Methodological Advances in the Examination of the Dopamine System in Brain

by

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Stockholm 2005
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ABSTRACT

The dopamine (DA) system in brain has attracted considerable attention in neuroscience due to its involvement in fundamental brain functions and its postulated role in the pathophysiology of several neuropsychiatric disorders. Radioligand binding techniques in vitro and in vivo, such as receptor binding autoradiography and positron emission tomography (PET) have substantially contributed to our understanding of the DA system. With the aid of these methodologies the distribution, density and affinity of dopamine receptors can be examined in the brain at physiological conditions and in neuropsychiatric disorders. More recent aspects of research on DA receptors are the examination of coupling between DA receptors and G proteins, selective labelling of different DA receptor subtypes and in vivo examination of DA receptors in small regions of the human brain with minute receptor densities. Examination of the aforementioned aspects of the DA system necessitates the establishment of new techniques to study DA receptors. The aim of the present thesis was to apply and evaluate new in vitro and in vivo imaging methods for the examination of dopamine-D2/D3 receptors in the brain with special attention to the following three topics: (i) examination of the interaction between D2/D3 receptors and G proteins in human brain in vitro, (ii) selective visualization of D3 receptors with PET, and (iii) improvement of the accuracy in PET imaging of D2/D3 receptors by correction for partial volume effects (PVEs).

In the first study agonist stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding autoradiography was established for the examination of D2/D3 receptors in the postmortem human brain. The functional response to DA, the physiological agonist, and quinpirole, a prototype D2/D3 agonist was described in human whole hemisphere cryosections. The stimulatory effect of DA was primarily mediated by D2/D3 receptors. Both DA and quinpirole stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding to the highest level in the striatum. Moderate to low stimulation was observed in other brain regions, such as substantia nigra, thalamus, amygdala, hippocampus and anterior cingulate. The results indicate that this method could be a suitable tool for examination of coupling between D2/D3 receptors and G proteins in neuropsychiatric diseases.

The aim of the next two studies was to develop a radioligand for selective in vivo labelling of D3 receptors by PET. As currently no D3-selective radioligands are available for in vivo examinations, the binding of the putative D3-selective radioligand, \(^{11}\text{C}\)RGH-1756, was evaluated in the monkey brain. Despite the promising in vitro characteristics of the molecule, \(^{11}\text{C}\)RGH-1756 yielded very low signal for specific D3 binding in the monkey brain. Pretreatment experiments with unlabelled RGH-1756 and raclopride showed some, albeit low, saturable binding of the radioligand. It has previously been suggested that endogenous DA occupies D3 receptors to a high degree, which could prevent binding of \(^{11}\text{C}\)RGH-1756. To test this hypothesis the effect of reserpine induced DA depletion was examined on the binding of \(^{11}\text{C}\)RGH-1756 in the monkey brain. Following reserpine treatment there was no consistent increase in specific binding of \(^{11}\text{C}\)RGH-1756. This observation does not support the assumption that binding of \(^{11}\text{C}\)RGH-1756 to D3 receptors is inhibited by high occupancy of D3 receptors by endogenous DA. The most likely reason for low specific binding of \(^{11}\text{C}\)RGH-1756 is therefore the insufficient in vivo affinity of the radioligand.

In the final two studies the influence of PVE was estimated in PET studies using the D2/D3 selective radioligands, \(^{11}\text{C}\)FLB 457 and \(^{11}\text{C}\)raclopride in human subjects. Kinetic rate constants, binding potential (BP) and total volume of distribution (DV\text{tot}) were derived from the standard two-tissue compartment model before and after PVE correction. The results demonstrated that underestimation of regional radioactivity concentration and contamination of time activity curves by spill-in of radioactivity from neighbouring regions have substantial effects on quantitative PET measurements with \(^{11}\text{C}\)FLB 457 and \(^{11}\text{C}\)raclopride. PVE correction can therefore contribute to the accuracy of quantitative PET measurements both by compensating for loss of activity (spill-out) and influence from neighbouring regions (spill-in). Based on the results initial recommendations were formulated for the application of PVE correction particularly in clinical PET studies on disorders with structural brain abnormalities.
„Mondottam ember: küzdj’ és bízva bízzál!”
Madách Imre

