From THE DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

ASSESSMENT OF DOPAMINE AND SEROTONIN RELEASE IN THE NON-HUMAN PRIMATE BRAIN USING PET

Sjoerd J. Finnema

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Cover illustration:
MRI-coregistered PET summation images obtained after intravenous injection of the dopamine D_2/D_3 receptors agonist radioligand $[^{11}C]$MNPA into a cynomolgus monkey during baseline and post-amphetamine (1.0 mg/kg) conditions.

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ABSTRACT

The molecular imaging technique positron emission tomography (PET) allows for non-invasive examination of biochemical markers in the living brain. For over three decades PET studies have provided important insight into the relationship of monoaminergic neurotransmitter systems to brain functioning and psychiatric disorders. A more recent application of PET is the study of endogenous neurotransmitter release *in vivo*. Clinical relevance of such methods is found in studies demonstrating enhanced amphetamine-induced dopamine release in schizophrenia patients, whereas PET studies in non-human primates provide a translational model for evaluation of the pharmacological mechanisms before initiation of studies in man.

The first aim of this thesis was to develop improved methods for measurement of endogenous dopamine levels. In study I the potent D_2/D_3 receptors agonist (*R*)-(-)-2-methoxy-*N*-*n*-propyl-norapomorphine (MNPA) was radiolabeled with carbon-11 and found suitable for *in vivo* characterization of the high affinity state. In study II, amphetamine-induced displacement of [^{11}C]MNPA binding by dopamine was ~1.8 fold higher at four different doses than for the antagonist [^{11}C]raclopride and demonstrated that an agonist radioligand has improved sensitivity to endogenous neurotransmitter level. Study III aimed to further obtain *in vivo* support for the existence of two affinity states for the D_2/D_3 receptors. Receptor occupancy of the exogenous agonist apomorphine was determined with [^{11}C]MNPA and [^{11}C]raclopride. Binding of [^{11}C]MNPA and [^{11}C]raclopride was inhibited monophasic and approached full saturation. ID_{50} and K_i values of apomorphine were indistinguishable when measured with the agonist or antagonist radioligand. Study III did not support the existence of two affinity states and a possible explanation could be that all D_2/D_3 receptors are in the high affinity state *in vivo*. In study IV, the new D_1/D_5 receptors partial agonist radioligand (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 was found insensitive to dopamine levels, and receptor binding was inferior to previously developed antagonist radioligands. Moreover, a COMT formed radiometabolite was found to enter the brain but the formation could be prevented with the use of a COMT inhibitor. COMT inhibition provides a methodology enabling quantitative PET measurements with (*S*)-[^{11}C]*N*-methyl-NNC 01-0259.

The second aim of this thesis was to evaluate the sensitivity of the new 5-HT_{1B} receptor radioligand [^{11}C]AZ10419369 to alterations in endogenous serotonin concentration. Previous serotonergic PET radioligands have ambiguously shown sensitivity to serotonin level. In study V the effective serotonin releaser fenfluramine decreased the binding of [^{11}C]AZ10419369 in a dose-dependent manner. In study VI the effect of fenfluramine on [^{11}C]AZ10419369 binding was confirmed using an equilibrium approach with a bolus infusion protocol. The further developed methodology is suitable for exploring the sensitivity limit to serotonin levels as measured using [^{11}C]AZ10419369 and PET.

In conclusion, the present thesis demonstrates that the D_2/D_3 receptors agonist radioligand [^{11}C]MNPA is an improvement for measurement of dopamine release, when compared to previously used antagonist radioligands. Moreover, a novel methodology, using the 5-HT_{1B} receptor antagonist [^{11}C]AZ10419369 and PET, was developed for measurement of serotonin release in the living brain. These newly developed methodologies may help to further understand the treatment and pathophysiology of several major neurological and psychiatric disorders.

"*Zing, vecht, huil, bid, lach, werk en bewonder*"

[Sing, fight, cry, pray, laugh, work and admire]

Ramses Shaffy, 1933-2009

LIST OF PUBLICATIONS

This thesis is based on six studies performed at the Department of Clinical Neuroscience, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden. The studies are presented in the following papers and will be referred to in the text by their roman numerals:

I. **Finnema SJ**, Seneca N, Farde L, Shchukin E, Sóvágó J, Gulyás B, Wikström HV, Innis RB, Neumeyer JL and Halldin C. (2005) A preliminary PET evaluation of the new dopamine D_2 receptor agonist [^{11}C]MNPA in cynomolgus monkey. *Nuclear Medicine and Biology* 32:353-360.

II. Seneca N, **Finnema SJ**, Farde L, Gulyás B, Wikström HV, Halldin C* and Innis RB*. (2006) Effect of amphetamine on dopamine D_2 receptor binding in nonhuman primate brain: a comparison of the agonist radioligand [^{11}C]MNPA and antagonist [^{11}C]raclopride. *Synapse* 59:260-269.

III. **Finnema SJ**, Halldin C, Bang-Andersen B, Gulyás B, Bundgaard C, Wikström HV and Farde L. (2009) Dopamine $D_{2/3}$ receptor occupancy of apomorphine in the nonhuman primate brain – a comparative PET study with [^{11}C]raclopride and [^{11}C]MNPA. *Synapse* 63:378-389.

IV. **Finnema SJ**, Bang-Andersen B, Jørgensen M, Gulyás B, Wikström HV, Farde L and Halldin C. Inhibition of catechol-*O*-methyltransferase enhances visualization of (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 binding to dopamine D_1-like receptors in monkey brain. Manuscript.

V. **Finnema SJ**, Varrone A, Hwang TJ, Gulyás B, Pierson ME, Halldin C* and Farde L*. (2010) Fenfluramine-induced serotonin release decreases [^{11}C]AZ10419369 binding to 5-HT_{1B}-receptors in the primate brain. *Synapse* 64:573-577.

VI. **Finnema SJ**, Varrone A, Hwang TJ, Halldin C* and Farde L*. Confirmation of fenfluramine effect on 5-HT_{1B} receptor binding of [^{11}C]AZ10419369 using an equilibrium approach. Manuscript.

*Authors contributed equally to this work.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HTP	5-hydroxy-*L*-tryptophan
AADC	Aromatic *L*-amino acid decarboxylase
AC	Adenylate cyclase
ADHD	Attention deficit hyperactivity disorder
ALDH	Aldehydedehydrogenase
AMPT	Alpha-methyl-para-tyrosine
ATP	Adenosine-5'-triphosphate
B_{max}	Receptor density
BBB	Blood-brain-barrier
BP	Binding potential
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
COMT	Catechol-*O*-methyltransferase
DAT	Dopamine transporter
ex vivo	"Out of the living", i.e. *in vivo* tissue studied outside an organism
FOV	Field of view
FWHM	Full width half maximum
G-protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HPLC	High-performance liquid chromatography
HRRT	High resolution research tomograph
i.m.	Intramuscular
in vitro	"Within the glass", i.e. in a test tube
in vivo	"Within the living", i.e. in an organism
i.v.	Intravenous
K_{D}	Dissociation constant at equilibrium
K_{i}	Inhibition constant at equilibrium
L-DOPA	*L*-3,4-dihydroxyphenylalanine
LogD	Logarithm of partition coefficient of octanol/water at pH 7.4
MAO	Monoamine oxidase
MNPA	(*R*)-(-)-2-methoxy-*N*-n-propyl-norapomorphine
MRI	Magnetic resonance imaging
MRTM	Multilinear reference tissue model
PET	Positron emission tomography
PKA	Protein kinase A
ROI	Region of interest
SPECT	Single photon emission computerized tomography
SRTM	Simplified reference tissue model
SUV	Standard uptake value
TH	Tyrosine hydroxylase
TP	*L*-Tryptophan
TPH	Tryptophan hydroxylase
TYR	Tyrosine

1 INTRODUCTION

1.1 MAJOR APPLICATIONS OF PET

Molecular imaging is a rapidly expanding field and covers techniques that allow for the visualization of biochemical processes in living organisms. Prominent modalities are magnetic resonance imaging (MRI), optical imaging and nuclear imaging, with combination of techniques currently under development[30,77]. Positron emission tomography (PET) is a nuclear imaging technique that allows for quantitative measurement of binding to specific proteins in tissue. The first PET studies of the human brain were performed in the early 1980s and focused on the study of neurotransmitter systems[341].

The dopamine and serotonin neurotransmitter systems have for several reasons attracted key interest in relation to the pathophysiology of several neurological and psychiatric disorders including, Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD) and depression. PET studies have provided the first quantification of receptors in the living brain and thereby allowed for studies of receptor density in relation to pathophysiology[69,71,347]. A significant enhancement in the clinical utility of brain PET imaging is soon anticipated with recent development of biomarkers of pathophysiology. First examples are radioligands that bind β-amyloid aggregates, which are proteins thought to be heavily deposited in the brains of Alzheimer patients[160,233].

A second utility of PET imaging is to understand drug action and to facilitate drug development[73,121,178]. Pioneering studies have explored dopamine receptor occupancy of antipsychotic drugs and examined the relationship between receptor occupancy, clinical efficacy and side-effects[74,75,235,237]. Another type of approach undertaken makes use of the "microdosing concept" (typically < 5μg) in which a drug is radiolabeled to obtain a detailed description of its distribution in the body[18]. More recently, the microdosing approach is gaining increasing acceptance and interest from governmental approving agents and pharmaceutical industries.

Another application which uses PET and neuroreceptor radioligands is to study the competition between radioligand and endogenous neurotransmitter. Pioneering studies have evaluated amphetamine-induced changes in synaptic dopamine level in the primate brain[57,74,139], and the clinical relevance of such methodology has been demonstrated by increased dopamine release in patients with schizophrenia[22,172]. Successful studies of endogenous neurotransmitter release have thus far been almost exclusively reported for dopamine, which is possibly related to the lack of radioligands sensitive to other endogenous neurotransmitters.

PET studies in non-human primates provide a translational model for evaluation of pharmacological mechanisms before initiation of studies in human subjects. Non-human primates are phylogenetically the closest relatives with humans: non-human primates share a large percentage of their DNA with humans and consequently yield physiological and neuroanatomical similarities. Similar complexities of adult human and monkey brains allow for translation from animal models to the human condition more readily than when using more phyologenetically distant animals[11,230].

1.2 PRINCIPLES OF PET

After intravenous (i.v.) administration of a substance labelled with a positron-emitting radionuclide (either radiotracer or radioligand), PET imaging allows for evaluation of regional distribution and quantification of radioactivity in the living body (*in vivo*). The radiolabeled substance contains a radionuclide, which at decay emits a

positron (β+). The positron passes through surrounding tissue (one to a few millimetres) until it annihilates with an electron, resulting in the emission of two 511 keV γ-particles (photons), which travel approximately 180˚ apart (Figure 1).

The two photons comprise high energy and have therefore high probability to escape from the body. When both photons hit two γ-ray detectors of the PET system within a predefined time window, a "coincidence" event occurs. The coincidence event can be used to locate the positron-electron annihilation, and this location closely approximates of where the positron was emitted. A typical PET measurement consists of a large collection of coincidence events occurring after i.v. administration of a radiolabeled substance to animal or human. Quantitative images are then obtained after reconstruction and appropriate correction for absorption, scatter and random coincidences[33,66].

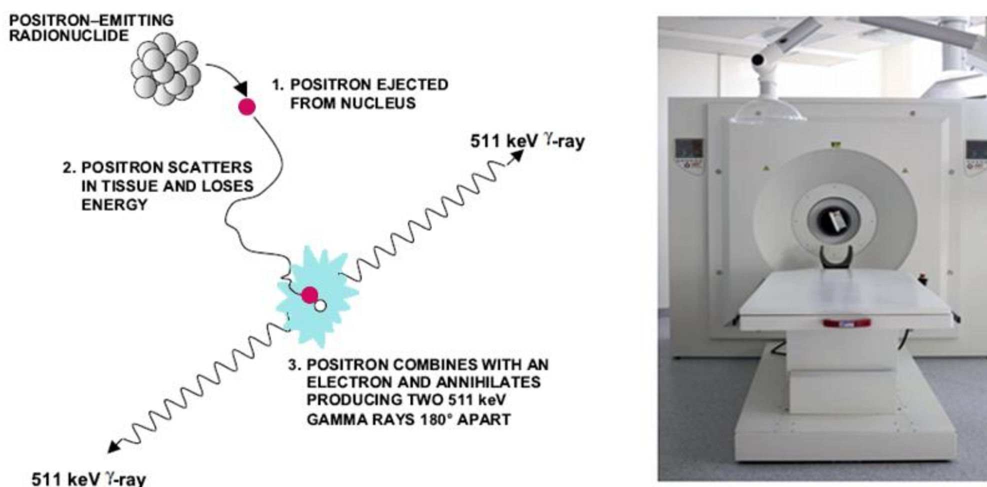


Figure 1. (Left) Summary of physics underlying the PET methodology, figure adopted from[33]. (Right) HRRT PET system as used in routine practice at the Karolinska Institutet since 2008.

PET systems must be designed with high sensitivity and high spatial resolution for obtaining acquisition data with high accuracy. Spatial resolution can be defined as a measure of the smallest image area identifiable as a separate unit. The resolution of a PET system is typically expressed in terms of full width at half maximum (FWHM). A Gaussian function is used to represent a perfect point source, and the resolution is defined as the distance between the maximum signal to where the signal intensity is half of the maximum[14]. Specific brain dedicated PET systems are available with the high resolution research tomograph (HRRT) being the most recently developed system[50] (Figure 1). In this thesis two PET systems were used, the ECAT exact HR 47, which has a spatial resolution of 3.8 mm FWHM, and the HRRT, which has a resolution of 1.5 mm FWHM[334,343].

1.3 DEVELOPMENT OF PET RADIOLIGANDS FOR CNS

One of the key advantages of PET is that several available short-lived radionuclides are isotopes of elements very typically seen in biological materials, elements such as nitrogen, oxygen and carbon. Incorporation of the radionuclides into molecules does not affect the biological activity of the molecule and thereby provides a unique opportunity to study biological processes in living subjects. Short-lived radionuclides are typically produced onsite by a particle accelerator (cyclotron): the radionuclide used in the present thesis was carbon-11 ($t_{1/2}$ = 20.4 min) (Table 1). The

short half-life of carbon-11 limits the possibility of transportation off site, but has the advantage to allow for multiple PET studies in the same subject on the same day.

Table 1. Main characteristics of frequently used radionuclides[121].

	^{15}O	^{11}C	^{18}F
Half-life (min)	2	20	110
Mode of decay (% β+)	100%	100%	97%
Maximal energy (MeV)	1.74	0.97	0.64
Penetration distance (mm)	8	4	2

The development of PET radioligands for imaging of new targets has often benefited from organic chemistry efforts in drug discovery. Whereas the properties of an optimal therapeutic drug and radioligand commonly differ, the drug programs provide large numbers of compounds, from structural different classes, of which some may eventually prove useful as drugs and others as radioligands. An example, relevant to this thesis, is the development of agonist radioligands selectively targeting D_1/D_5 or D_2/D_3 receptors. Dopamine agonists developed for the treatment of Parkinson's disease preferably have agonistic properties for both D_1 and D_2 receptor to achieve optimal treatment effect. A radioligand is in contrary preferably selective to one receptor subtype, thereby allowing evaluation of binding to the specific receptor subtype.

PET radioligands to be useful for the examination of targets in the central nervous system (CNS) must fulfil a range of criteria[122,253]. In short, radioligands should display high selectivity and receptor affinity (K_D in nM range) to the binding target as well as sufficient lipophilicity to enable passage of the blood-brain-barrier (BBB). Too high lipophilicity may result in high non-specific binding in brain. Suitable radioligands for CNS are not substrates for efflux-transporters, such as P-glycoprotein (Pgp), as Pgp can drastically limit accumulation of radioligand in brain[149]. In addition, radioligands should have appropriate metabolism avoiding formation of CNS-penetrating radiometabolites. For proper quantification of target binding, a suitable pharmacokinetic profile is of great importance. Optimal receptor binding kinetic properties for ^{11}C-labeled radioligands provide a peak in specific binding within 30-60 minutes after radioligand injection to provide reliable measurements. Finally, amenability for labelling with ^{11}C or ^{18}F with sufficient high specific radioactivity should ensure straightforward radiosynthesis and the administration of minimal mass of radioligand to fulfil the demands of tracer conditions and safety requirements.

