)

Deletion of the gene for dopamine β-hydroxylase (DBH) causes mice to become hypersensitive to the effects of psychostimulants, and *in vitro* radioligand binding studies suggested that this hypersensitivity is caused by an increased percentage of D_2 dopamine receptors in a high affinity state. Based on these findings, we thought that the DBH knockout mice would be an interesting animal model to study high affinity agonist binding *in vivo.* To determine whether DBH knockout mice display an increased percentage of D_2 receptors in the high affinity state *in vivo*, we scanned DBH knockout and control mice with the agonist PET radioligand [^{11}C]MNPA, which is thought to bind preferentially to the high affinity state of the D_2 receptor. In addition, we performed *in vitro* binding experiments on striatal membrane homogenates to measure B_{max} values and the percentage of D_2 receptors in the high affinity state.

We found no differences in [^{11}C]MNPA BP_{ND} between DBH knockout and control mice suggesting that DBH knockout mice do not display increased high affinity agonist binding *in vivo* (Fig. 10). In agreement with our PET studies, we found no differences in the percentage of D_2 dopamine receptors in the high affinity state between DBH knockout and control mice with binding studies on membrane homogenates. Our results are in contrast to two prior studies that reported an increased percentage of D_2 receptors in the high affinity state in DBH knockout mice, although these studies did not indicate statistical significance (Schank et al., 2006; Seeman et al., 2005). The reasons for these discrepant results are not clear, although they may be related to differences in methodology between the studies. In agreement with previous studies, we did not find any difference in D_2 receptor density (B_{max}). In summary, our results suggest that DBH knockout mice have normal density of D_2 receptors and do not display increased high affinity agonist binding *in vivo.*

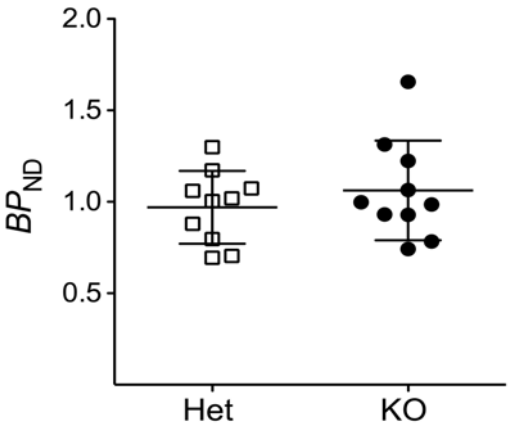

Fig. 10. [^{11}C]MNPA BP_{ND} measured in DBH heterozygous (Het) and knockout (KO) mice. BP_{ND} was insignificantly different between heterozygous controls (0.98 ± 0.2) and knockout (1.07 ± 0.3). Values represent mean ± SD, n = 10 mice in each group.

4.4. ARRESTIN3 MEDIATES D_2 RECEPTOR INTERNALIZATION (PAPER IV)

Although, agonist induced internalization of D_2 receptors has been extensively studied *in vitro*, its effect on *in vivo* receptor binding has largely been unexplored. The purpose of the current paper was to characterize and evaluate the arrestin3 knockout mice as a potential mouse model to study agonist induced internalization *in vivo.* We previously demonstrated the importance of arrestin proteins for D_2 receptor internalization with binding assays and confocal microscopy in cell cultures (paper II). In addition, Caron and coworkers suggested that arrestin3 interacts with the D_2 dopamine receptor in the brain (Beaulieu et al., 2005). Therefore, we speculated that arrestin3 deficient mice would be incapable of D_2 receptor internalization. To test this hypothesis we studied agonist induced internalization of the D_2 receptor using immunohistochemistry in striatal tissue slices from arrestin3 knockout and wild-type mice.

Briefly, membrane bound D_2 receptors were labeled with primary antisera raised against the amino terminal of the receptor and fluorescently labeled secondary antisera. D_2 receptor internalization was induced by incubating striatal tissue slices from arrestin3 knockout and wild-type mice in either MNPA (50 nM) or dopamine (100 μM) for 30 min before immunoassaying. To verify selective D_2 receptor internalization and immunostaining, control experiments were performed with the selective D_2 antagonist eticlopride to block D_2 internalization, and the selective D_1 agonist SKF-81297 to verify selective D_2 receptor staining. Subsequently, D_2 receptor internalization was quantified by measuring the fluorescence intensity on the tissue slices.