„Så hör min maning: Kämpa och var tröst!”
Översättning: Olof Lundgren
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<th>Definition</th>
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<tr>
<td>2-TCM</td>
<td>Two-tissue compartment model</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>Receptor density</td>
</tr>
<tr>
<td>BP</td>
<td>Binding potential</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>$D_1$-$D_5$</td>
<td>Dopamine receptor subtypes 1-5</td>
</tr>
<tr>
<td>DFV</td>
<td>Distance from vertex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>$DV_{\text{tot}}$</td>
<td>Total volume of distribution</td>
</tr>
<tr>
<td>e$^-$</td>
<td>Electron</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GM</td>
<td>Grey matter</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>[$^{35}$S]GTP$\gamma$S</td>
<td>Guanosine 5'-$\gamma$-thiotriphosphate</td>
</tr>
<tr>
<td>ICC</td>
<td>Intra-class correlation coefficient</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>$K_1$</td>
<td>Rate constant for ligand transfer from plasma to tissue</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Rate constant for ligand transfer from tissue to plasma</td>
</tr>
<tr>
<td>$k_3$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$k_4$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Equilibrium dissociation constant from saturation binding</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Equilibrium dissociation constant from competition binding</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>p$^+$ or $\beta^+$</td>
<td>Positron</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PSF</td>
<td>Point spread function</td>
</tr>
<tr>
<td>PVE</td>
<td>Partial volume effect</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SA</td>
<td>Specific radioactivity</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SPM2</td>
<td>Statistical parametric mapping, 2</td>
</tr>
<tr>
<td>SRTM</td>
<td>Simplified reference tissue model</td>
</tr>
<tr>
<td>TAC</td>
<td>Time activity curve</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
</tbody>
</table>

Abbreviations used in the figures are explained in the corresponding figure legends.
1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Of the several billions of neurons in the human brain only a very small fraction (0.0003%-0.0004%) use dopamine (DA) as neurotransmitter (Alvaro et al. 2001; Sedvall & Farde, 1995). Despite the small number of dopaminergic neurons in the brain, huge efforts have been focused on research on the DA system during the past half of a century. Many characteristics of the DA system have already been described, but many other aspects of DA signalling must still be examined to clarify the role of this neurotransmitter system for maintenance of normal brain function.

DA was initially considered merely as an intermediate in the biosynthesis of adrenaline, until Arvid Carlsson and co-workers showed that DA itself served as a neurotransmitter (Carlsson et al. 1958). Extensive research since then has shown that DA mediates a number of different functions in the brain including locomotor control, positive reinforcement, cognitive functions, personality traits and endocrine regulation (reviewed in Missale et al. 1998; Vallone et al. 2000). Moreover, alterations of dopaminergic neurotransmission have been implicated in the pathophysiology of neuropsychiatric disorders, such as Parkinson disease, schizophrenia and drug abuse (for additional reviews see Heimer, 2003; Kaasinen & Rinne, 2002; Sedvall & Farde, 1995; Wong, 2002).

Progress in research on the DA system is dependent on the development and validation of new methodologies that enable us to examine new aspects of this neurotransmitter system. Two approaches that revolutionized neurobiological research are receptor binding and molecular biological techniques. Several milestones in research on the DA system can be attributed to the application of both of these techniques. As our knowledge about the importance of DA system grows newer and newer questions are raised regarding unexplored but potentially important features of DA signalling. Finding an answer to these questions necessitates the establishment of new approaches for the examination of the DA system.

The focus of the present thesis is on radioligand binding techniques for examination of DA receptors \textit{in vitro} and \textit{in vivo}. The goals were to establish new imaging methods and to improve the existing techniques for the examination of DA receptors.

1.2. THE DOPAMINE SYSTEM

1.2.1. Dopaminergic pathways in the human brain

DA is synthesized in a few circumscribed cell groups in the mesencephalon, the diencephalon and the telencephalon. The projections originating from these nuclei form four major pathways in the brain (Figure 1).

1. The nigrostriatal pathway originates from the substantia nigra (SN) and innervates the dorsal striatum (caudate and putamen). This pathway contains about 80% of all dopaminergic projections. The nigrostriatal pathway is involved in the regulation of movement, and its degeneration causes Parkinson disease.

2. The mesocortical pathway contains axons of dopaminergic cell bodies in the ventral tegmental area (VTA), which project to different neocortical areas (frontal and temporal cortex) and anterior cingulate. This pathway is probably implicated in some aspects of learning and memory.