1.4 QUANTITATIVE PET MEASUREMENTS

1.4.1 Quantification of radioligand receptor binding

In pharmacology, the binding reaction between substance (drug or radioligand) and receptor is conventionally described according to equation 1, in which equilibrium exists between available receptors (*R*), concentration of free substance (*F*) and concentration of receptor bound substance (*B*).

$$\boldsymbol{R} + \boldsymbol{F} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftarrows}} \boldsymbol{B} \qquad (1)$$

At equilibrium conditions, the relationship between receptor binding and the concentration of substance can be described by the Michaelis-Menten equation, which

includes the receptor density (B_{max}) and the equilibrium dissociation constant (K_D) (equation 2).

$$B = \frac{B_{max}F}{K_D + F} \quad (2)$$

In applied studies this hyperbolic function can, for instance, be used to describe the relationship between receptor occupancy of drug, maximum receptor occupancy (Occ_{max}), drug concentration (C_D) and inhibition constant (K_i) (equation 3).

$$\text{Receptor occupancy} = \frac{Occ_{max}C_D}{K_i + C_D} \quad (3)$$

Neurochemical techniques using radioligands administered at minimal mass, such as PET, allow for redefinition of equation 2. During tracer conditions, often defined as less than 5% receptor occupancy, the concentration of free substance (F) is considered much smaller than the dissociation constant (K_D) resulting in the expression:

$$\frac{B}{F} = \frac{B_{max}}{K_D} \quad (4)$$

In 1984 Mintun and co-workers used established concepts for *in vitro* radioligand binding to introduce the term binding potential (BP) for quantification of radioligand receptor binding with PET[212]. The BP was defined as the ratio of B_{max} to K_D. At tracer dose conditions, the BP definition of Mintun equals the ratio of B/F at equilibrium. Recently, the nomenclature for *in vivo* imaging of radioligands with reversible binding was further established and the definition of BP was specified in relation to the reference concentration used to define an affinity constant[140]. Shortly, affinity can be expressed by comparison to three distinct reference concentrations, the free plasma concentration of radioligand (BP_F), the total plasma concentration of radioligand (BP_P) or the non-displaceable concentration of radioligand (BP_{ND}). The main outcome parameter in this thesis was BP_{ND}, which represents the product of receptor density (B_{max}), apparent affinity (K_D^{-1}) and the free fraction of radioligand in the non-displaceable tissue compartment (f_{ND})[140].

1.4.2 Quantification of neurotransmitter release

The application of PET to study neurotransmitter release was first proposed in 1984[84]. Investigations conducted since have resulted in a large number of studies confirming that modification of neurotransmitter concentration can be measured using this technology[175]. During PET measurements, the radioligand competes with the neurotransmitter for binding to receptors. When including the free neurotransmitter concentration (F_{NT}) and the neurotransmitter dissociation constant (K_{NT}) in equation 4 it can be rewritten as:

$$BP_{\text{ND Baseline}} = f_{ND}\frac{B_{max}}{K_D\left(1+\frac{F_{NT}}{K_{NT}}\right)} \quad (5)$$

A change in free neurotransmitter concentration (ΔF_{NT}), induced by a pharmacological or physiological intervention, results in a change in BP_{ND} due to the altered competition between radioligand and neurotransmitter to the receptor. In the competition model, it is assumed that the B_{max}, K_D, F_{NT} and K_{NT} are not modified when

compared to the baseline measurement. The BP_{ND} in the challenge study can accordingly be described as:

$$BP_{\text{ND Challenge}} = f_{\text{ND}} \frac{B_{\max}}{K_{\text{D}}(1+\frac{F_{\text{NT}}+\Delta F_{\text{NT}}}{K_{\text{NT}}})} \quad (6)$$

Comparison of the BP_{ND} obtained during baseline and challenge conditions provides initial evaluation of the sensitivity of a radioligand to neurotransmitter release. By combining equation 5 and 6 the relative change in BP_{ND} (Δ) observed in the challenge condition can be expressed as:

$$\Delta = \frac{BP_{\text{ND Baseline}} - BP_{\text{ND Challenge}}}{BP_{\text{ND Baseline}}} = \frac{\Delta F_{\text{NT}}}{K_{\text{NT}}+F_{\text{NT}}+\Delta F_{\text{NT}}} \quad (7)$$

From equation 7 it becomes evident that a significant change in BP_{ND} (Δ) can be expected when the change in neurotransmitter concentration (ΔF_{NT}) is much larger than the sum of the affinity of the neurotransmitter (K_{NT}) and the baseline neurotransmitter concentration (F_{NT}). In addition, it can be concluded that the observed Δ is independent of radioligand characteristics and solely depends on the affinity of the neurotransmitter to the receptor. Development of radioligands suitable for study of neurotransmitter release should therefore preferably target receptors for which the neurotransmitter has high affinity[248].

It has previously been argued that "low-affinity" radioligands could be more suitable tools for measurement of neurotransmitter release[290]. As seen from equation 7, the affinity of the radioligand to the target receptor (K_D) does however not influence the change in BP_{ND} (Δ)[72]. A prerequisite of the validity of equation 7 is that receptor binding is obtained during equilibrium and at tracer dose conditions. Some PET experiments are, on the other hand, performed under rapid changes in neurotransmitter concentration and, under these conditions, it has been suggested that the K_D, or more specifically the K_{off}, is an important parameter allowing for rapid adjustment to the changes in neurotransmitter concentration[64]. Also, the rate constant k_2 has been suggested to be an important radioligand characteristic when the radioligand is to be used for measurement of neurotransmitter release during dynamic conditions[64,187,216]. No systematic study has thus far been conducted to support these theories with experimental results.

1.5 G-PROTEIN COUPLED RECEPTORS

1.5.1 Families of G-protein coupled receptors

G-protein coupled receptors (GPCRs) are the largest family of membrane proteins and mediate the majority of cellular responses to hormones and neurotransmitters. All GPCRs contain seven membrane-spanning segments, which are separated by alternating intra- and extracellular loop regions. In vertebrates, GPCRs are commonly divided into five families: rhodopsin, secretin, glutamate, adhesion and Frizzled/Taste2[276].

1.5.2 G-protein coupled receptor signalling

It has been estimated that more than half of available drugs on the market target GPCRs[81]. Despite intensive academic and industrial research efforts, the structural basis of GPCR functioning is still not fully understood. Guanine nucleotide binding proteins (G-proteins) are heterotrimeric and exist out of three subunits, α, β and γ, with

the α-subunit containing a guanine nucleotide binding site. In the ternary models, agonist binding is thought to modulate the proportion of receptors that are in an active conformation to those that are inactive and not signalling. An agonist induced conformational change results in activation of the associated heterotrimeric G-protein, involving exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by the α-subunit and thereby causing dissociation of the heterotrimeric complex. The dissociated α- and βγ-subunits separately promote cellular signalling by second messenger systems, such as adenylate cyclase (AC). Signal transduction is terminated when bound GTP is hydrolyzed to GDP, and the heterotrimeric complex is reunited and coupled back to the receptor[241,242,275] (Figure 2).

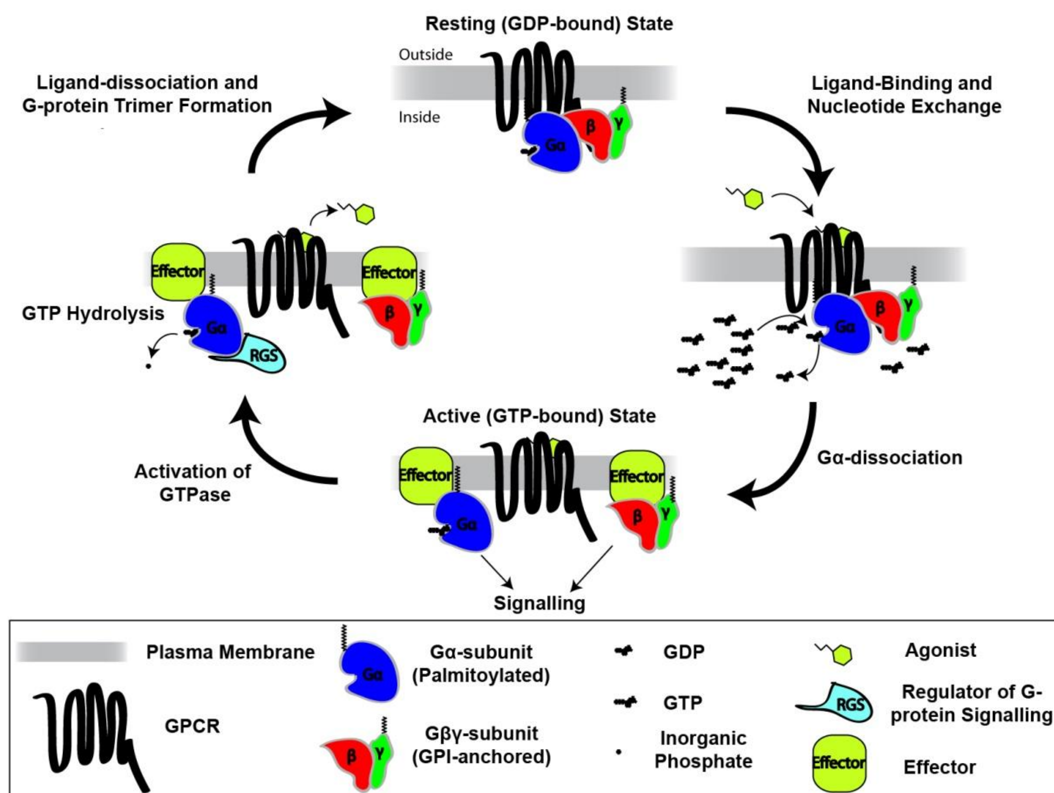


Figure 2. Schematic representation of GPCR activation and deactivation[266].

1.5.3 The high affinity binding state

Three decades ago, *in vitro* binding studies on tissue homogenate indicated that GPCRs exist in two affinity states for agonist binding. Saturation studies using an antagonist radioligand showed biphasic displacement after addition of increasing concentrations of an agonist. The high affinity state was thought of as coupled to the G-protein, since the addition of guanine nucleotide altered the biphasic displacement curve into a monophasic curve with similar affinity as the low affinity state. Conversely, antagonist binding was shown as being insensitive to GTP addition and as binding with only one affinity[52,300,342]. Moreover, Scatchard analyses demonstrated different receptor densities (B_{max}) when obtained with agonist and antagonist D_2/D_3 receptors radioligands[17]. The proportion of receptors in the respective states may have implications for function and disease. For instance, the high affinity state of D_2 receptors has been suggested to represent the functional state of the receptor[92], and it has been proposed that proportions may vary in such CNS-disorders as schizophrenia[294,296].

1.5.4 GPCR models

Several models have been used to describe the agonist activation of GPCRs (Figure 3). The ternary model was proposed in 1980 and described the interaction between agonist (A), receptor (R) and G-protein (G)[51]. In this model, agonists promote and stabilize the ternary complex (ARG). The model accounts for the heterogeneity of agonist binding and for the high affinity receptor state being sensitive to guanine nucleotides.

In 1993, an extension of the ternary model was proposed. The extension was based on observations that GPCRs have basal activity in the absence of an agonist, and that mutant GPCRs can enhance the agonistic-independent activity[34,181,281]. In the extended ternary complex, an equilibrium is described between the inactive receptor state (R) and the active receptor state (R*). Agonistic efficacy was thought to be reflected in the ability to modify the equilibrium between R and R*, with R* having potential to bind the G-protein. The extended ternary complex can account for different classes of drug activity, including full agonists, partial agonists, neutral antagonists and inverse agonists.

Over the last two decades it has become clear that not all GPCR properties can be explained by the extended ternary complex[155,156]. The existence of multiple conformational states has gained support and in this model the inactive receptor state R is, after agonist binding, proposed to gradually conform into the active R* state by intermediate states R' and R''[93]. Several functional and biophysical studies now support that most GPCRs sample multiple conformations[161]. The existence and functional relevance of multiple conformational states has been mainly demonstrated *in vitro* and awaits further *in vivo* support.

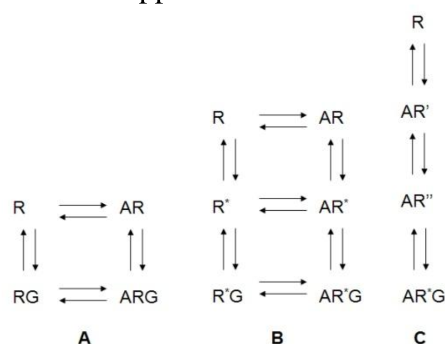


Figure 3. Three models describing GPCR activation by agonist (A), receptor (R) and G-protein (G). (A) Classical ternary complex. (B) Extended ternary complex. (C) Multistate model[51,93,181].

1.5.5 Receptor internalization and β-arrestin

After agonist-induced G-protein dissociation, the receptor can undergo desensitization by phosphorylation and β-arrestin binding to the receptor. This initial desensitization is followed by receptor internalization in which β-arrestin functions as a scaffolding protein. Receptor internalization is a process in which cells internalize cell surface located receptors into plasma membrane vesicles, a process considered to maintain cellular homeostasis. After internalization, the receptor may be recycled or degraded[78,79]. Recent work in cell lines suggests that β-arrestin not only regulates desensitization, but is a multifunctional adaptor protein also involved in the signalling cascade. Receptor ligands have been shown as unbalanced in stimulating the G-protein and β-arrestin signalling pathways, with some receptor systems activating only one pathway; this mechanism is referred to as "biased agonism"[192,261,338]. These new insights in GPCR signalling pathways should be taken into account when studying neurotransmitter release with PET.

1.6 THE DOPAMINE SYSTEM

Arvid Carlson discovered in 1958 that dopamine is of great importance for the functioning of the healthy brain[27]. Dopamine was characterized as a catecholamine with neurotransmitter function in the CNS. The dopamine system is one of the most widely studied neurotransmitter systems, and has been examined in an extensive number of studies with molecular imaging techniques[43]. Dopamine is thought to play an important role in physiological functions such as cognition, movement, reward, emotional expression, prolactin secretion and cardiovascular function. The dopamine system is degenerated in Parkinson's disease and Huntington's disease and dopamine dysfunction has been postulated to play a role in ADHD and schizophrenia.

1.6.1 Dopaminergic neurotransmission

Dopamine does not pass the BBB and is synthesised in brain from the essential amino acid tyrosine (TYR). TYR is converted by tyrosine hydroxylase (TH) into *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and subsequent decarboxylation by the *L*-aromatic acid decarboxylase-enzyme (AADC) results in dopamine. After synthesis dopamine is stored in terminal vesicles and upon neurological firing dopamine is released into the synaptic cleft. Activation by dopamine is primarily terminated by reuptake by the dopamine transporter (DAT). Degradation of dopamine occurs by three enzymes, monoamine oxidase (MAO), aldehydedehydrogenase (ALDH) and catechol-*O*-methyltransferase (COMT)[215]. MAO and ALDH are mainly membrane bound enzymes and are predominately located on the outer layer of mitochondria in neurons and glial cells[20]. COMT is present in periphery and CNS and located in the cytoplasm of neurons and glial cells in brain[196] (Figure 4).

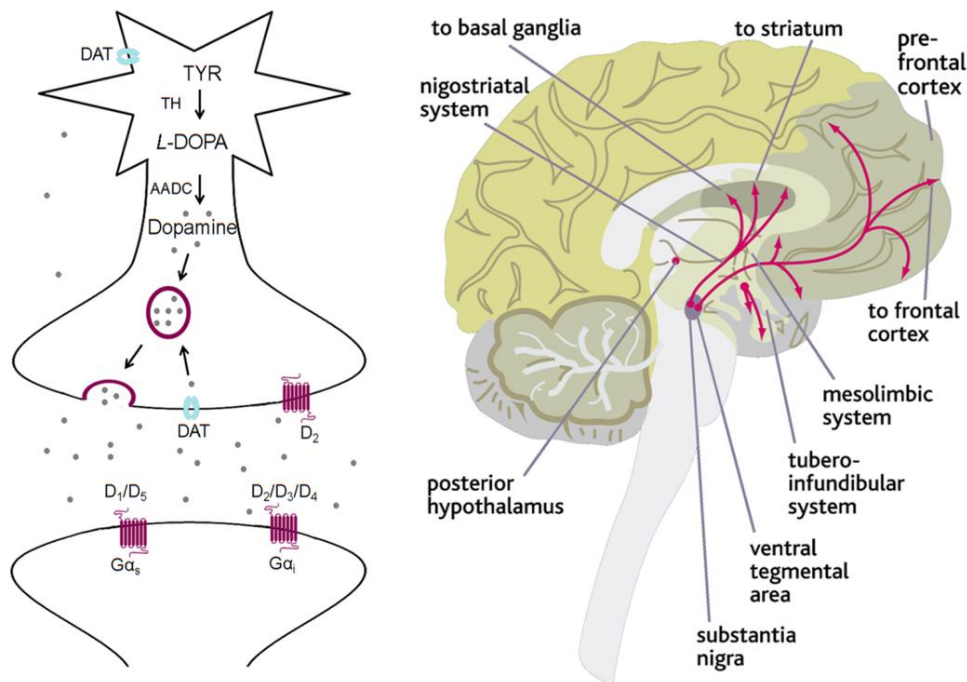


Figure 4. (Left) Schematic representation of a dopaminergic neuron with cell body (top) and the synaptic terminal region, including synaptically located dopamine receptors (bottom). (Right) Dopaminergic pathways in the human brain, modified from[133].

1.6.2 Dopaminergic pathways

The dopaminergic neurons in the midbrain (substantia nigra and ventral tegmental area) and the hypothalamus give origin to the four main dopaminergic pathways (Figure 4). The nigrostriatal pathway projects from the substantia nigra to the dorsal striatum, is important for the control of movement and is the system primarily involved in movement disorders such as Parkinson's disease. The mesocortical and mesolimbic pathways originate from the ventral tegmental area and project, respectively, to the neocortex and to limbic structures, such as the nucleus accumbens, amygdala and hippocampus. Both these pathways have been suggested to have a role in memory, reward and desire, as well as addiction and emotion, and have been proposed to be affected in the pathophysiology of schizophrenia. The fourth pathway is the tuberinfundibular pathway between the hypothalamus and the pituitary gland. The tuberinfundibular pathway plays a role in hormonal regulation, maternal behaviour and sensory processes[330]. In recent years, the existence of a novel dopaminergic system targeting the thalamus has been described for macaques and humans using immunolabelling techniques[90,91,282].