Both dopamine and MNPA significantly reduced immunofluorescence staining of the D_2 receptor by ~35% in striatal tissue slices from wild-type mice. In contrast, immunofluorescence staining was unaffected in tissue slices from arrestin3 knockout mice suggesting that D_2 receptor internalization was blocked in these mice (Fig. 11). The selective D_2 antagonist eticlopride completely blocked dopamine induced internalization of the D_2 receptor and the selective D_1 agonist SKF-81297 had no effect on immunofluorescence staining, thus verifying D_2 subtype specific internalization and immunostaining (Fig. 11).

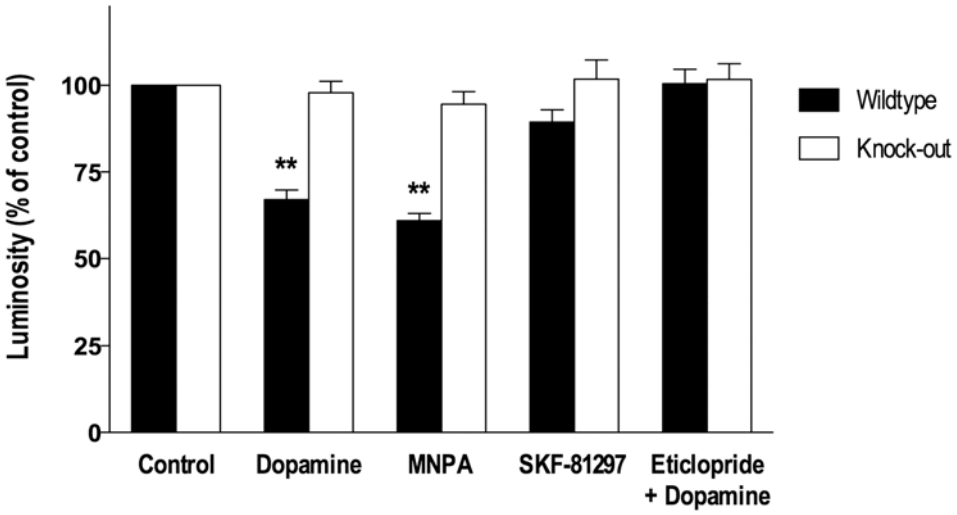

Fig. 11. Agonist induced internalization of the D_2 receptor measured by loss of immunofluorescence staining in striatal tissue slices from arrestin3 wild-type (closed bars) and knockout (open bars) mice. Both dopamine and MNPA significantly (***P*<0.001) reduced of D_2 immunostaining in wild-type tissue but had no effect in arr3 knockout tissue. The selective D_1 agonist SKF-81297 had no effect on D_2 immunostaining and the selective D_2 antagonist, eticlopride completely blocked agonist induced internalization. Bars represent mean ± SEM expressed as percentage of control (100%). Experiments were performed two to five times with three slices per slide.

Our results suggest that D_2 receptor internalization is primarily mediated by arrestin3 in the striatum and are in agreement with those previously reported (Beaulieu et al., 2005). However, our results conflict with those of Macey et al. (2004), who reported that D_2 receptor internalization was mediated by arrestin2 in cultured neostriatal neurons. These discrepancies may be related to developmental differences. Whereas Macey et al. (2004) used neostriatal neurons from E15 and E18, we used striatal tissue from adult mice. However, more studies are needed to clarify a possible developmental involvement in the interactions of the two arrestin subtypes with the D_2 receptor. In summary, this study demonstrates that arrestin3 mediates D_2 receptor internalization in adult striatal tissue slices and furthermore suggest that arr3 knockout mice can be used as an animal model to study D_2 receptor internalization *in vivo.*