3. The mesolimbic pathway, which also arises from the VTA, projects to several limbic areas of the brain, including nucleus accumbens, amygdala, anterior perforated substance, piriform and entorhinal cortices. The mesolimbic pathway was proposed to be
**1.2.2. Dopamine receptors: the discovery of multiple subtypes**

DA exerts its effect by binding to specific membrane bound receptors, which belong to the family of G protein coupled receptors (GPCR). Initially two distinct subtypes of DA receptors were distinguished: D$_1$ receptors, which increased cyclic AMP synthesis, and D$_2$ receptors, which were believed not to be associated with adenylyl cyclase (for an early review see Kebabian & Calne, 1979). Soon it was demonstrated that D$_2$ receptors were negatively coupled to adenylyl cyclase, and receptor stimulation reduced cAMP formation (Stoof & Kebabian, 1981). Based on pharmacological evidence the existence of several types of DA receptors was suggested as early as 1979 (Kebabian & Calne, 1979). This assumption was verified by molecular biological techniques about a decade later. The cDNA for the D$_3$ receptor subtype was cloned and expressed in different cell lines by Sokoloff and co-workers in 1990 (Sokoloff et al. 1990). Discovery of the D$_3$ receptor was soon followed by the identification of two other DA receptor subtypes, the D$_4$ and D$_5$ receptor (Sunahara et al. 1991; Van Tol et al. 1991).

Based on biochemical and pharmacological properties and on sequence homology the five DA receptor subtypes are classified as D$_1$-like (D$_1$ and D$_3$ receptors) and D$_2$-like (D$_2$, D$_3$ and D$_5$ receptors). The five DA receptor subtypes differ in their pharmacological and biochemical characteristics, as well as in their anatomical distribution in the human brain (reviewed in Hall, 1994; Missale et al. 1998). Each DA receptor subtype is assumed to be involved in the mediation of a different set of functions in brain, and to play a role in the pathophysiology of distinct neuropsychiatric disorders, which set the need for selective examination of the functional role of DA receptors.

The present thesis focuses on two of the five identified dopamine receptor subtypes: the D$_2$ and the D$_3$ receptors.
1.2.3. Brain distribution of dopamine-D$_2$/D$_3$ receptors

1.2.3.1. Methodological considerations

Several studies, using in vitro or in vivo receptor ligand binding techniques have been performed to describe the regional distribution of D$_2$ and that of D$_3$ receptors in the brain. Selective labelling of D$_2$ and that of D$_3$ receptors is, however, hampered by the lack of selective radioligands.

Most in vitro and in vivo studies, aiming at describing the distribution and density of D$_2$ receptors, have utilized radioligands, which had about equal affinity for D$_2$ and D$_3$ receptors (e.g. $^3$H or $^{11}$C labelled raclopride, $[^{11}]$FLB 457, $[^{125}]$lpidepride). In addition, all radioligands used for visualization of D$_3$ receptors also have some affinity for D$_2$ receptors (e.g. $[^3]$H7-OH-DPAT, $[^3]$HPD 128907). A possible solution for selective in vitro labelling of D$_3$ receptors is co-incubation of the tissue samples with a radioligand for D$_2$/D$_3$ receptors and a non-labelled ligand, which has higher affinity for D$_2$ than for D$_3$ receptors. Thereby the cold ligand blocks radioligand binding to D$_2$ receptors. Reciprocal approach can also be applied for selective visualization of D$_2$ receptors (Table 1). The issue of D$_2$ and D$_3$ selective radioligands is further discussed in detail below (see page 14).

In vitro receptor-binding studies can be complemented with in situ hybridisation experiments for the detection of mRNA. A limitation of that method is that only the somata of neurons are visualized. Another limitation is that it does not show whether the transcript is translated into the encoded protein. Therefore, mRNA mapping may show a mismatch with results of radioligand binding studies to the receptor protein.

1.2.3.2. Striatum

Within the brain the highest concentration of dopamine-D$_2$/D$_3$ receptors is expressed in the striatum. The density of D$_2$ receptors is similar in caudate and putamen, and the receptors are distributed evenly through the dorsoventral extent of the striatum (Gurevich & Joyce, 1999; Murray et al. 1994; Piggott et al. 1999). D$_2$ receptor mRNA is expressed at evenly high levels throughout the entire striatum by medium spiny neurons and large, presumably cholinergic interneurons (Gurevich & Joyce, 1999).