1.6.3 Dopamine receptor subtypes

The effects of dopamine are mediated through five receptor subtypes, divided into two families, the D_1-like receptors (D_1 and D_5) and the D_2-like receptors (D_2, D_3 and D_4), based on pharmacological and structural properties[152,309,330]. Two splice variants of the D_2 receptor exist, $D_{2\text{-short}}$ and $D_{2\text{-long}}$ which differ by an insertion of 29 amino acids[103]. The $D_{2\text{-short}}$ is mainly located presynaptically and proposed to function as autoreceptor, while the $D_{2\text{-long}}$ is mainly located postsynaptically[329].

Activation of the D_1-like receptor class results in the stimulation of $G\alpha_s$ or $G\alpha_{olf}$, which induces activation of AC. AC catalyzes the conversion of adenosine-5'-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and consequently activates the disinhibition of protein kinase A (PKA). PKA in turn causes phosphorylation of several downstream targets, including CREB and DARPP-32. *Post-mortem* autoradiography studies have shown that the D_1 receptor is the most abundant dopamine receptor in the human brain, with high densities in striatum and moderate density in substantia nigra and neocortex[113,114]. Currently, there are no pharmacological tools available that differentiate between the D_1 and D_5 receptor. Comparison of mRNA levels in monkey brain shows high levels for the D_1 receptor in striatum with lower levels in cortex and amygdala, whereas the D_5 receptor expression is high in cortex[35,184]. Studies using subtype-specific antibodies have mapped D_5 receptors in several brain regions, including striatum, hippocampus and substantia nigra in rat and human brain[159].

Activation of the D_2-like receptor class results in stimulation of $G\alpha_i$ or $G\alpha_o$, and inhibition of cAMP production[49]. Consequently, downstream effects of cAMP, such as PKA and phosphorylation of DARPP-32, are thereby inhibited. While D_1 and D_2 receptors have opposite effects at the molecular level, they often have a synergistic action when more complex outputs are considered. *Post-mortem* autoradiography studies have shown that the D_2 receptor density is high in striatum[113,114] with low levels in neocortex and thalamus[115,158]. The D_3 receptor has been shown mainly located in the ventral striatum, including the nucleus accumbens, although most studies have been performed with D_2/D_3-receptors unselective radioligands making selective study of the less abundant D_3 receptor cumbersome[116]. In rodents, the D_4 receptor has been shown to be located in limbic and motor areas using autoradiography[53]. A recent PET study indicates, however, high D_4 receptor binding in retina but low binding in the monkey brain[167].

1.6.4 PET studies on dopamine release

1.6.4.1 D_2/D_3 receptors antagonist radioligands

PET radioligands developed for imaging of the D_2/D_3 receptors in brain have primarily been antagonist radioligands, such as [^{11}C]raclopride[61,68] and [^{11}C]FLB 457[76,118]. A large number of studies have shown that dopamine release induced by a pharmacological or cognitive/behavioural intervention can be studied using D_2/D_3 receptors radioligands and PET or single photon emission computerized tomography (SPECT)[59,175]. Typically, amphetamine has been used to enhance and reserpine or alpha-methyl-para-tyrosine (AMPT) to decrease dopamine levels, thereby respectively decreasing or increasing D_2/D_3 receptors binding of radioligand in primates[175]. Two early observations in human subjects greatly stimulated the use of this methodology for further applications. First an enhanced amphetamine effect on [^{123}I]IBZM and [^{11}C]raclopride binding was observed in patients with schizophrenia[22,172]. Secondly, videogame playing was shown to cause a significant decrease in [^{11}C]raclopride binding[59,163]. It has more recently been shown that dopamine release can be detected in extra-striatal regions in some studies using [^{18}F]fallypride[40,218,219,269] or [^{11}C]FLB 457[2,3,31,83,228], but not when using [^{11}C]fallypride[228].

1.6.4.2 D_1/D_5 receptors antagonist radioligands

Previous studies using D_1/D_5 receptors antagonist radioligands, such as (*R*)-[^{11}C]SCH 23390 and (+)-[^{11}C]NNC 112 did not show changes in *BP* after modification of dopamine levels[4,36]. These observation were not anticipated as electrical induced dopamine release did decrease [^{3}H]SCH 23390 binding *in vitro*[95]. Several reasons have been proposed to explain this discrepancy.

Firstly, dopamine affinity to the D_1 receptor has been reported to be of the same order as for the D_2 receptor (30 vs. 6 nM, $K_{i\ high}$)[295], but more recent studies have shown lower D_1 receptor affinity (897 vs. 64 nM, $K_{i\ high}$)[113]. The large variation between results obtained from different assays indicates the dependence to assay conditions. Secondly, the D_1/D_5 receptors have been found to be located predominantly extra-synaptically[128,183], which is possibly a mismatch when amphetamine increases dopamine mainly in the synapse. This possible mismatch does not, however, explain the lack of effect on radioligand binding after the long-lasting effect of reserpine. Thirdly, a rather small percentage of D_1 receptors are in the high affinity state, *in vitro* measured as 20-40%[195,201,271]. An agonist radioligand selectively binding the high affinity state may therefore provide a more sensitive approach to further understand the reported lack of dopamine sensitivity of D_1/D_5 receptors antagonist radioligands.

1.6.4.3 Ceiling effect

The interpretation of the neurotransmitter release studies along the competition model was supported by the relationship between changes in dopamine release measured with microdialysis and observed changes in *BP* with PET[22,62,173,319,320]. Enhanced dopamine level, however, only modified *BP* values to a small extent, as well as a 44% increase in dopamine level, causing 1% change in *BP*. This modest displacement suggested a maximum effect smaller than that observed for antipsychotics, which could completely inhibit radioligand binding. This effect became referred to as "ceiling effect". The ceiling effect was proposed to be related to the two affinity states of the D_2/D_3 receptors and the displaceable part of the antagonist radioligand binding was described as binding to the high affinity state. Agonist radioligands, specifically targeting the high affinity state were therefore expected to be more effectively competing with endogenous dopamine than antagonist radioligands,

thereby providing more sensitive tools for measurement of dopamine release (Figure 5)[175].

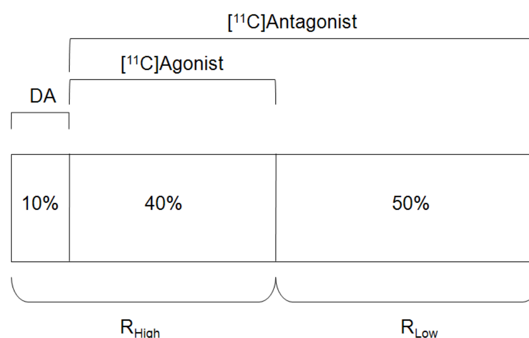


Figure 5. The proposed pharmacological model for agonist and antagonist radioligand binding to D_2/D_3 receptors, modified from[175].

1.6.5 PET imaging with dopamine agonist radioligands

PET imaging with agonist radioligands was expected to confirm the existence of the high affinity state *in vivo*. In addition, the agonist radioligands were suggested as superior tools for measurement of changes in endogenous dopamine level.

1.6.5.1 Agonist radioligands for the D_1/D_5 receptors

The development of PET radioligands targeting the D_1/D_5 receptors has thus far focused on the 1-phenyl-3-benzazepines scaffold[8,231], as other potent D_1/D_5 receptors agonists, such as dihydrexidine[188] and dinapsoline[94,302], cannot be easily radiolabeled. DaSilva and co-workers compared (+)-[^{11}C]SKF 75670 and (±)-[^{11}C]SKF 82957[44] and found (±)-[^{11}C]SKF 82957 to be more suitable[45]. Several studies using [^{11}C]SKF 82957 have been reported, including dopamine challenge studies in rat and baboon[46,109,174,283,314] as well as pioneering PET studies in humans[47]. However, in 2003, it was shown that a lipophilic radiometabolite accumulated in rat brain after i.v. injection of (+)-[^{11}C]SKF 82957[48]. The formation of this lipophilic radiometabolite most likely confound the quantification of D_1/D_5 receptors binding in brain, and therefore drastically limited the further application of (+)-[^{11}C]SKF 82957 in PET studies[48].

1.6.5.2 Agonist radioligands for the D_2/D_3 receptors

The development of an agonist radioligand for D_2/D_3 receptors has been pursued for about two decades and includes the radiolabeling of compounds originating from numerous scaffolds, such as the ergolines, aporphines, 2-aminotetralins, benzoquinolines and naphthoxazines. In Appendix I a detailed review is provided on all D_2/D_3 receptors agonist PET radioligands reported in the literature. This section only shortly discusses the three D_2/D_3 receptors agonist radioligands that have been evaluated in human subjects, [^{11}C]PHNO, [^{11}C]NPA and [^{11}C]MNPA (Figure 6).

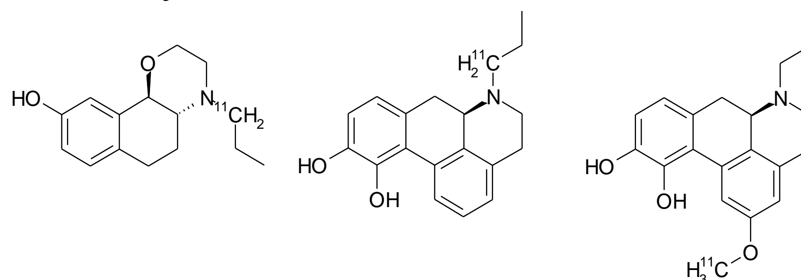


Figure 6. The three most widely used D_2/D_3 receptors agonist PET radioligands. From left to right: [^{11}C]PHNO, [^{11}C]NPA and [^{11}C]MNPA.

[^{11}C]PHNO binding has been studied in rat[85], cat[100], non-human primate,[225] and human subjects[101,105,344]. [^{11}C]PHNO has high D_3 receptor affinity and several studies have confirmed that the [^{11}C]PHNO signal in brain mainly represents D_3 binding[107,260,289,325]. [^{11}C]PHNO provides a very good binding signal in striatum (BP_{ND} ~3). One disadvantage of [^{11}C]PHNO, however, is the radiolabeling by ^{11}C-propyionylation,[346] which typically results in a mass administrated of >1 µg. This mass can be considered as a non-tracer dose and may sometimes induce adverse events, such as transient nausea (mass dose of >0.03 µg/kg)[213]. [^{11}C]PHNO has already been used in clinical studies evaluating D_3 receptor function in neurologic and psychiatric disorders[19,106]. An optimization of labelling strategy is ongoing and may further advance the use of [^{11}C]PHNO[60,89].

Another potent class of D_2/D_3 receptors agonists is the aporphines scaffold. [^{11}C]NPA[136] was the first aporphine extensively studied in non-human primates[137,223,224] and has now been evaluated in humans[177,227,229]. [^{11}C]MNPA is the 2-methoxy derivative of NPA and has been reported to have higher D_2 receptor binding affinity (K_i, 0.17 vs. 0.80 nM)[87]. PET studies have, however, reported comparable striatal BP_{ND} values (0.8-1.0) for [^{11}C]MNPA and [^{11}C]NPA in monkey[297,315] and human subjects[162,246], as possibly partly explained by higher non-displaceable binding due to the higher lipophilicity of [^{11}C]MNPA (3.69 vs. 3.37 cLogD). A clear advantage of [^{11}C]MNPA is the radiolabeling with ^{11}C-methylation, when compared to ^{11}C-propionylation. ^{11}C-Methylation is more routinely used, more automated, more GMP-compliant and achieved with higher specific radioactivity. The agonist radioligand of choice in this thesis has therefore been [^{11}C]MNPA.

1.6.5.3 PET imaging of the high affinity agonist binding site

During the time period of this thesis work several approaches have been undertaken to demonstrate that the D_2/D_3 receptors exist *in vivo* in two affinity states. Scatchard analyses have been performed directly comparing the B_{max} for agonist and antagonist radioligands. Two studies reported no major differences in B_{max} using two-point Scatchard plots in cat and baboon[100,224]. A different approach in rodents made use of dopaminergic supersensitivity models, in which altered fractions of receptors in the high affinity state were reported using *in vitro* binding techniques. Among four different models, no differences were observed between agonist and antagonist radioligand binding measured either *ex vivo*[203] or *in vivo*[304]. A third approach taken was to use saturation studies to examine different doses of exogenous dopamine receptor agonists that displace antagonist and agonist radioligand binding. Receptor occupancy of exogenous agonists was found monophasic when measured with [^{11}C]/[^{3}H]raclopride with indistinguishable K_i values as obtained with agonist radioligands[164,202,250]. Despite this variety of studies attempting different approaches, the existence of the high and low affinity state *in vivo* has not been conclusively disputed or confirmed.

1.6.5.4 Agonist radioligands as improved tools for study of dopamine release

A second rationale for the development of agonist radioligands is to obtain tools that are more sensitive to changes in endogenous dopamine level than the already available D_2/D_3 receptors antagonist radioligands. PET studies using dopamine level altering drugs, such as amphetamine, have consistently reported a more pronounced effect on agonist radioligand binding than on antagonist radioligand binding[41,42,100,223]. Interestingly, the amphetamine studies showed a similar, amphetamine-dose-consistent ratio of improved sensitivity for agonist radioligands, when compared to antagonist radioligands. Based on the consistent ratio observed, it was suggested that a constant

fraction of receptors was in the high affinity state (60-80%)[223]. The findings stand in contrast to earlier mentioned studies directly investigating the existence of two affinity state *in vivo*.

Importantly, a small number of studies have now shown that anaesthesia and stress modify agonist radioligand binding to D_2/D_3 receptors[204,240,322]. Direct comparison in humans is required to fully understand the confounding effects of anaesthesia. The first comparative study in man used [^{11}C]NPA and [^{11}C]raclopride, and indicates that the agonist radioligand [^{11}C]NPA was more sensitive to amphetamine-induced changes in dopamine concentration[229]. Amphetamine has already been shown to displace [^{11}C]PHNO binding in man[345]. Although a full comparative study with [^{11}C]PHNO and [^{11}C]raclopride in human subjects has thus far only been reported in abstract form, the preliminary results confirm improved sensitivity to dopamine concentration for the agonist [^{11}C]PHNO[299]. Confirmation of the enhanced sensitivity of agonist radioligands to endogenous dopamine level in man will encourage the development and future application of agonist radioligands.

1.6.5.5 PET studies supporting the internalization model

Agonist induced internalization of the D_1 and D_2 receptor has been consistently demonstrated *in vitro*[16,58,318]. Three *in vivo* observations suggest a contribution of internalization and raise concern about the validity of the competition model for interpretation of dopamine challenges studies[99,175]. First, a temporal discrepancy has been observed between the amphetamine-induced dopamine pulse and the prolonged effect on agonist and antagonist radioligand binding[26,100,131,173,226]. Second, amphetamine does not decrease receptor binding of all D_2/D_3 receptors radioligands (e.g., butyrophenones)[125,244]. Third, amphetamine has been shown to decrease [^{11}C]raclopride B_{max} values *in vivo* in cat[98] and *ex vivo* in rat[311].

To account for these observations, part of the change in radioligand binding may be related to receptor internalization[175]. The internalization model was initially proposed by Chugani and co-workers for paradoxical observations using [^{3}H]spiperone[37]. The model proposes that internalization of receptors includes a relocalisation of receptors into an intracellular compartment resulting in reduced accessibility of radioligands which is reflected in a decrease in B_{max}. Conversely, an elegant *in vitro* study recently conducted observed a decrease in receptor affinity for radioligands after receptor internalization[111]. Whereas the exact contributing factors remain unclear, the internalization model can help to explain the three previously mentioned inconsistencies of the competition model observed in PET studies. Recently, the contribution of internalization in dopamine challenge studies was confirmed using PET. Four hours after amphetamine administration, a decrease in D_2/D_3 receptors radioligand binding was still observed in wild-type mice, but not in arrestin-3 knockout mice, which are animals that lack the capacity to internalize D_2/D_3 receptors[303].

1.7 THE SEROTONIN SYSTEM

Serotonin (5-hydroxytryptamine, 5-HT) was first identified in serum as a vasoconstrictive substance. In 1953, serotonin was found to be present in the mammalian brain[324], and its function in brain has since then been widely studied and an extensive number of studies with molecular imaging techniques have been performed[335]. Serotonin is thought to play an important role in normal physiological processes such as appetite, pain perception, sleep and thermoregulation, and serotonin dysfunction has been implicated in addiction, anxiety, depression, migraine, obsessive compulsive disorders and schizophrenia.

1.7.1 Serotonergic neurotransmission

Serotonin does not pass the BBB and is therefore synthesised in serotonergic neurons in brain. The biosynthesis starts from *L*-tryptophan (TP): the initial step is conversion to 5-hydroxy-*L*-tryptophan (5-HTP) by tryptophan hydroxylase (TPH). 5-HTP is then consecutively converted by the enzyme AADC to 5-HT. Both *L*-tryptophan and 5-HTP can pass the BBB, and are integral to our dietary intake. After synthesis, serotonin is stored in terminal vesicles, and upon neurological firing released into the synaptic cleft. Activation by serotonin is terminated by reuptake by the serotonin transporter (SERT), and degradation occurs by MAO and ALDH (Figure 7).