4.5. D_2 RECEPTOR INTERNALIZATION PROLONGS THE DECREASE OF RADIOLIGAND BINDING AFTER AMPHETAMINE (PAPER V)

Dopamine released by amphetamine decreases the *in vivo* binding of PET radioligands to the dopamine D_2 receptor. Although concentrations of extracellular dopamine largely return to baseline within one to two hours after amphetamine treatment, radioligand binding remains decreased for several hours. The purpose of this study was to determine whether the prolonged decrease of radioligand binding after amphetamine administration is caused by D_2 receptor internalization. To distinguish dopamine displacement from receptor internalization, we used wild-type and arrestin3 knockout mice, which we previously demonstrated are incapable of internalizing D_2 receptors in striatum (Paper IV). As mentioned in the introduction, agonist radioligands at the D_2 receptor are more sensitive to dopamine displacement than antagonist radioligands. Therefore, we used both the D_2 agonist radioligand, [^{11}C]MNPA and the D_2 antagonist radioligand, [^{18}F]fallypride for the PET studies. After an initial baseline scan the mice were divided into three groups for a second scan: either 30 min or 4 hours after amphetamine administration (3 mg/kg, i.p.) or as retest with no treatment. We expected that the 30 min group would represent primarily direct dopamine displacement and the 4 hour group would represent primarily internalization, whereas the retest group served as a control to verify that baseline radioligand binding did not change from the first to the second scan. The data were analyzed with repeated measures analysis of variance (rmANOVA) with BP_{ND} as the dependent variable, treatment as the within-subject variable and time and genotype as between-subject variables.

We found a significant 3-way interaction of treatment × genotype × time for both [^{11}C]MNPA ($F= 4.32$, $P = 0.026$) and [^{18}F]fallypride ($F = 4.79$, $P = 0.018$). That is, amphetamine significantly decreased radioligand binding, and its effect was dependent upon genotype and time. To determine the different effect of amphetamine on the genotype, we analyzed the groups at separate time points (retest, 30 min and 4 hours). As expected, no difference was observed from the first to the second baseline scan in the test-retest group. At 30 min, BP_{ND} was reduced to a similar degree in both knockout and wild-type mice, suggesting that the decrease of radioligand binding at 30 min was primarily due to dopamine displacement. In agreement with previous studies, the agonist [^{11}C]MNPA showed greater sensitivity to amphetamine than the antagonist

[^{18}F]fallypride (Fig. 12). Consistent with our hypothesis, BP_{ND} was reduced only in

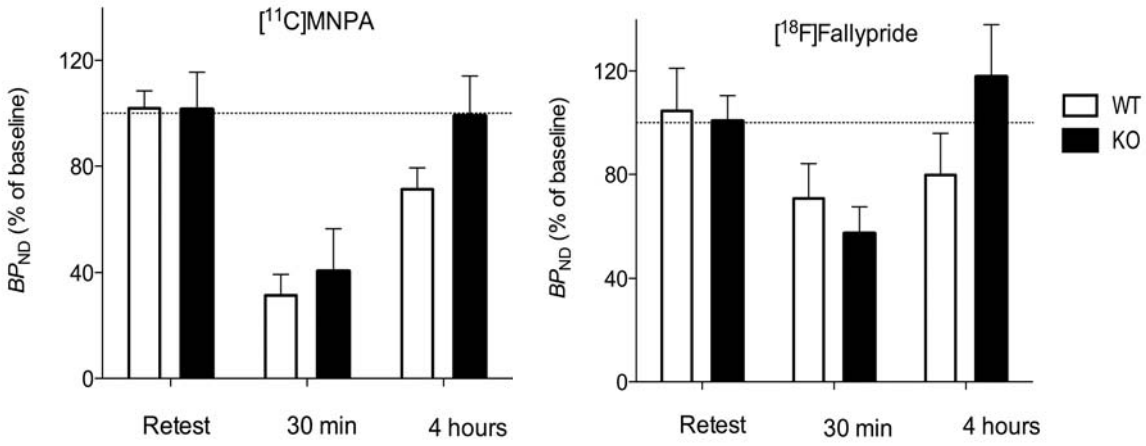

Fig. 12. Striatal BP_{ND} of [^{11}C]MNPA and [^{18}F]Fallypride in arr3 wild-type (open bars) and knockout (solid bars) mice expressed as percentage of the baseline BP_{ND} (baseline = 100%, dashed line) for retest and at 30 min and 4 hours after amphetamine. BP_{ND} was unchanged for retest groups. At 30 min after amphetamine, BP_{ND} was reduced to the same extent in both knockout and wild-type mice. At 4 hours after amphetamine, BP_{ND} was reduced in only wild-type mice. Bars represent mean ± SD, n=5 of each genotype in each group.

wild-type mice in the 4-hour group, strongly suggesting that the prolonged decrease of radioligand binding was primarily due to D_2 receptor internalization (Fig. 12). Similar to the 30 min group, the reduction of radioligand binding at 4 hours was greater for the agonist [^{11}C]MNPA than for the antagonist [^{18}F]fallypride suggesting differential sensitivity to internalization. One explanation to this could be that the two radioligands have differential abilities to cross the membrane and bind to internalized receptors due to different lipophility. Indeed the antagonist [^{18}F]fallypride has been reported to bind to internalized receptors, however with a 2-fold lower affinity, which would affect the radioligand binding to internalized receptors (Laruelle et al., 2008). Assuming that agonist radioligands bind to a high affinity site of the D_2 receptor in vivo, another explanation for this differential sensitivity could be that internalized receptors are separated from G-proteins, which prevent high affinity binding. Nevertheless, our results suggest that the prolonged decrease of radioligand binding after amphetamine challenge is caused by receptor internalization.