D$_3$ receptors are more abundant in the nucleus accumbens and in the ventral parts of caudate and putamen than in the dorsal part of the striatum. D$_3$ receptors are concentrated in the acetylcholinesterase-poor striosomal compartment within the striatum (Gurevich & Joyce, 1999; Murray et al. 1994; Piggott et al. 1999). D$_3$ mRNA is expressed predominantly in medium spiny neurons.

1.2.3.3. Thalamus

There are over twenty major nuclei in the thalamus. The nuclei differ in their histological, functional and biochemical characteristics, including density of D$_2$/D$_3$ receptors.

D$_2$ receptors are expressed at the highest density in the envelope of the thalamus, i.e. in parafascicular, parataenial, paracentral, centrolateral and centromedian nuclei (Gurevich & Joyce, 1999; Hall et al. 1996a; Rieck et al. 2004). Somewhat lower densities of D$_2$ receptors are expressed in the principle anterior and in the mediodorsal nuclei (Hall et al. 1996a; Kessler et al. 1993; Rieck et al. 2004). The density of D$_2$ receptors in the pulvinar is somewhat lower than D$_2$ density in the mediodorsal nucleus (Hall et al. 1996a; Kessler et al. 1993). The ventral nuclei have low-to-moderate densities of D$_2$ receptors (Gurevich & Joyce, 1999; Rieck et al. 2004). The expression of D$_2$ mRNA corresponds to that of the D$_2$ receptor protein (Gurevich & Joyce, 1999).

D$_3$ receptors are also expressed in the thalamus, and their distribution pattern is different from that of the D$_2$ receptors. D$_3$ receptors are expressed in highest density in the ante-
Methodological advances in the examination of the dopamine system in brain

Moderate densities of D$_3$ receptors are found in the mediodorsal and the ventral posterior nuclei, whereas no D$_3$ receptor binding has been detected in the centromedian and centrolateral nuclei. Localization of D$_3$ mRNA expressing cells in the thalamus has matched that of the receptor sites. Double labelling for D$_2$ and D$_3$ mRNA has revealed the presence of neurons that expressed both mRNA species throughout the thalamus (Gurevich & Joyce, 1999).

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Dopamine-D$_2$ receptors</th>
<th>Dopamine-D$_3$ receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$[^{125}\text{I}]$Epidepride$^A$</td>
<td>$[^{125}\text{I}]$Epidepride$^B$</td>
</tr>
<tr>
<td>Blocker</td>
<td>$B_{\text{max}}$ (pmol/g tissue)</td>
<td>$B_{\text{max}}$ (pmol/g protein)</td>
</tr>
<tr>
<td>Putamen</td>
<td>16.6</td>
<td>88-101</td>
</tr>
<tr>
<td>Caudate</td>
<td>16.5</td>
<td>117-155</td>
</tr>
<tr>
<td>Nucl. accumbens</td>
<td>7.2</td>
<td>79-101</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.9</td>
<td>5-39</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Ant. cingulate</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>VTA</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>Islands of Calleja</td>
<td>-</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 1. Density of dopamine-D$_2$/D$_3$ receptors measured by in vitro radioligand binding studies in selected regions of the human brain. Experimental data from: A (Kessler et al. 1993); B (Murray et al. 1994); C (Lahti et al. 1995); - Not examined.

1.2.3.4. Cortex

The density of D$_2$ receptors in the cerebral cortex is generally low and only about 0.2-1.5% of the D$_2$ receptor density in the striatum. D$_2$ receptors are expressed throughout the neocortex (Camus et al. 1986; Hall et al. 1996a; Kessler et al. 1993; Olsson et al. 2004), with highest density in the temporal cortex (Kessler et al. 1993; Olsson et al. 2004).

D$_3$ receptors have not been detected in the neocortex (Hall et al. 1996b; Lahti et al. 1995).

1.2.3.5. Cerebellum

In the cerebellum minute densities of D$_2$ receptors have been reported in several studies (Camps et al. 1989; De Keyser et al. 1988; Hall et al. 1988, 1994, 1996a). In accordance with the expression of D$_2$ receptor protein, in situ hybridisation has revealed very low level of D$_2$ mRNA expression in the cerebellum (Hurd et al. 2001).