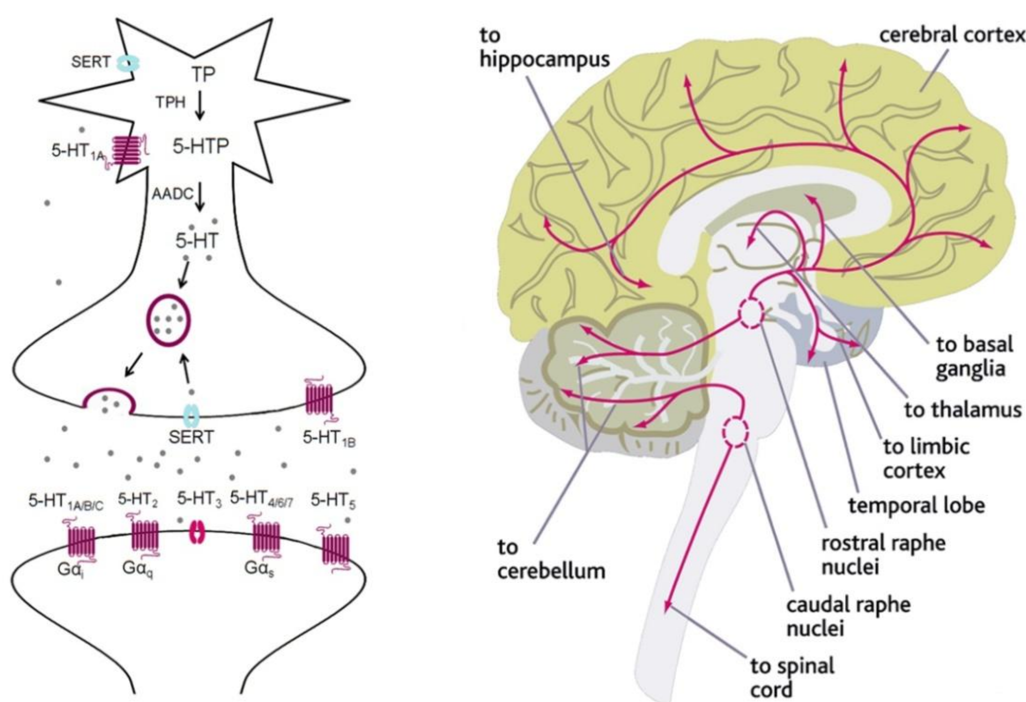


Figure 7. (Left) Schematic representation of a serotonergic neuron with cell body in the raphe nuclei (top) and the synaptic terminal region, including synaptically located 5-HT receptors (bottom). (Right) Serotonergic pathways in the human brain, modified from[133].

1.7.2 Serotonergic pathways

Serotonergic innervations in brain arise from cell bodies concentrated in the raphe nuclei. Two distinct subdivisions of raphe nuclei are recognized: the rostral nuclei (located in midbrain and rostral pons) and the caudal nuclei (located primarily in the medulla oblongata). Most brain serotonergic innervations originate from the rostral raphe nuclei, which includes the dorsal raphe nucleus and the median raphe nucleus. From the rostral raphe nuclei, axons ascend to the cerebral cortex, the limbic regions and to the basal ganglia. Serotonergic nuclei in the caudal raphe nuclei give rise to descending axons, some of which terminate in the medulla, while others descend to the spinal cord (Figure 7)[11,317].

1.7.3 Serotonin receptor subtypes

The serotonin system is one of the oldest phyologenetic neurotransmitter/ hormone systems, and includes a diverse group of receptors. Thus far, fourteen mammalian receptor subtypes have been characterized based on distinct structural and pharmacological properties. The receptor subtypes are assigned to seven families, 5-$HT_{1\text{-}7}$ and all serotonin receptors are G-protein coupled, except for the 5-HT_3

receptor[15,132]. The receptors can be further categorized into four groups according to their main second messenger system: the 5-HT_1 receptors coupled to $G\alpha_i/G\alpha_o$ proteins (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}), the 5-HT_2 receptors coupled to $G\alpha_q$ proteins (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}), the 5-HT_4, 5-HT_6, and 5-HT_7 receptors coupled to $G\alpha_s$ proteins, and the 5-HT_5 receptors (5-HT_{5A} and 5-HT_{5B}) for which the coupling is still uncertain[264].

1.7.4 PET studies on serotonin release

Whereas PET imaging of dopamine release has been successful, this approach has not been successfully extended to the serotonin system[248]. PET has long been recognized as a promising methodology for measuring serotonin release, thereby providing further understanding of the pathophysiology and treatment of such common CNS-diseases as mood, anxiety, sleep and food disorders. Several suitable radioligands have been developed for PET imaging of the serotonin system, specifically for the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_4 receptor and SERT. To date, most of these radioligands have been tested for sensitivity to serotonin level, but with limited success.

1.7.4.1 5-HT$_{1A}$ receptor radioligands

An extensive number of radioligands have been developed for the 5-HT_{1A} receptor, with the antagonist [*carbonyl*-^{11}C]WAY-100635 being the most commonly used[165,245,252]. Several studies have attempted to evaluate the effect of pharmacological-induced changes in serotonin release on [^{11}C]WAY-100635 binding, but have shown inconsistent effects in rodents[129,135,193,270] and no effect in man[259]. The development of new radioligands having potential for enhanced sensitivity to serotonin has resulted in [^{18}F]FPWAY, [^{18}F]FCWAY and [^{18}F]MPPF, of which [^{18}F]MPPF is perhaps the most promising with regards to serotonin sensitivity[102,142,298].

Although initial studies with [^{18}F]MPPF indicated serotonin susceptibility in rat and cat[12,13,265,268,327,351-353], the effect has not been consistently confirmed in non-human primates[328] or humans[56,256,301,326]. The contradictory findings are suggested to partly originate from differences in regional response to serotonin release. 5-HT_{1A} autoreceptors in the raphe nuclei were shown to undergo internalization, while postsynaptic receptors in the hippocampus and cortex do not internalize[12,268]. A report on fluoxetine effect on serotonin level being restricted to raphe nuclei in man is in line with these regional differences in the serotonin response[301]. The raphe nuclei is, however, a small region, and determination of *BP* has proven challenging and has thus far not resulted in optimal reproducibility[147,189].

1.7.4.2 5-HT$_{2A}$ and 5-HT$_4$ receptor radioligands

Radioligands selectively targeting 5-HT_{2A} or 5-HT_4 receptors have also been tested for sensitivity to modified serotonin release: radioligands targeting 5-HT_{2A} receptors, for example, include the non-selective radioligands [^{18}F]setoperone, [^{3}H]NMSP and the selective radioligands [^{18}F]altanserin and [^{11}C]MDL-100907. However, PET studies have shown no evident susceptibility of these radioligands to modified serotonin level in rat[130,270] and human[169,199,207,254,306,349]. Currently, only [^{11}C]SB207145 is available for imaging of 5-HT_4 receptors using PET[197], but citalopram did not modify [^{11}C]SB207145 binding in control subjects[198]. It can therefore be concluded that none of the serotonin receptor targeting radioligands has unambiguously been shown to be sensitive to serotonin level.

1.7.4.3 SERT radioligands

Another approach for measurement of serotonin release is the use of radioligands targeting SERT. This approach is more challenging as most serotonin challenge studies utilize drugs acting on SERT. However, several attempts have been reported using a non-selective MAO inhibitor, 5-HTP, or TP depletion. After elevation of serotonin level, consistent decreases in [^{11}C]DASB binding has been shown in rat[190,191], cat[97] and non-human primate[191,348]. Milak and colleagues, however, reported that tryptophan depletion decreased *BP* values, possibly related to serotonin induced SERT internalization[208]. In two studies in control subjects no effect of TP depletion on [^{11}C]DASB binding has been shown[257,313]. In summary, although elevation of serotonin levels did effect [^{11}C]DASB binding in animals, this approach has not been found suitable for humans.

1.7.4.4 State of the art for PET imaging of serotonin release

It can be concluded that after two decades of serotonergic radioligand development, no radioligand has been found suitable for study of modified serotonin release in clinical studies in man. Potential reasons for this lack of success were recently reviewed[248]. Considering the basal serotonin concentration and receptor affinity, imaging of serotonin release may be feasible, but more cumbersome than for dopamine. Comparison of the fraction of receptors in high affinity state has indicted lower fractions for the 5-HT_{1A} (20-40%) and 5-HT_{2A} receptor (40-60%) when compared to D_2 dopamine receptors (70%), possibly explaining the observed low sensitivity to serotonin for radioligands targeting these receptors. Moreover, the internalized receptor pool may vary between receptors, thereby limiting endogenous serotonin competition with the radioligand binding. This may be a particular problem for the 5-HT_{2A} receptor (80-90% internalized).

1.7.4.5 PET imaging of 5-HT_{1B} receptors

Radioligands targeting the 5-HT_7 or 5-HT_{1B} receptor have been suggested as more promising because endogenous serotonin has relative high affinity to these receptor subtypes. Initially, it was claimed that the 5-HT_{1B} receptor only existed in rodents[249] but was later demonstrated to be the homologous species of the human 5-$HT_{1D\beta}$ receptor[6,123,132]. Several physiological functions have been shown to be mediated through 5-HT_{1B} receptors, and animal studies have shown a role in aggression[243,262,287], feeding[157,179], learning[7] and locomotion[262]. The 5-HT_{1B} receptors are also implicated in the pathophysiology and potential treatment of several neuropsychiatric disorders, including anxiety disorders[185], depression[279], migraine[337], anorexia[157] and substance abuse[39,273,274].

The 5-HT_{1B} receptor acts as autoreceptor[65,104,200,307] and heteroreceptor[80] when located at serotonergic or non-serotonergic neurons, respectively. On serotonergic neurons the 5-HT_{1B} receptor is not located on cell bodies, like the 5-HT_{1A} autoreceptors, but on terminals[21,267,285,286]. Endogenous serotonin has relative high 5-HT_{1B} receptor affinity (~1 nM)[211] and a large proportion of receptors has been shown to be G-protein coupled and thus in the high affinity state[10,25,108,211,239]. Radioligands targeting the 5-HT_{1B} receptor may thus have potential for measurement of serotonin release. However, until recently, the role of 5-HT_{1B} receptors could not be studied in the living human brain because no suitable PET radioligand was available.

During the time of this thesis work, two novel radioligands suitable for study of the 5-HT_{1B} receptor were reported, [^{11}C]AZ10419369[251] and [^{11}C]P943[86]. [^{11}C]AZ10419369 is a selective antagonist radioligand for the 5-HT_{1B} receptor subtype

(K_D is 0.8 nM) as confirmed by autoradiography and PET studies in monkey and human[194,251,333]. [^{11}C]AZ10419369 binding is high in 5-HT$_{1B}$ receptor rich regions, including the globus pallidus, the substantia nigra and occipital cortex. The receptor binding of [^{11}C]AZ10419369 has been shown to be reversible, making this radioligand suitable for quantitative PET measurements of 5-HT$_{1B}$ receptors and drug occupancy *in vivo*[251,333].

[^{11}C]P943 is a selective antagonist radioligand for the 5-HT$_{1B}$ receptor and the brain distribution of [^{11}C]P943 has been shown to be very similar to [^{11}C]AZ10419369 in primates[86,221]. Though, no direct comparison of [^{11}C]P943 and [^{11}C]AZ10419369 has thus far been reported, the reported BP_{ND} values of [^{11}C]AZ10419369 are approximately 30-40% higher[333]. Initial clinical studies using [^{11}C]P943 indicate the clinical relevance of 5-HT$_{1B}$ receptor imaging in alcohol-dependence[134] and depression[220]. The 5-HT$_{1B}$ receptor radioligand of choice in this thesis is [^{11}C]AZ10419369.

2 AIMS

The overall aim of the present thesis was to evaluate drug-induced changes in dopamine and serotonin release in the non-human primate brain using PET.

The specific aims of the program were as follows:

1. To develop improved methods for measurement of endogenous dopamine level, with the following sub-aims:
    a. To radiolabel and to perform an *in vivo* PET evaluation of the new D_2/D_3 receptors agonist radioligand $[^{11}C]$MNPA.
    b. To compare the sensitivity of $[^{11}C]$MNPA and $[^{11}C]$raclopride to stimulant-induced dopamine release.
    c. To apply $[^{11}C]$MNPA to further obtain *in vivo* support for the existence of two affinity states for the D_2/D_3 receptors.
    d. To evaluate the sensitivity of the newly developed D_1/D_5 receptors partial agonist (*S*)-$[^{11}C]$*N*-methyl-NNC 01-0259 to alterations in endogenous dopamine concentration.

2. To evaluate the sensitivity of the new 5-HT_{1B}-receptor radioligand $[^{11}C]$AZ10419369 to alterations in endogenous serotonin concentration.

3 MATERIALS AND METHODS

The present chapter provides a description of the general methods used during this thesis work. For complete details of the experimental work, the reader is referred to the full papers and manuscripts as indicated elsewhere in this book.

3.1 RADIOCHEMISTRY

All irradiations were performed on a GEMS PETtrace cyclotron (GE, Uppsala, Sweden) equipped with a [^{11}C]methane ([^{11}C]CH_4) target filled with nitrogen gas containing 10% hydrogen. [^{11}C]Methyl iodide ([^{11}C]CH_3I) was prepared according to previously reported methods[9,170,284]. In short, [^{11}C]CH_4 was collected in a Porapak Q trap cooled with liquid nitrogen and subsequently released into a recirculation system. The [^{11}C]CH_4 was mixed with vapours from iodine crystals at 60˚C and then reacted at 720˚. After the reaction, [^{11}C]CH_3I was collected in a Porapak Q trap at room temperature and the unreacted [^{11}C]CH_4 was recirculated for three minutes. [^{11}C]Methyl triflate ([^{11}C]CH_3OTf) was prepared by sweeping [^{11}C]CH_3I vapour through a heated glass column containing silver-triflate-impregnated graphitized carbon, as previously described[222].

The produced [^{11}C]CH_3I ([^{11}C]MNPA and (*S*)-[^{11}C]*N*-methyl-NNC 01-0259) or [^{11}C]CH_3OTf ([^{11}C]raclopride and [^{11}C]AZ10419369) was trapped into a reaction vessel containing the corresponding mixture of precursor, solvent and base. After possible heating, the reaction mixture was diluted with mobile phase and injected into the semi-preparative HPLC system for purification. The fraction from the semi-preparative HPLC that contained the product was evaporated to dryness under reduced pressure and the residue dissolved into 8 mL sterile physiological phosphate buffer solution (pH = 7.4). The solution was finally filtered through a Millipore Millex®GV filter unit (0.22 μm).

3.2 IN VITRO RECEPTOR ASSAYS

All *in vitro* receptor affinity and efficacy assays were performed at H. Lundbeck A/S, Valby, Denmark, except for the 5-HT_{2A} receptor assay (Cerep, Paris, France). Data obtained in the assays at H. Lundbeck A/S were measured in a minimum of two full concentration-response curves using 10 concentrations of drugs (covering 4 decades). The results are provided as K_i values (nM) derived from computer fitted IC_{50} values converted to K_i values using the Cheng-Prusoff equation ($K_i = IC_{50}/(1+(L/K_D))$).

3.3 PET MEASUREMENTS IN NON-HUMAN PRIMATES

3.3.1 PET experimental procedures

Cynomolgus monkeys (*Macaca fascicularis*) are housed in the Astrid Fagraeus Laboratory of the Swedish Institute for Infectious Disease Control (SMI), Solna, Sweden. All studies were approved by the Animal Ethics Committee of the Swedish Animal Welfare Agency (Dnrs: 245/04, 147/05, 16/06, 260/07, 145/08 and 399/08) and were performed according to "Guidelines for planning, conducting and documenting experimental research" (Dnr 4820/06-600) of the Karolinska Institutet as well as the "Guide for the Care and Use of Laboratory Animals"[38].

On all experimental days, anaesthesia was initiated by intramuscular (i.m.) injection of ketamine hydrochloride (~10 mg/kg, Ketaminol®, Intervet AB). Following transportation to the PET facility, the anaesthesia was maintained in the research setting by either a mixture of ketamine and xylazine or by sevoflurane. For study I-IV, anaesthesia was maintained for the duration of the experiment with i.m. injections of a

mixture of ketamine hydrochloride (3.75 mg/kg/h, Ketalar®, Pfizer) and xylazine hydrochloride (1.5 mg/kg/h, Rompun® Vet., Bayer). In study V and VI, the anaesthesia was maintained for the duration of the experiment by a mixture of sevoflurane (1.5-8%, Abbott Scandinavia AB), oxygen (~30%) and medical air after endotracheal intubation.

During all experimental days, the head was immobilized with a fixation device[151]. Body temperature was maintained by Bair Hugger Model 505 (Arizant Healthcare Inc., Eden Prairie, MN, USA) and continuously monitored by an oral or rectal thermometer. Cardiac and respiratory rates were initially monitored manually at least every 20 minutes during the duration of the experiment (study I-IV). For study V and VI, the ECG, heart rate, respiratory rate and blood gasses were continuously monitored throughout the experiment and blood pressure was monitored at least every 15 minutes.

3.3.2 PET acquisition in HR and HRRT

In study I-V, each PET measurement used a sterile physiological phosphate buffer solution (pH = 7.4) containing radioligand that was injected as a bolus (4 mL) into a surreal vein during 5 seconds with simultaneous start of the PET acquisition. As described in study VI, a bolus plus constant infusion approach was applied and included in addition to the bolus injection an infusion of radioligand with a speed of ~10 mL/hour at different K_{bol} values.