5. SUMMARY OF FINDINGS

The present thesis focused on high affinity agonist binding and agonist induced D_2 receptor internalization using *in vitro* analysis and *in vivo* PET imaging.

- PET imaging of rats at baseline and under dopamine depletion suggested that about 50% of the D_2 dopamine receptors are occupied by endogenous dopamine at baseline. In addition, the novel agonist radioligand [^{11}C]MNPA was tested for its selectivity at the D_2 and the D_3 receptor and found to primarily bind to the D_2 receptors *in vivo.*
- The pharmacological characterization of MNPA demonstrated that MNPA is a full agonist at both the D_2 and the D_3 dopamine receptor. MNPA was 50 times more potent than dopamine at the D_2 receptor but equally as potent as dopamine at the D_3 receptor. The pharmacological profile showed that MNPA binds with high and low affinity to the D_2 receptor in membrane homogenates, but only with low affinity in intact cells. In addition, MNPA was able to induce D_2 receptor internalization.
- Dopamine-β-hydroxylase (DBH) knockout mice had normal percentages of D_2 dopamine receptors in the high affinity state measured with *in vivo* PET imaging and *in vitro* binding experiments.
- Agonist induced internalization of the D_2 dopamine receptor is mediated by arrestin3. Receptor internalization measured by immunohistochemistry was detectable in striatal tissue slices from wild-type but not from arrestin3 knockout mice. Arrestin3 knockout mice were characterized and validated as a useful model to study agonist induced receptor internalization *in vivo.*
- *In vivo* PET imaging of arrestin3 knockout and wild-type mice demonstrated that the prolonged decrease of radioligand binding after amphetamine is due to receptor internalization rather than dopamine displacement.

6. FUTURE PROSPECTS

High affinity agonist binding in vivo

Several psychiatric disorders have been linked to dysfunction of the dopamine system and altered dopamine receptor signaling but the exact pathophysiology for these disorders is still poorly understood. If high affinity agonist binding *in vivo* can be used to measure dopamine receptor signaling (i.e. G-protein coupling and activation), development of agonist PET radioligands to study the pathophysiology of psychiatric disorders will be a highly desired goal.

The present thesis studied high affinity agonist binding at the D_2 dopamine receptor. We found that the novel PET ligand MNPA was a potent and full agonist at the D_2 receptor and a suitable agonist radioligand *in vivo.* Although MNPA bound to both a high and a low affinity state of the D_2 receptor in membrane homogenates, the evidence for high affinity agonist binding *in vivo* is still elusive. Firstly, because MNPA bound with one affinity, corresponding to the low affinity, to the D_2 receptor in intact live cells. Secondly, because DBH knockout mice, which were reported to display and increased percentage of D_2 receptor in the high affinity state, were insignificantly different from the control mice. In conclusion, more studies are needed in order to fully elucidate whether agonists bind to a high affinity site of the D_2 receptor. Clarification of this complex issue is of utmost importance for further development of PET radioligands with the purpose of studying and diagnosing psychiatric disorders.

Agonist induced receptor internalization

The present thesis demonstrated that the prolonged decrease of radioligand binding after amphetamine is due to agonist induced receptor internalization. Although our results would need to be replicated by other independent investigators, they provide important information for further studies of receptor trafficking *in vivo* using PET imaging. For example, recent studies suggest that inhibition of arrestin recruitment at the D_2 receptor may be important for the clinical effectiveness antipsychotic drugs (Klewe et al., 2008; Masri et al., 2008). Notably, inhibition of arrestin recruitment would interfere with receptor internalization, suggesting that receptor trafficking may be altered in schizophrenia. In conclusion, further studies of receptor trafficking using PET imaging are strongly encouraged and would provide valuable information about diseases associated with altered receptor trafficking.

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