In study I-IV, radioactivity in brain was measured continuously with the Siemens ECAT EXACT HR system (Siemens, Knoxville, TN, USA). All acquisitions were acquired in 3D-mode[343]. A three-ring detector block architecture gives a 15-cm wide field of view. The transversal resolution in the reconstructed image is about 3.8 mm FWHM and an axial resolution of 3.125 mm. The attenuation correction of the data was obtained with three rotating ^{68}Ge rod sources. Raw PET data were then reconstructed using standard filtered back projection consisting of the following reconstruction parameters: 2-mm Hanning filter, scatter correction, a zoom factor of 2.17, and a 128 3 128 matrix size[343]. Emission data were collected continuously for 93 min, according to a pre-programmed series of 20 frames starting immediately after i.v. injection of radioligand.

In study V and VI, PET measurements were conducted using the High Resolution Research Tomograph (HRRT) (Siemens Molecular Imaging, Knoxville, TN, USA). List-mode data were reconstructed using the ordinary Poisson-3D-ordered subset expectation maximization (OP-3D-OSEM) algorithm, with 10 iterations and 16 subsets including modelling of the point spread function (PSF). The corresponding in-plane resolution with OP-3D-OSEM PSF was 1.5 mm FWHM in the centre of the field of view (FOV) and 2.4 mm at 10-cm off-centre directions[334]. Attenuation correction data was acquired before every PET measurement, with a six minutes transmission measurement using a single ^{137}Cs source. List mode data were acquired continuously for 125 or 155 minutes starting at the injection of [^{11}C]AZ10419369 and PET images were then reconstructed with a series of frames.

3.3.3 Determination of radiometabolites in plasma

The evaluation of metabolism of radioligands measured in plasma was performed with slight modification of HPLC methods which were previously described[119]. In short, venous blood samples (1-2 mL) were obtained from the monkey at several time points after injection of radioligand. After centrifugation at 2000 *g* for 2 min, plasma was obtained (0.5 mL) and mixed with acetonitrile (0.7 mL). The mixture was centrifuged at 2000 *g* for 2 minutes and the supernatant (1 mL) was injected to a HPLC system. The radioactivity in blood and plasma were measured in a sodium iodide (NaI)

well counter. The unchanged radioligand fraction was calculated by the integration of the corresponding radioactivity peak and its area was expressed as a percentage of the sum of the areas of all radioactive peaks.

3.3.4 Regions of interest

In study I-III, a preliminary set of anatomical regions of interest (ROIs) for striatum and cerebellum was manually defined on summation images, representing mean radioactivity measured between 9 and 93 min during baseline conditions. Preliminary parametric images of BP_{ND}[140] and relative blood flow (R_1) were generated from the original reconstructed PET data by use of the two-parameter multilinear reference tissue model (MRTM2)[138]. The final set of ROIs was manually delineated on the fused preliminary R_1 and BP_{ND} images, according to an atlas of a cryosected cynomolgus monkey head in situ[151]. The final set of ROIs was verified by visual inspection of the delineation on the summation images and applied to all PET studies performed on the same day. ROI volumes of all regions were kept similar in size between experimental days.

In study IV-VI, magnetic resonance images were available of the individual monkeys. Brain magnetic resonance imaging was performed in a 1.5-T GE Signa system (General Electric, Milwaukee, WI, USA). A T1 weighted image was obtained for co-registration with PET and delineation of anatomic brain regions. The T1 sequence was a 3D spoiled gradient recalled (SPGR) protocol with the following settings: repetition time (TR) 21 ms, flip angle 35˚; FOV 12.8; matrix 256x256x128; 128x1.0 mm slices; 2 NEX. The sequence was optimized for trade-off between a minimum of scanning time and a maximum of spatial resolution and contrast between gray and white matter.

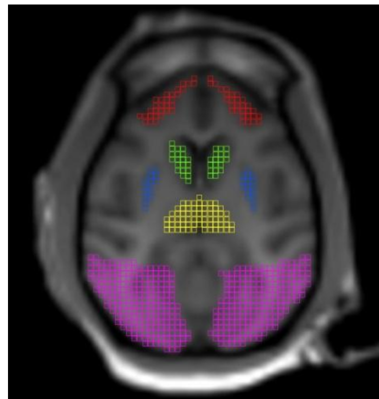


Figure 8. ROIs delineated on an individual monkey MRI orientated in the horizontal projection. Presented ROIs: dorsal lateral prefrontal cortex (red), caudate nucleus (green), putamen (blue), thalamus (yellow) and occipital cortex (pink).

Before delineation of ROIs, the orientation of the brain was spatially normalized by having the high-resolution T1-weighted magnetic resonance images reoriented according to the line defined by the anterior and posterior commissures being parallel to the horizontal plane and the interhemispheric plane being parallel to the sagittal plane. The standardized T1-weighted MR images were then resliced and used as an individual anatomical template for each monkey. ROIs were defined manually on the reoriented MR images (Figure 8). Mean PET images representing mean radioactivity between 0-57 minutes of the baseline PET measurement were co-registered to the magnetic resonance images using PMOD (PMOD technologies Ltd, Zurich, Switzerland). The generated transformation matrices were then applied to the dynamic emission data sets of all PET measurements obtained on the same day.

3.3.5 Quantitative PET data analysis

All quantitative analyses were based on the assumption that all radioactivity in brain represents unchanged radioligand[212]. Time activity curves were obtained by calculation of regional radioactivity for each frame, corrected for decay and plotted versus time. For quantification of receptor binding, a mathematical model is often required. Typically, this model describes the relationship between input to brain and the brain response to the input. The input can be directly measured using arterial blood sampling or indirectly estimated using a reference region approach. A well accepted mathematical model is the compartmental model (Figure 9). The compartmental model assumes homogenous pools of radioligand concentration, which are assumed to exchange radioligand according to a prescribed set of equations. The values of the parameters of the model can then be estimated by fitting of the data. Several approaches can be used for fitting of the data, including kinetic, equilibrium and graphical analysis. In this thesis work, no arterial blood data was obtained from the monkeys. The kinetic models used for quantification of PET data are therefore reference tissue models.

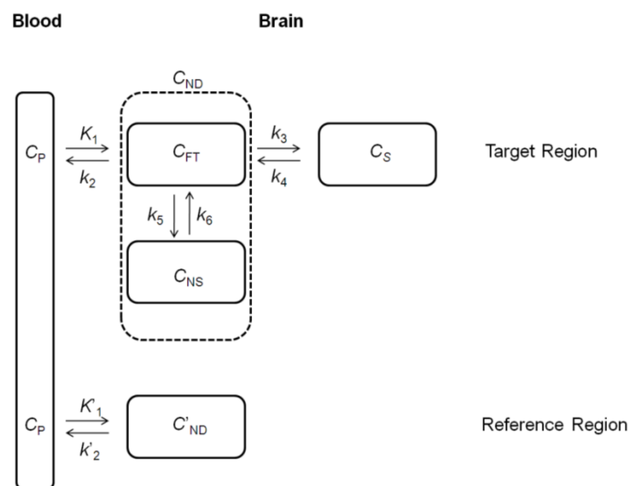


Figure 9. Multicompartment model. C refers to concentration of radioligand in the defined compartment. C_P = plasma, C_{FT} = free in tissue, C_{NS} = non-specifically bound, C_S = specifically bound, C_{ND} = non-displaceable. K_1-k_6 refer to kinetic rate constants.

3.3.5.1 The simplified reference tissue model

In study IV the BP_{ND} values were estimated with the Simplified Reference Tissue Model (SRTM), using the cerebellum as reference region. The SRTM model contains only three parameters (R_1, k_2 and BP_{ND}) in which R_1 is introduced to control for difference in K_1 and K_1'. The kinetic model is based on two main assumptions. First, the volume of distribution of the non-displaceable bound radioligand is the same in both target and reference region ($C_{ND} = C'_{ND}$). Second, the free concentration (C_{FT}) and non-specific concentration (C_{NS}) equilibrate quickly and can therefore be fitted satisfactorily to a single tissue compartment model. The final expression includes the BP_{ND} and is solved in a convolution manner using least square fitting of the data[168].

3.3.5.2 The multilinear reference tissue model

In study II and III, BP_{ND} values were estimated with MRTM2. This approach is a variation of the graphical method of Logan[186] which derives the ratio of radioligand distribution volumes[138]. The method makes use of linear least squares estimation

algorithms, which is a less computer consuming approach than using non-linear square fitting (such as SRTM) and MRTM is therefore well suited for parametric imaging. Preliminary application of MRTM was used to estimate the cerebellum clearance rate (k'_2). The k'_2 value was then fixed for all voxels (MRTM2) and BP_{ND} and R_1 maps were generated[138]. Final BP_{ND} values were obtained by ROI analysis of the BP_{ND} maps.

3.3.5.3 The transient and late equilibrium approach

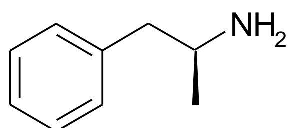
In study I and V, the transient and late equilibrium approach were applied, respectively. The occurrence of the transient equilibrium is defined as when the derivative of the specific binding equals zero and at this specific time point the ratio C_S/C_{ND} equals BP_{ND}[72]. To improve reliability of quantification, a time interval method has been used in which the time interval includes a short period before and after the occurrence of the transient equilibrium[236]. Moving the time interval to the late part of the time activity curves[24,272,339] has been referred to as the late time method[141].

3.3.5.4 The continuous infusion equilibrium approach

In study VI a continuous infusion approach was applied. When using this method the BP_{ND} is defined by the ratio of C_S and C_{ND} during the time interval of equilibrium[28,176]. In study VI C_{CER}(t) was used as estimate for C_{ND}(t). Calculations were performed for a time period at which C_{CER}(t) and C_S(t) were constant.

3.4 EXPERIMENTAL DRUGS

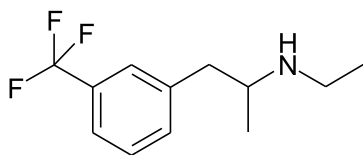
3.4.1 *D*-Amphetamine



Dextroamphetamine (*D*-amphetamine) and substituted amphetamines, such as methamphetamine (METH) and methylenedioxyamphetamine (MDMA, ecstasy), are widely abused psychostimulant drugs. Amphetamines are substrates for the transporters of the monoamines dopamine, norepinephrine and serotonin. Administration of amphetamines promotes the release of monoamines by several pathways, mainly along two primary mechanisms: reversal of monoamine transporter function and modification of monoamine redistribution between synaptic vesicles and cytosol[310].

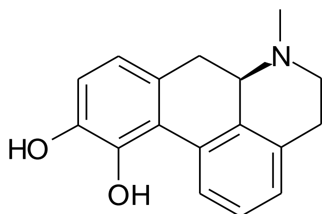
Microdialysis studies in rodents have shown that *D*-amphetamine mainly increases dopamine levels[148]. *D*-amphetamine is still widely prescribed for ADHD[127] and narcolepsy[232] and has negligible affinity to the D_2 receptor[139] and could therefore be safely used as a test drug in PET studies evaluating dopamine release with D_2/D_3 receptors radioligands in non-human primates[22,29,36,57,96,125,139,173] and human subjects[22,73,171,172]. Microdialysis studies in non-human primates[22,214,288] have shown a dose-dependent relation between amphetamine and increase in dopamine levels, ~500% at 0.27 mg/kg, ~1300% at 0.6 mg/kg, ~1600% at 1.0 mg/kg and ~1800% at 1.5 mg/kg[173]. In study II, *D*-amphetamine was administered intravenously approximately 20 minutes prior to radioligand injection at four doses (0.1, 0.2, 0.5 and 1.0 mg/kg). *D*-amphetamine was obtained from Apoteket, Sweden.

3.4.2 (±)-Fenfluramine



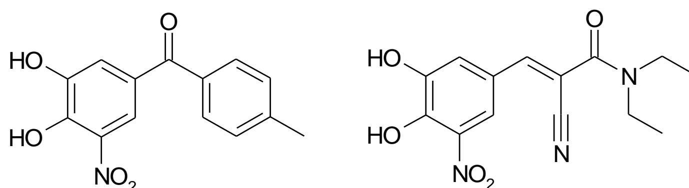
(±)-Fenfluramine is generally considered a substrate for the SERT and may increase extracellular serotonin levels by SERT inhibition and by promotion of a process of carrier-mediated exchange[88,278]. Microdialysis studies in unanaesthetized monkeys have shown that an i.v. dose of 5 and 10 mg/kg (±)-fenfluramine increases serotonin levels by 20- and 35–fold, respectively[328]. Fenfluramine has previously been on the market for treatment of obesity but has been withdrawn due to increased risks of valvular heart disease, possibly related to 5-HT_{2B} receptor affinity[277]. PET studies evaluating the sensitivity to serotonin levels of 5-HT_{1A} receptor radioligands have previously made use of fenfluramine in rodents and non-human primates[135,193,351]. (±)-Fenfluramine has been reported to have lower affinity to the 5-HT_{1B} receptor than to the 5-HT_{1A} receptor (16.3 vs. 3.7 µM, IC_{50})[205]. In study V and VI (±)-fenfluramine was i.v. administered to the monkeys at doses of 1.0 and 5.0 mg/kg. (±)-Fenfluramine was provided by H. Lundbeck A/S.

3.4.3 (*R*)-Apomorphine



(*R*)-Apomorphine is an exogenous dopamine agonist and has *in vitro* high affinity for the D_4 receptor, moderate affinities for D_2, D_3, and D_5 and similar or lower affinity for the D_1 receptor [210]. It has been shown that (*R*)-apomorphine is a full agonist for both splice variants $D_{2\text{-short}}$ and $D_{2\text{-long}}$[112,206], and competition studies have demonstrated two binding sites with 34–58 times selectivity to the high affinity state. This selectivity is similar to that reported for dopamine *in vitro* (65–79)[52,300]. (*R*)-Apomorphine is currently indicated for symptomatic treatment of recurring episodes of hypomotility ("off" episodes) in patients with advanced Parkinson's disease[55]. Importantly, by contrast to dopamine, (*R*)-apomorphine readily passes the BBB. (*R*)-Apomorphine was, therefore suitable as a test drug for *in vivo* studies and was administered in study III at i.v. doses of 0.01, 0.05, 0.15, 0.5, 1.0 and 3.0 mg/kg. (*R*)-Apomorphine was obtained from Apoteket, Sweden.

3.4.4 Tolcapone and entacapone



Tolcapone and entacapone are COMT inhibitors[146] and are currently used in conjunction with dopamine agents in the treatment of Parkinson's disease[180]. COMT inhibitors are used to decrease metabolism of *L*-DOPA and thereby to improve the efficacy of *L*-DOPA treatment[144,145,280]. Tolcapone and entacapone inhibit equally rat liver COMT but tolcapone has better brain penetration than entacapone[82]. The effect of COMT inhibitors *in vivo* has been widely studied using 6-[^{18}F]fluoro-*L*-DOPA[110,124,182,258] and these PET studies have here been used for dose-selection for study IV. Tolcapone was i.v. administered at 1, 5, 10 and 30 mg/kg and entacapone at 1 and 10 mg/kg. Tolcapone and entacapone were provided by H. Lundbeck A/S.

3.5 STATISTICAL ANALYSIS

In study II, IV and VI, analyses of variance (ANOVA) or repeated measures analyses of variance (RM ANOVA) were performed to test for group differences in BP_{ND} values. In study VI, subsequent paired *t*-tests were performed to test individual regions for fenfluramine effect. The minimum level of significance was designated as $P < 0.05$.

4 RESULTS AND COMMENTS

4.1 STUDY I: D_2/D_3 AGONIST PET RADIOLIGAND DEVELOPMENT

In this study, an agonist radioligand was prepared to evaluate the high affinity state of the D_2/D_3 receptors in the living brain using PET. (*R*)-(-)-2-methoxy-*N*-*n*-propyl-norapomorphine (MNPA) is a potent D_2/D_3 receptors agonist (K_i = 0.17 nM) with high selectivity over the D_1 receptor (K_i = 1.8 μM, 10500 fold)[87]. [^{11}C]MNPA was prepared by direct methylation using [^{11}C]CH_3I and (*R*)-(-)-2-hydroxy-*N*-*n*-propyl-norapomorphine as precursor. Derivatization of [^{11}C]MNPA and comparison to reference standards on HPLC confirmed a selective labelling of the 2-hydroxy-position. An improved radiosynthesis was more recently developed in which [^{11}C]MNPA is produced in a two-step synthesis starting from (*R*)-(-)-2-hydroxy-10,11-acetonide-*N*-*n*-propyl-noraporphine[308].

PET measurements after i.v. injection of [^{11}C]MNPA in cynomolgus monkey showed high uptake in D_2/D_3 receptors rich regions, such as putamen and caudate nucleus, and striatum to cerebellum ratios reached a maximum value of 2.2 (Figure 10 and 11).

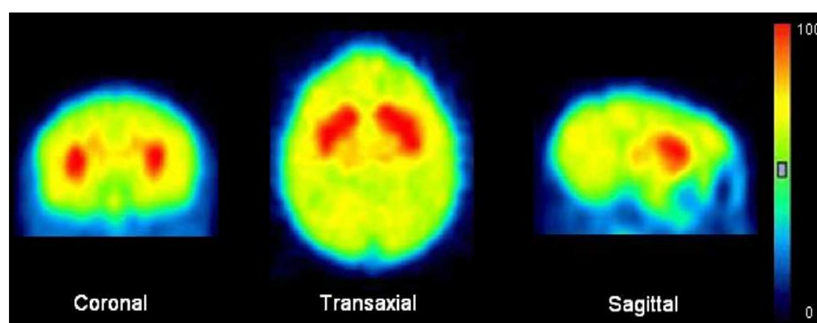


Figure 10. Colour-coded PET images showing the distribution of radioactivity in the monkey brain after i.v. injection of about 57 MBq [^{11}C]MNPA (summation image of 9-93 min).

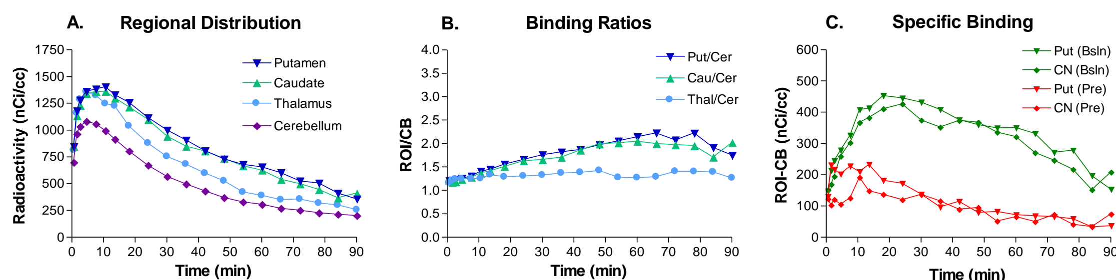


Figure 11. (A) Time course for regional radioactivity (nCi/cc) in the brain of a cynomolgus monkey after i.v. injection of [^{11}C]MNPA and corresponding binding ratios (B). (C) Time course for specific binding (nCi/cc) in the brain of a cynomolgus monkey after i.v. injection of [^{11}C]MNPA in baseline and pretreatment conditions. In the pretreatment measurement, raclopride (1 mg/kg) was administered i.v. ten minutes before injection of [^{11}C]MNPA.

Administration of raclopride reduced the specific binding in striatum (Figure 11C) and striatum to cerebellum ratios decreased to 1.3 confirming specific binding of [^{11}C]MNPA to D_2/D_3 receptors. The fraction of total radioactivity in monkey plasma representing unchanged [^{11}C]MNPA was 20% at 45 minutes after injection of radioligand and no radiometabolites were observed more lipophilic than [^{11}C]MNPA, as measured with gradient HPLC. This initial characterization demonstrated that [^{11}C]MNPA has potential as an agonist radioligand for examination of D_2/D_3 receptors in human subjects.

4.2 STUDY II: DOPAMINE RELEASE MEASURED WITH $[^{11}C]$MNPA

Experimental studies using pharmacological challenges and PET allow for an *in vivo* assessment of synaptic neurotransmitter levels in a non-invasive manner. Amphetamine is a strong dopamine releaser and has been consistently shown to decrease D_2/D_3 receptors binding of some antagonist radioligands in animals and humans. The purpose of this study was to compare the effect of amphetamine-enhanced dopamine level on D_2/D_3 receptors binding of the agonist radioligand $[^{11}C]$MNPA and the antagonist radioligand $[^{11}C]$raclopride. Four cynomolgus monkeys were examined with both radioligands before and after i.v. administration of a single dose of amphetamine. Finally, the results obtained were used in an attempt to estimate the proportion of D_2/D_3 receptors in the high and low affinity state.

During baseline conditions, i.v. injection of $[^{11}C]$MNPA and $[^{11}C]$raclopride resulted in high accumulation of radioactivity in striatum while concentrations of radioactivity in cerebellum were lower. Comparison of striatal BP_{ND} values showed significantly higher values for $[^{11}C]$raclopride (5.76 ± 0.95, n = 8) when compared to $[^{11}C]$MNPA (1.31 ± 0.21, n = 8) (RM ANOVA, $P = 0.00$). Comparison of the BP_{ND} values between the four monkeys showed no significant inter-subject differences for $[^{11}C]$MNPA and $[^{11}C]$raclopride (ANOVA, $P > 0.10$). Administration of amphetamine caused a dose dependent reduction in $[^{11}C]$MNPA striatal BP_{ND} values of 4% at 0.1 mg/kg, 23% at 0.2 mg/kg, 25% at 0.5 mg/kg, and 46% at 1.0 mg/kg. Reductions in $[^{11}C]$raclopride striatal BP_{ND} values were less than for $[^{11}C]$MNPA, 2% at 0.1 mg/kg, 16% at 0.2 mg/kg, 15% at 0.5 mg/kg, and 23% at 1.0 mg/kg. The amphetamine effect was found significantly greater for $[^{11}C]$MNPA than compared to $[^{11}C]$raclopride (ANOVA, $P = 0.024$) (Figure 12).

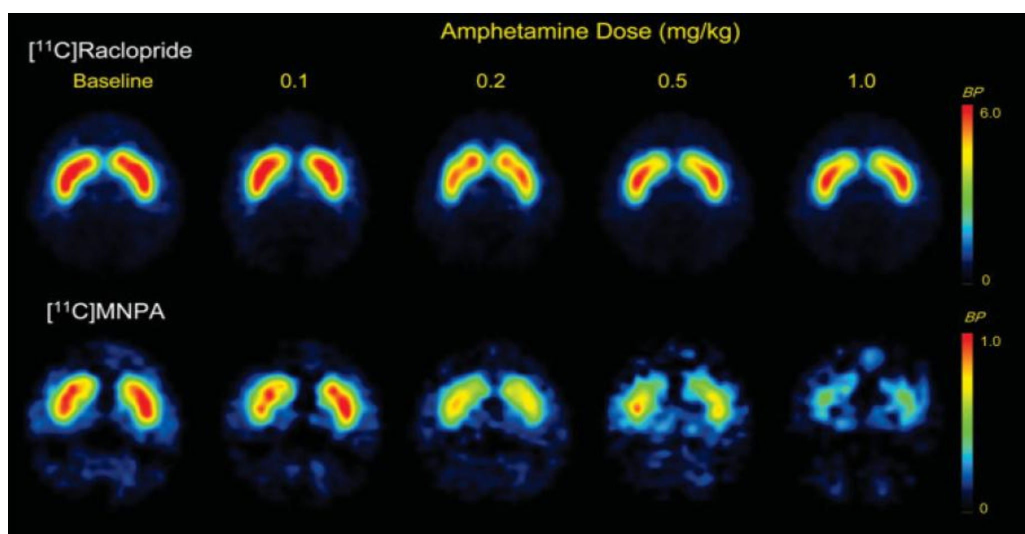


Figure 12. BP_{ND} maps of $[^{11}C]$raclopride and $[^{11}C]$MNPA estimated by MRTM2 at baseline and post-amphetamine conditions.

The observed improvement in sensitivity to endogenous dopamine level for $[^{11}C]$MNPA was consistent at all evaluated amphetamine doses, illustrated by the ratio of change in striatal BP_{ND} of $[^{11}C]$MNPA and $[^{11}C]$raclopride being 1.85 at 0.1 mg/kg, 1.51 at 0.2 mg/kg, 1.70 at 0.5 mg/kg, and 2.03 at 1.0 mg/kg, with an average of 1.77. Previously, Narendran et al. have proposed that this ratio of changes in BP_{ND} of both tracers could be used to calculate the fraction of receptors in the high affinity state according to equation 8[223].

$$\frac{\Delta BP\ Rac}{\Delta BP\ MNPA} = \frac{Rhigh\ avail}{Bmax\ avail} \quad (8)$$

Assuming that 10% of the D_2/D_3 receptors are occupied by endogenous dopamine, it was calculated for the current study that 61% of the D_2/D_3 receptors were configured in the high affinity state. Furthermore, by assuming that escalating doses of amphetamine would not further decrease radioligand binding, it was calculated that only 23% of the total pool of D_2/D_3 receptors was in the high affinity state and susceptible to dopamine, so called “synaptic” located receptor sites.

4.3 STUDY III: APOMORPHINE DECREASES D_2/D_3 RADIOLIGAND BINDING

Binding studies *in vitro* have indicated that the D_2 receptor may exist in two affinity states for agonists[52,300]. *In vitro* studies have further shown that agonists induce measurable D_2 receptor occupancy at clinically relevant concentrations, but only when measured at the high affinity state[293,295]. Recently developed PET-radioligands, such as [^{11}C]MNPA, have now made it possible to directly study agonist binding *in vivo*. The aim of this study was to obtain further *in vivo* support for the existence of two affinity states for the D_2/D_3 receptors, by comparison of the inhibition by apomorphine of agonist and antagonist radioligand binding *in vivo*. A total of 36 PET measurements were performed with [^{11}C]raclopride or [^{11}C]MNPA in two cynomolgus monkeys. On each study day, a baseline measurement was followed by two consecutive pretreatment studies with rising doses of apomorphine (0.01, 0.05, 0.15, 0.5, 1.0, and 3.0 mg/kg). BP_{ND} values were calculated for the striatum with cerebellum as reference region.

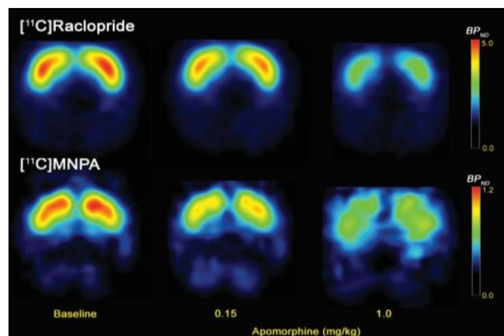


Figure 13. BP_{ND} maps of [^{11}C]raclopride and [^{11}C]MNPA estimated by MRTM2 at baseline and post-apomorphine conditions.

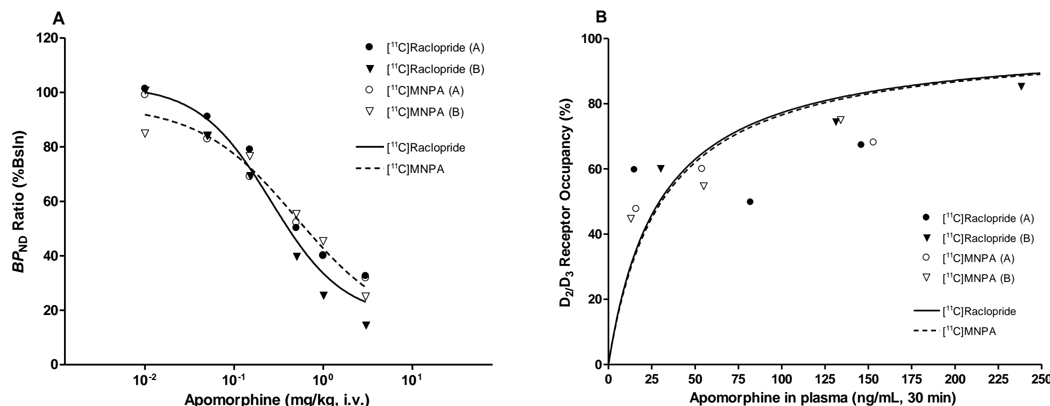


Figure 14: (A) Inhibition of striatal BP_{ND} of [^{11}C]raclopride and [^{11}C]MNPA by pretreatment with apomorphine in two monkeys (A and B). The lines represent an unconstrained four-parameter logistic fit. (B) D_2/D_3 receptors occupancy of apomorphine as a function of plasma concentration 30 min after radioligand injection (ng/mL). The lines represent a one-site binding fit constrained to B_{max} of 100%.

The binding of the antagonist [^{11}C]raclopride as well as the agonist [^{11}C]MNPA was inhibited to a high degree and approached full saturation (Figure 13 and 14). Multifarious analysis methods indicated that the inhibition by apomorphine was monophasic, and the Hill slope coefficients were close to unity. Brain homogenate binding studies *in vitro* have previously demonstrated that apomorphine binds with 30 to 60-fold selectivity to the high affinity state over the low affinity state[52,300].

In this study, we obtained non-distinguishable ID_{50} and K_i values of apomorphine for the D_2/D_3 receptors when measured with the antagonist or the agonist radioligand (0.26 and 0.50 mg/kg and 29 and 31 ng/mL, respectively). This PET study provides no support for the existence of two affinity states of the D_2/D_3 receptors. A possible explanation is that almost all D_2/D_3 receptors are in the high affinity state *in vivo*. The results of this study are not directly in line with the outcome of study III. The differences observed could possibly be related to dissimilarity in cellular concentration of agonist or by differences in agonist-induced receptor internalization.

4.4 STUDY IV: D_1/D_5 AGONIST PET RADIOLIGAND DEVELOPMENT

A radioligand with D_1 receptor agonistic properties may provide new understanding of the reasons for lack of sensitivity to dopamine shown for D_1/D_5 receptors antagonist radioligands, such as (*R*)-[^{11}C]SCH 23390[54,70,117,263] and (+)-[^{11}C]NNC 112[120]. The utility of the previous reported partial D_1/D_5 receptors agonist radioligand (+)-[^{11}C]SKF 82957[44,45] has been limited due to a brain-penetrant radiometabolite, being formed by COMT[48]. In this study, we radiosynthesised and performed an extensive *in vitro* and *in vivo* evaluation of the new D_1/D_5 receptors partial agonist radioligand (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 ((*S*)-[^{11}C]**1**) (Figure 15).

(*S*)-**1** has high affinity to the D_1 receptor (K_i = 4.9 nM), which is in a similar range as for the antagonists (*R*)-SCH 23390 and (+)-NNC 112 (K_i = 2.1 and 1.5 nM, respectively). A functional D_1 receptor assay confirmed high potency of (*S*)-**1**, but indicated only partial agonistic activity (EC_{50} = 1.9 nM, 35% of dopamine).

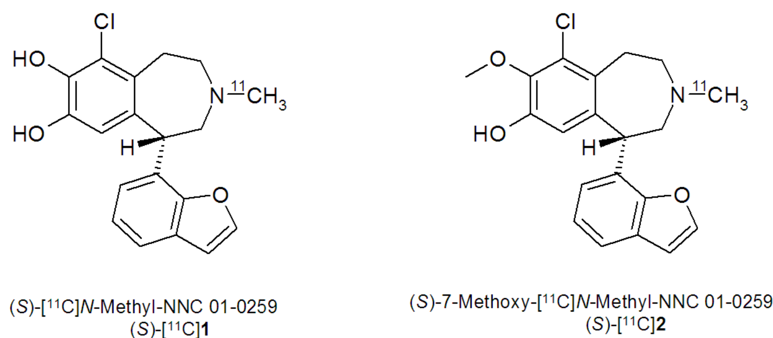


Figure 15. Two compounds synthesised in the current study, the partial D_1/D_5 receptors agonist [^{11}C]*N*-methyl-NNC 01-0259 ((*S*)-[^{11}C]**1**) and the 7-methoxy analogue of (*S*)-[^{11}C]*N*-methyl-NNC 01-0259, (*S*)-[^{11}C]**2**.

The regional distribution of radioactivity after injection of (*S*)-[^{11}C]**2** was in accordance with the known distribution of the D_1/D_5 receptors, with high concentration of radioactivity in striatum, moderate in neocortex and lowest in cerebellum. The peak in specific binding in the striatum was observed at ~30 minutes and specific binding ratios reached a maximum value of about 2.0 approximately sixty minutes after radioligand injection (Figure 16).

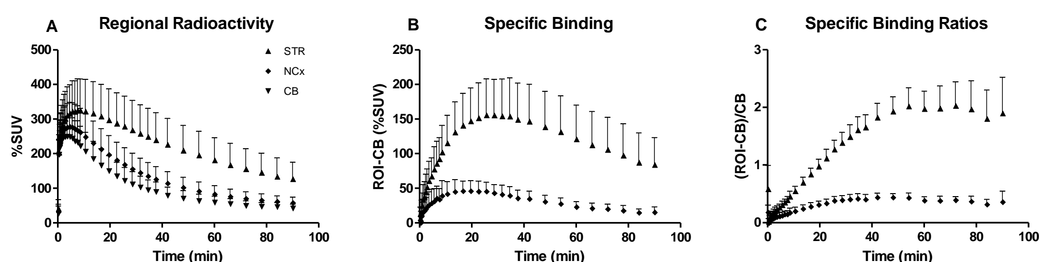


Figure 16. (A) Regional brain radioactivity (%SUV) after i.v. administration of (*S*)-[^{11}C]1 in cynomolgus monkey. (B) Specific binding in striatum and neocortex. (C) Specific binding ratios in striatum and neocortex. All values represent the mean and standard deviation (presented to above) of nine measurements.

Administration of the dopamine releaser *D*-amphetamine did not have a significant effect on receptor binding of (*S*)-[^{11}C]**1**. The specificity and selectivity of (*S*)-[^{11}C]**1** receptor binding was tested with pretreatment studies using the D_1/D_5 receptors antagonist (*R*)-SCH 23390 and the selective 5-HT_{2A} receptor antagonist MDL-100907. (*R*)-SCH 23390 decreased BP_{ND} in the striatum with 97% to 0.06, while the BP_{ND} value in neocortex only decreased with 77% to 0.12. The mean decrease in BP_{ND} value after MDL-100907 was 19% in striatum and 30% in neocortex.

During the PET measurements in monkeys radiometabolism of (*S*)-[^{11}C]**1** was measured in plasma and demonstrated the formation of a radiometabolite, which was more lipophilic than (*S*)-[^{11}C]**1**. The lipophilic radiometabolite was identified by HPLC as (*S*)-7-methoxy-[^{11}C]*N*-methyl-NNC 01-0259 ((*S*)-[^{11}C]**2**) (Figure 15). An *in vitro* binding assay demonstrated that (*S*)-**2** has affinity for D_1 receptors (K_i = 52 nM). A PET study after injection of (*S*)-[^{11}C]**2** in monkey confirmed that (*S*)-[^{11}C]**2** passes the BBB and concentrates in D_1/D_5 receptors rich brain regions (BP_{ND} in striatum is 0.33). The second part of this project therefore aimed for avoidance of the formation of the confounding lipophilic radiometabolite (*S*)-[^{11}C]**2** by the use of COMT inhibitors tolcapone and entacapone. COMT inhibition indeed reduced the formation of (*S*)-[^{11}C]**2** measured in plasma and tolcapone administration significantly increased the regional BP_{ND} values, measured after injection of (*S*)-[^{11}C]**1** in monkey (unpaired *t*-test, $p < 0.05$) (Figure 17). These results are consistent with a recent study showing that systemic COMT inhibition enabled (+)-[^{11}C]SKF 82957 receptor studies in rodents[247].

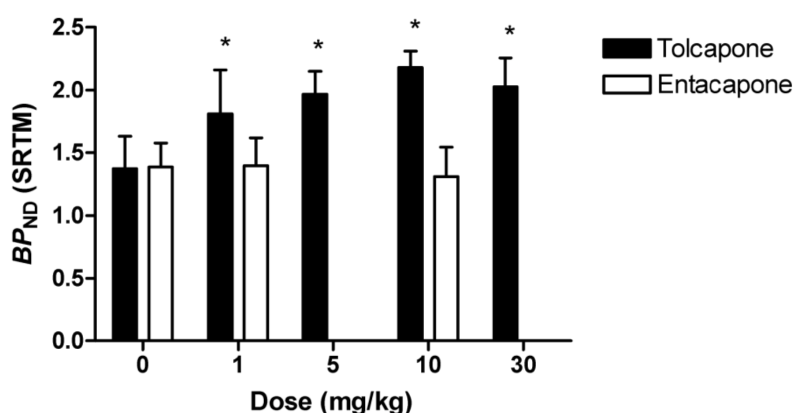


Figure 17. Striatal BP_{ND} values measured after i.v. injection of (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 in monkeys pre-treated with COMT inhibitor entacapone or tolcapone. *Indicates significant difference compared to BP_{ND} of baseline ($p < 0.05$, unpaired *t*-test).

From this study, two main conclusions can be made. Firstly, (*S*)-[^{11}C]**1** receptor binding is insensitive to altered dopamine level and (*S*)-[^{11}C]**1** is an inferior radioligand for imaging of D_1/D_5 receptors when compared to previous reported antagonist radioligands. Secondly, quantification is complicated by the *in vivo* formation of (*S*)-

[^{11}C]**2** but quantitative measurements with PET and (*S*)-[^{11}C]**1** are feasible when performed in combination with a COMT inhibitor. As a catechol group is most often critical for D_1 receptor agonism, the COMT inhibition approach may indeed be needed as a more general approach when using a D_1/D_5 receptors agonist radioligand and, importantly, the approach seems suitable for extension to humans.

4.5 STUDY V: [^{11}C]AZ10419369 IS SENSITIVE TO SEROTONIN RELEASE

The need for PET-radioligands that are sensitive to changes in endogenous serotonin levels in brain is recognized in experimental and clinical psychiatric research. Of available serotonergic radioligands, the 5-HT_{1A} receptor antagonist [^{18}F]MPPF has been the most promising with regard to changes in endogenous serotonin levels in the rat and cat brain[12,351,352], but the findings have not been consistently confirmed in monkey and human subjects[56,256,301,326,328]. Examination of serotonin levels *in vivo* by PET has therefore possibly been precluded by the lack of suitable radioligands. [^{11}C]AZ10419369 is a novel PET radioligand highly selective for the 5-HT_{1B} receptor and recently developed by our group in collaboration with AstraZeneca[194,251,333]. Of the fourteen 5-HT-receptor subtypes, the 5-HT_{1B} receptor is of particular interest for radioligand development since this primarily presynaptic autoreceptor regulates the release of serotonin[65,104,200] and thus should be sensitive to serotonin levels.

In this PET study the sensitivity of [^{11}C]AZ10419369 to altered endogenous serotonin levels was examined in cynomolgus monkeys. Serotonin levels were enhanced with the test compound fenfluramine which has been shown to increase basic serotonin levels by 20-fold (5 mg/kg) in monkey[328]. The effect of fenfluramine on [^{11}C]AZ10419369 receptor binding was studied in three monkeys using a displacement paradigm. Fenfluramine (1 and 5 mg/kg) was administered in the displacement study during the period of 15 and 20 minutes after radioligand injection.

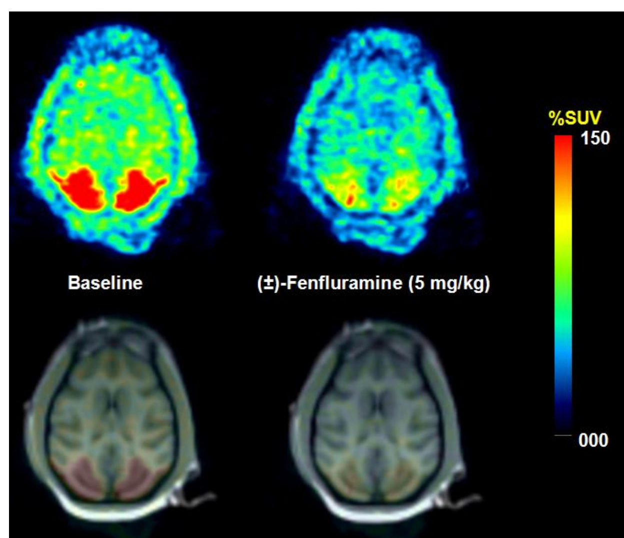


Figure 18. PET images of mean radioactivity 45–123 min after i.v. injection of [^{11}C]AZ10419369 in a cynomolgus monkey at baseline (top left) and after displacement with 5 mg/kg (±)-fenfluramine (top right). In the bottom corresponding MR images overlaid with PET images.

After administration of fenfluramine there was an evident decrease in [^{11}C]AZ10419369 binding. There was no evident effect of fenfluramine on the radioactivity concentration in the reference region cerebellum, whereas there was a rapid decrease in specific [^{11}C]AZ10419369 binding in all other regions examined. The mean specific binding ratio (SBR) was calculated by use of the area under the curve for time interval 45-123 min (Figure 18 and 19).

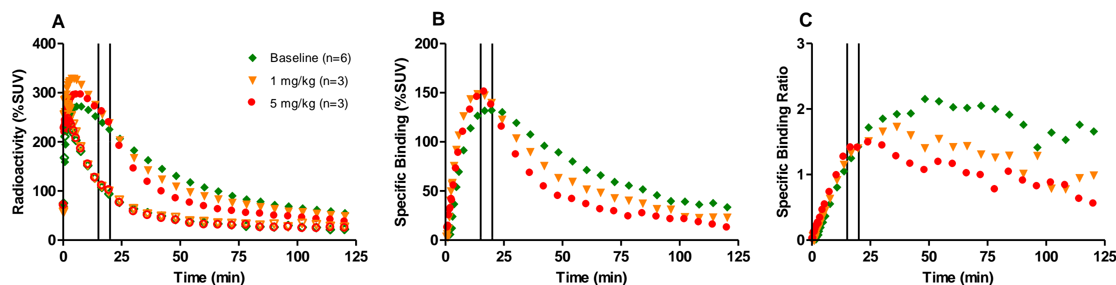


Figure 19. (A) Mean radioactivity in the occipital cortex (closed symbols) and cerebellum (open symbols) at baseline and displacement with fenfluramine (1.0 and 5.0 mg/kg). (B) Specific [^{11}C]AZ10419369 binding in the occipital cortex. (C) Specific binding ratios in the occipital cortex. Fenfluramine was administered between 15 and 20 min after radioligand injection and the time interval is illustrated by two vertical lines.

The fenfluramine effect appeared dose-dependent because the decrease was more pronounced after a dose of 5 mg/kg than after 1 mg/kg. Fenfluramine-induced serotonin release decreased the SBR in a dose-dependent fashion with a regional average of 27% after 1 mg/kg and 50% after 5 mg/kg (Figure 20).

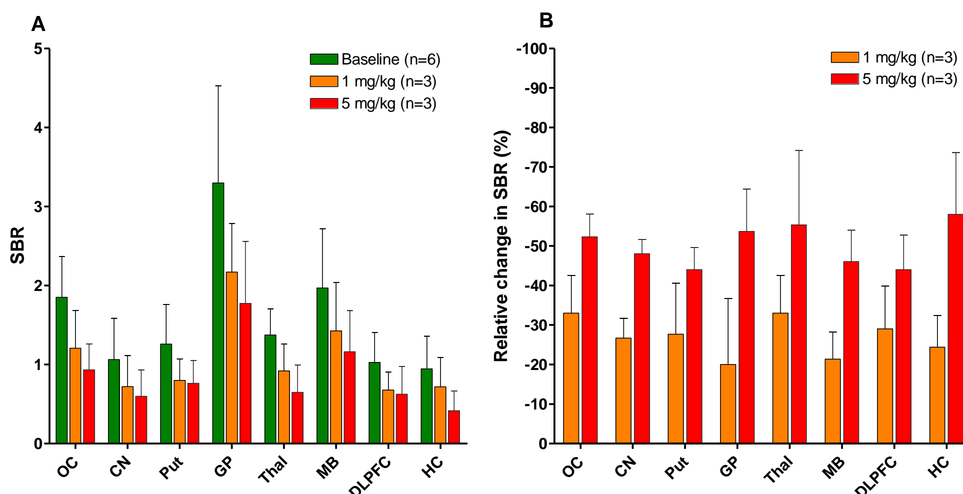


Figure 20. (A) Regional mean specific binding ratios (SBR) during baseline and after displacement with fenfluramine (1 and 5 mg/kg). (B) Relative change in SBR after fenfluramine. Bars represent mean ± SD.

This preliminary study supports that [^{11}C]AZ10419369 is sensitive to endogenous serotonin levels *in vivo* and may serve as a tool to examine the pathophysiology and treatment of major psychiatric disorders.

4.6 STUDY VI: CONFIRMATION OF SEROTONIN SENSITIVITY OF [^{11}C]AZ10419369

The displacement paradigm is attractive for quantification of neurotransmitter release because it allows for direct observation of changes in neuronal activity during pharmacological or physiological stimulation, which are reflected in modified radioligand binding. A methodology considered suitable for quantification of neurotransmitter release, when using a displacement paradigm, is the equilibrium approach, which utilizes the administration of radioactivity by a bolus and constant infusion (BI-protocol)[28]. The aim of the present study was to develop an updated methodology for measurement of drug-induced serotonin release using [^{11}C]AZ10419369 and PET in non-human primates and to apply this setup to confirm

our previous findings of fenfluramine-induced decreases in [^{11}C]AZ10419369 receptor binding.

A total of 24 PET measurements were conducted, including six preparative PET measurements to assess suitable K_{bol} values. During baseline conditions the C_S/C_{ND} ratios became stable after approximately 50 minutes in the occipital cortex as well as in all other brain regions. K_{bol} values of 180-240 minutes allowed for rapid achievement of steady state and BP_{ND} was calculated between 50-80 minutes (equilibrium before fenfluramine) and between 117-153 minutes (equilibrium after fenfluramine) (Figure 21).

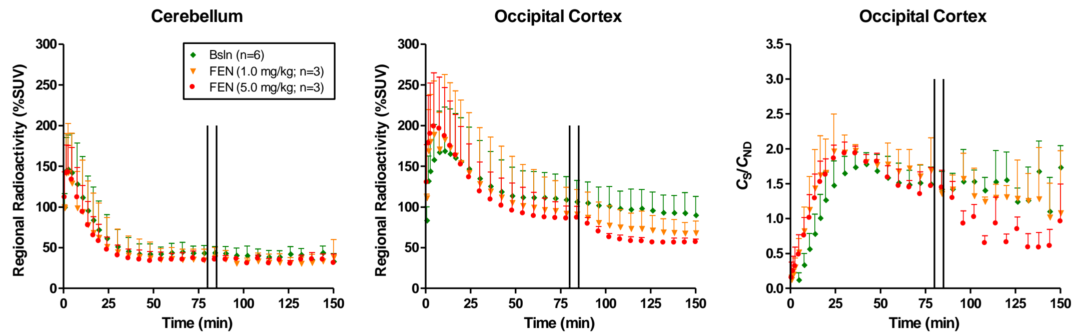


Figure 21. Time activity courses of radioactivity in cerebellum (left) and occipital cortex (middle) after i.v. administration of [^{11}C]AZ10419369 using a BI-protocol in monkey during baseline and displacement conditions with fenfluramine (1.0 and 5.0 mg/kg, FEN). (Right) Corresponding time activity courses for the C_S/C_{ND} values for the occipital cortex are shown on the right. All values represent the mean and standard deviation (presented to above) of three or six measurements. In the displacement studies fenfluramine was administered during the time period indicated by the vertical lines (80-85 min).

During baseline measurements (n=3x2) [^{11}C]AZ10419369 BP_{ND} values were stable over time and did not significantly differ during time frames 51'-81' vs. 117'-153' (RM ANOVA, P = 0.2842). [^{11}C]AZ10419369 BP_{ND} values (n=3x2) of baseline and displacement measurements obtained during 51'-81' showed no significant difference (RM ANOVA, P = 0.9929), thus demonstrating good test-retest variability (Figure 22).

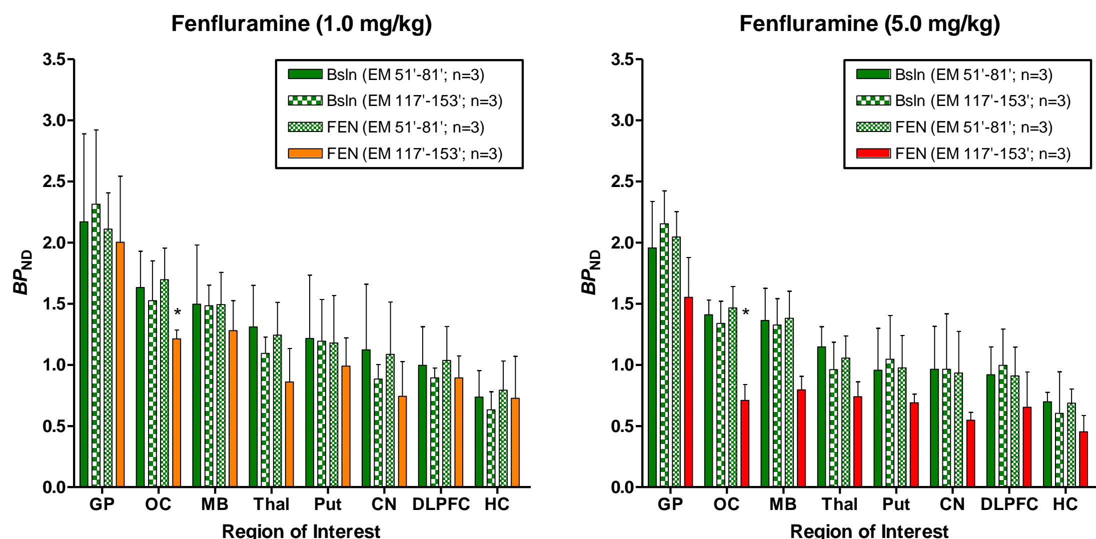


Figure 22. BP_{ND} values obtained after i.v. administration of [^{11}C]AZ10419369 using a BI-protocol in monkey during baseline and displacement conditions with fenfluramine (1.0 mg/kg (left) and 5.0 mg/kg (right), FEN). All values represent the mean and standard deviation of three subjects. In the displacement studies fenfluramine was administered during the time period of 80-85 min after the start of BI-protocol. The effect of fenfluramine is therefore only represented in the BP_{ND} values corresponding to FEN (EM 117'-153') values. *Indicates significant difference compared to BP_{ND} of method FEN (EM 51'-81') (p < 0.05, two-tailed paired t-test).

Fenfluramine caused a dose-dependent decrease in [^{11}C]AZ10419369 binding in all evaluated regions except for in the cerebellum. In the displacement paradigm fenfluramine significantly reduced [^{11}C]AZ10419369 BP_{ND} (FEN EM 117'-153') when compared to control BP_{ND} (FEN EM 51'-81'), with no significant ROI effect, at both 1.0 mg/kg and 5.0 mg/kg, respectively (RM ANOVA, $P < 0.0001$). Among the different regions, the effect of fenfluramine was significant in the occipital cortex (paired *t*-test, $p < 0.05$) when [^{11}C]AZ10419369 BP_{ND} (FEN EM 117'-153') were compared to control BP_{ND} (FEN EM 51'-81') for both fenfluramine doses.

To further improve counting statistics, pretreatment studies with fenfluramine were performed, which enabled the use of the majority of the PET measurement for quantification of BP_{ND} values. In the pretreatment paradigm, fenfluramine (5.0 mg/kg) significantly reduced [^{11}C]AZ10419369 BP_{ND} (FEN EM 51'-123') when compared to baseline BP_{ND} (Bsln EM 51'-123') with a significant ROI effect (RM ANOVA, $P < 0.0001$). A significant effect of 5.0 mg/kg fenfluramine on [^{11}C]AZ10419369 BP_{ND} (FEN EM 51'-123') was found in the globus pallidus, occipital cortex, midbrain, thalamus and hippocampus (paired *t*-test, $p < 0.05$) when compared to baseline BP_{ND} (Bsln EM 51'-123') (Figure 23).

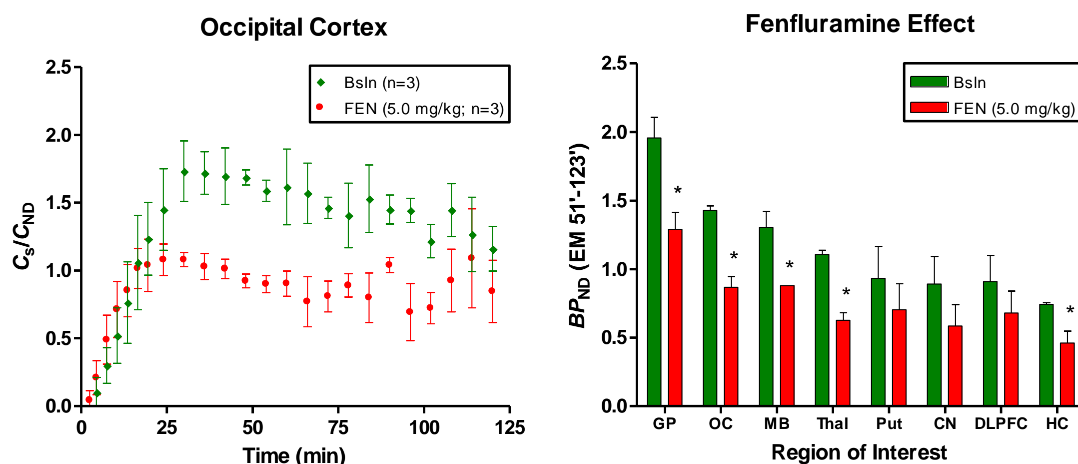


Figure 23. (Left) Time activity courses of C_S/C_{ND} values for the occipital cortex after i.v. administration of [^{11}C]AZ10419369 using a BI-protocol in monkey during baseline and pretreatment conditions with fenfluramine (5.0 mg/kg, FEN). All values represent the mean and standard deviation (presented to both sides) of three subject. (Right) Effect of fenfluramine on BP_{ND} values obtained for eight different brain regions of three subjects. *Indicates significant difference compared to baseline measurements ($p < 0.05$, two-tailed paired *t*-test).

This study confirms that the new 5-HT_{1B}-receptor radioligand [^{11}C]AZ10419369 is sensitive to fenfluramine-induced changes in endogenous serotonin levels *in vivo*. The effect of fenfluramine on [^{11}C]AZ10419369 BP_{ND} was dose-dependent in the displacement paradigm, but more reliably estimated with the pretreatment paradigm. After pretreatment administration of fenfluramine (5.0 mg/kg), the mean BP_{ND} of the occipital cortex decreased by 39%, from 1.43±0.04 to 0.87±0.08.

The further developed methodology is suitable for exploring the sensitivity limit to serotonin release as measured using [^{11}C]AZ10419369 and PET. The developed methodology can accordingly be applied to further examine the effect of drugs on brain endogenous serotonin level, and to study serotonin-related brain functions and psychiatric disorders in man.

5 METHODOLOGICAL CONSIDERATIONS

5.1 RESOLUTION OF THE PET SYSTEM

When considering the small volume of the cynomolgus monkey brain (~65 cc), partial volume and spillover effects must be taken into account, in particular in small brain regions.

In this thesis, baseline and drug-challenge PET studies were performed on the same day. Due to the use of a head fixation device the monkey head was maintained in the same position during the day. This approach makes it possible to use the same ROIs for sequentially performed PET measurements. Importantly, potential differences in partial volume and spill over effects were therefore minimized between PET measurements and should therefore not influence the calculated effects significantly.
Over the course of the thesis work, two PET systems were used. The ECAT EXACT HR has a in plane resolution of 3.8 mm FWHM[343] and the HRRT system has an in plane resolution of 2.3 mm FWHM[334]. The resolution of the HRRT has been further enhanced to 1.5 mm FWHM by software advancements which include the point spread function (PSF) of the PET system[334]. The use of a combination of the HRRT and PSF reconstruction provides imaging with improved quantification and reduced partial volume effects[312,334]. Future studies will benefit from this improvement in methodology.

5.2 REFERENCE TISSUE MODELS

PET measurements in cynomolgus monkeys were performed without arterial cannulation. The quantification of radioligand receptor binding has therefore been limited to the use of reference tissue models. Studies performed with [^{11}C]MNPA, [^{11}C]raclopride, (*S*)-[^{11}C]*N*-methyl NNC 01-0259 and [^{11}C]AZ10419369 have all used cerebellum as a reference region. This approach is based on two main assumptions: that cerebellum contains negligible specific binding and that the non-displaceable component is comparable in cerebellum and other brain regions.

The cerebellum has been shown to contain a negligible density of D_1/D_5 and D_2/D_3 receptors in *post-mortem* autoradiography[114-116]. The use of the cerebellum as a reference region for [^{11}C]raclopride has previously been extensively discussed[63,72]. The binding of [^{11}C]MNPA in cerebellum was evaluated in study I and III. The absence of specific binding in the cerebellum was confirmed by the lack of change in [^{11}C]MNPA binding in cerebellum after injection of raclopride (study I) and apomorphine (study III). Recent PET studies in non-human primates[297] and humans[246] further confirmed with kinetic modelling that [^{11}C]MNPA has no specific binding in the cerebellum and that cerebellum can therefore be used as a suitable reference region.

Previous studies using the D_1/D_5 receptors antagonist (*R*)-[^{11}C]SCH 23390 have shown that the cerebellum functions as a suitable reference region[32,70]. The lack of specific binding in cerebellum after injection of (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 was confirmed in study IV, in which injection of (*R*)-SCH 23390 did not decrease radioligand binding in cerebellum. In addition, administration of the inactive enantiomer (*R*)-[^{11}C]*N*-methyl NNC 01-0259 resulted in radioactivity levels in striatum and neocortex similar to observed as in the cerebellum after injection of (*S*)-[^{11}C]*N*-methyl-NNC 01-0259.

[^{11}C]AZ10419369 has recently been developed[251] and the complete validation of quantitative methods is currently emerging. Autoradiography studies have previously indicated a negligible 5-HT_{1B} receptor density in cerebellum[331,332]. The use of the cerebellum as a reference region has been confirmed in studies demonstrating that 5-

HT_{1B} receptor antagonists do not effect binding in cerebellum, when measured with PET in monkey[251] and with autoradiography in guinea pig[194]. Full kinetic modelling in humans has demonstrated that $[^{11}C]$AZ10419369 binding in cerebellum can be described by a one tissue compartment model[333], further confirming no specific binding in cerebellum. In study V and VI, fenfluramine did not cause changes in $[^{11}C]$AZ10419369 binding in cerebellum thereby further confirming the use of cerebellum as a reference region.

5.3 SAMPLE SIZE AND TEST-RETEST REPRODUCIBILITY

A limitation of PET studies is that they typically do not include a large sample size. In this thesis, studies were performed in two or three monkeys per investigated condition.

A small sample size can be accepted when reproducibility between PET measurements is high. Typically, an experimental day included a baseline measurement followed by one or two drug-challenge measurements. To obtain good reproducibility, a head fixation device was used and the monkey head was thereby located in the same position during sequential PET measurements during the day. For PET data analysis the same ROIs could also be applied for both measurements. In a small test-retest study, four cynomolgus monkeys were examined two times after administration of $[^{11}C]$MNPA two hours apart. The individual test-retest difference in striatal BP_{ND} values ranged from -6.4 to +4.2%, with a mean difference of -1.1 ± 4.6% (unpublished results). The high reproducibility is in line with previous reported reproducibility of 3-7% for measurements with (+)-$[^{11}C]$NNC 112 in monkey[36] and with $[^{11}C]$raclopride[234] and (*R*)-$[^{11}C]$SCH 23390 in man[32]. This observation in a small number of animals supports that the measurement of $[^{11}C]$MNPA binding is reliable.

During the time period of this thesis, MR images of the individual monkeys were obtained. Individual MR image-based ROI templates were generated and used for data analysis in study IV-VI. Co-registration of the baseline PET measurements to the MR images allowed for determination of the transformation matrix for all PET measurements performed on the same day. This method allows for the use of the same ROIs for PET measurements performed on separate days. The reproducibility of $[^{11}C]$AZ10419369 binding during baseline conditions was examined for six experimental days in one monkey using a BI-protocol. The individual occipital cortex BP_{ND} value differences to the mean value ranged from -3.1% to +4.6%, with a mean difference of 0.0 ± 3.2% (unpublished results). These results indicate that measurement of $[^{11}C]$AZ10419369 binding is reliably estimated between experimental days.

5.4 EFFECTS OF ANEASTHESIA

Study I-IV used radioligands targeting dopamine receptors and were performed using i.m. injections of a mixture of ketamine and xylazine. Ketamine is an antagonist for the *N*-methyl-*D*-aspartate (NMDA) receptor and has been reported to bind to the $D_{2\text{-long}}$ receptor (K_i = 55 nM) *in vitro*[292]. Ketamine has been reported to exhibit partial or full agonistic properties[150], although this has not been confirmed by others[143,238]. In addition to direct effects on the receptor, ketamine has been found to increase extracellular dopamine concentrations in the prefrontal cortex in conscious rat, but this increase was blunted or not observed in the striatum of conscious rat and monkey[5,320,336].

Further support of the lack of ketamine effect on D_2/D_3 receptors antagonist radioligand binding can be found in PET and SPECT studies. Although initial studies indicated a small reduction in $[^{11}C]$raclopride binding after ketamine administration[23,305,320,340] more recent studies have shown no effect on $[^{123}I]$IBZM or

[^{11}C]raclopride binding[1,126,153,154]. Taken together there are several but inconsistent observations suggesting that ketamine may influence antagonist radioligand binding to the D_2/D_3 receptors.

Ketamine/xylazine anaesthesia has, however, more recently been shown to increase D_2/D_3 receptors binding of the agonist radioligand [^{11}C]MNPA in monkeys[240]. In addition, the enhanced sensitivity of [^{11}C]MNPA binding to dopamine release was not observed in unanaesthetized monkeys. In a following study, the authors demonstrated that D_2/D_3 receptors radioligand binding in unanaesthetized animals is related to stress, but differs in direction when measured with an agonist or antagonist radioligand[323]. Taken together it can be concluded that the effect of ketamine and stress on agonist radioligands is complex and warrants further investigation.

The use of another anaesthetic could have been considered for the dopamine studies but, at clinical concentrations, several anaesthetics (e.g., isoflurane, halothane, ketamine and ethanol) have already been shown to inhibit the high affinity states of the D_2 receptor, as well as other GPCRs *in vitro* and *ex vivo*[291]. Moreover, isoflurane was shown to facilitate the actions of various dopamine related pharmacological stimulants, such as amphetamine, cocaine, and nicotine *in vivo*[202,204,319,321]. Further studies in man are warranted to fully address the possible differential effects of anaesthesia on agonist and antagonist radioligand binding.

Study V and VI evaluated serotonin release in sevoflurane anesthetized monkeys. Microdialysis studies have demonstrated that serotonin level is lower during sleep than during awake conditions[255]. Comparison of serotonin level during slow-wave sleep and isoflurane anaesthesia showed a similar reduction in serotonin level, to 21-44% of awake condition, when measured with microdialysis in rats[217]. Serotonin levels are therefore likely decreased in anaesthetized animals, independent of the type of anaesthesia. Importantly, in study VI no significant differences were observed between [^{11}C]AZ10419369 BP_{ND} values at baseline and during displacement measurements obtained during 51-81 minutes, indicating no differences in anaesthesia effects during the experimental day.

5.5 ETHICAL CONSIDERATIONS

The use of non-human primates in medical research is under constant debate. These unique experiments should be conducted with the highest possible quality and have therefore inherently been subject of improvements during the time of this work. The monkeys were always continuously monitored during and between PET measurements. Introduction of tracheal intubation and gas anaesthesia techniques has further enhanced animal safety and welfare and allows for detailed adjustments in anaesthesia. Temperature, ECG, heart rate, respiratory rate and blood gasses are now being continuously monitored during experimental days and provide valuable information. Out of 150 monkey PET measurements in the current work, no complications were related to experimental procedures.

6 SUMMARY OF FINDINGS

The present thesis focused on drug-induced changes in endogenous neurotransmitter release measured with PET in the living non-human primate brain.

The successful development of the agonist radioligand [^{11}C]MNPA allowed for *in vivo* evaluation of the high affinity state of D_2/D_3 receptors with PET. [^{11}C]MNPA was found more sensitive than the antagonist radioligand [^{11}C]raclopride to amphetamine-induced changes in dopamine level. The improved sensitivity was ~1.8 fold and consistent across four doses. It was estimated that about 60% of the total pool of D_2/D_3 receptors was in the high affinity state.

The existence of two affinity states for D_2/D_3 receptors *in vivo* was further studied using the exogenous agonist apomorphine. D_2/D_3 receptors occupancy was found indistinguishable when measured with [^{11}C]MNPA and [^{11}C]raclopride. This study provided therefore no support for the existence of two affinity states for D_2/D_3 receptors and it was speculated that all receptors are in the high affinity state at *in vivo* conditions. The differences observed between the amphetamine and apomorphine study could possibly be related to dissimilarity in cellular concentration of agonist or by differences in agonist-induced receptor internalization.

To further understand the study of dopamine release with PET, the D_1/D_5 receptors partial agonist (*S*)-[^{11}C]*N*-methyl-NNC 01-0259 was developed. Evaluation in monkey indicated that the radioligand is insensitive to dopamine level and inferior to previous reported antagonist radioligands in respect to binding signal. COMT inhibition was developed as a method to allow for proper quantification of receptor binding.

Studies on serotonin release made use of the newly developed 5-HT_{1B} receptor antagonist radioligand [^{11}C]AZ10419369. It was demonstrated that the effective serotonin releasing agent fenfluramine caused a dose-dependent reduction in [^{11}C]AZ10419369 receptor binding. The effect of fenfluramine on [^{11}C]AZ10419369 receptor binding was further confirmed in a study with an equilibrium approach using a bolus infusion protocol. If confirmed in human subjects, the developed methodology can be used to study the treatment and pathophysiology of major psychiatric disorders.

7 FUTURE PERSPECTIVE AND CHALLENGES

PET assessment of neurotransmitter release

The study of neurotransmitter release with PET is rapidly gaining interest and benefits from recent improvements in PET methodology and available radioligands. Although the studies have been mainly limited to dopamine, an extension to other neurotransmitter systems is expected in the near future. Understanding of the molecular underpinning of neurotransmitter-induced changes in radioligand binding has proven challenging, and including biological and chemical aspects. The combination of microPET imaging with molecular-modified animals provides new pathways to evaluate underlying mechanisms. Finally, for the methodology to provide clinical utility, improvements in sensitivity are required to allow for investigation of dynamic neurotransmitter concentrations at more physiological relevant levels.

Based on this thesis some specific challenges can be discussed:

Two affinity states in vivo

The existence of two affinity states for the D_2/D_3 receptors has not been demonstrated *in vivo* consistently and additional studies are required. Recent development of radioligands targeting the 5-HT_{1A}, 5-HT_{2A} and opioid κ-receptors make it possible to extent comparison of agonist and antagonist radioligand binding to other neurotransmitter systems[67,166,209,316,350].

D_2/D_3 agonist radioligands as improved tools for measurement of dopamine release

PET studies in anaesthetized animals have consistently reported a more pronounced effect of dopamine concentration on D_2/D_3 agonist radioligand binding than on D_2/D_3 antagonist radioligand binding. Future studies are required to understand the mechanisms of enhanced dopamine level sensitivity of agonist radioligands. Finally, studies in human subjects are needed to confirm that agonist radioligands have increased sensitivity to dopamine levels and to elucidate the potential contribution of stress.

Improvements for agonist radioligands targeting D_2/D_3 receptors

Existing D_2/D_3 receptors agonist PET radioligands are not optimal yet. For further improvement, several aspects can be considered including: receptor selectivity, receptor affinity, receptor kinetics, non-specific binding, specific radioactivity and ease of radiolabelling.

Development of a full D_1 receptor agonist radioligand

As a catechol group is most often critical for D_1 receptor agonism, the developed COMT inhibition approach provides a more general method for full quantitative measurement with D_1/D_5 receptors agonist radioligands. A full D_1 agonist radioligand is not yet available and is required to further understand the lack of sensitivity of antagonist and partial agonist radioligands to changes in endogenous dopamine level. The so far radiolabeled D_1/D_5 receptors agonist and antagonist PET radioligands almost all originate from the 1-phenyl-3-benzazepines scaffold. A radiolabeled agonist originating from another scaffold may be of interest to rule out possible scaffold-related insensitivity to dopamine level.

Measurement of serotonin release using [^{11}C]AZ10419369 and PET

The results of study V and VI demonstrate that [^{11}C]AZ10419369 binding is sensitive to major changes in endogenous serotonin level. Challenges causing smaller changes in serotonin concentration are required to explore the sensitivity of the methodology. Finally, the findings need to be confirmed in control human subjects to explore the possibilities of prospective clinical studies.

Improvements for radioligands targeting 5HT$_{1B}$ receptors

The development of a 5-HT$_{1B}$ receptor agonist radioligand would be of interest, as it may have enhanced sensitivity to serotonin concentration. In addition, radioligands with higher affinity could allow for study of serotonin concentration in 5-HT$_{1B}$ receptor low regions, such as the hippocampus.

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