Observations regarding the expression of D$_3$ receptors in the cerebellum have mostly been made in species other than human. Hall and co-workers detected no binding of $[^3\text{H}]$PD 128907 to human cerebellar cortex, whereas $[^3\text{H}]$7-OH-DPAT showed low binding (Hall et al. 1994).
Moderate density of D₃ receptors has been measured in lobules 9-10 in the cerebellum of the rat brain using in vitro autoradiography (Bancroft et al. 1998; Levant, 1998; Levesque et al. 1992; Stanwood et al. 2000a). In other mammalian species, the density of D₃ receptors is much lower in lobule 9-10 of the cerebellum as compared to the rat (Levant, 1998). The role of D₃ receptors in cerebellum is controversial since this region does not receive dopaminergic innervation. D₃ receptors are presumably involved in volume transmission, and may respond to DA diffusing extrasynaptically (Diaz et al. 1995).

### Other brain regions

Moderate to low level D₂ receptor expression has been observed in several other brain regions, including amygdala, hippocampus, hypothalamus, VTA, SN and globus pallidus. Within the amygdala the highest density of D₂ receptors is expressed in the basolateral and basomedial nuclei (Hall et al. 1996a; Kessler et al. 1993; Murray et al. 1994). D₂ receptors are expressed in the pars compacta of the SN and absent from the pars reticulata, whereas D₂ mRNA expression has been detected in both parts (Gurevich & Joyce, 1999). D₂ receptors in VTA and SN function as autoreceptors.

D₃ receptors are expressed in highest density in the basal forebrain, i.e. in islands of Calleja, septal nucleus and nucleus basalis (Gurevich & Joyce, 1999; Murray et al. 1994). D₃ receptors are also present in the amygdala, but their distribution pattern is different from that of the D₂ receptors, as the central nucleus and the amygdalostriatal transition zone contain more D₃ receptors than do the basal nuclei.

### Signal transduction of D₂/D₃ receptors

#### D₂/D₃ receptors belong to the family of G protein coupled receptors

G protein coupled receptors (GPCR) have been named on the basis of their ability to recruit and regulate the activity of heterotrimeric G proteins. However, GPCR may not solely act via G proteins. A common structural feature of all GPCRs is the presence of seven transmembrane α-helical domains connected by alternating intracellular and extracellular loops (reviewed in Baldwin, 1993; Gether, 2000). The transmembrane helices form a crevice, which is the binding site for the physiological agonist. Small-molecule transmitters, like DA bind deep within the binding crevice, which results in activation of the receptor. The activation signal is thereafter conveyed from the GPCR to the G protein. Studies with D₂/D₃ receptor chimeras have verified the central role of the second and third intracellular loops of the receptors in the coupling to G proteins (reviewed in Baldwin, 1993; Robinson & Caron, 1996).

#### Interaction of D₂/D₃ receptors with G proteins

##### The structure and function of G proteins

The name ‘G protein’ refers to the ability of these molecules to bind guanine nucleotides, such as guanosine-triphosphate (GTP) and guanosine-diphosphate (GDP). Their task is to couple GPCRs to intracellular effector systems.

A heterotrimeric G protein is composed of three different polypeptide chains termed α, β and γ subunits. The α subunit binds guanine nucleotides, but all subunits can participate in signal transduction (Logothetis et al. 1987). Four major classes of G proteins are distinguished upon the α subunit: Goₛ, Goₛ₁₅₁, Goₐ and Go₁₂ (reviewed in Neer, 1995; Pennington, 1995). In the inactive state GDP is bound to the α subunit. Upon activation by the receptor the conformation of the α subunit is changed, which in turn leads to the release of GDP followed by binding of GTP. Subsequently the α subunit dissociates from the βγ-dimer.
and the receptor. G proteins stay activated and can influence second messenger systems until the GTP is hydrolysed by the α subunit (Gether, 2000; Hamm, 1998, Figure 2).

![Figure 2. Schematic drawing of GPCR activation. A. Agonist (DA) binds to the uncoupled, low-affinity state of the receptor (R), and in turn promotes and stabilises the formation of the high-affinity state, the ternary complex (B, with lightly shaded receptor). C. Activation of the receptor in turn results in activation of the G protein (shown here as α and βγ subunits), which releases GDP and binds GTP instead. D. The activated G protein dissociates from the complex and interacts with effectors. The G protein is ‘turned off’ by hydrolysis of GTP.](image)

1.2.4.2.2. Models of receptor activation

Activation of GPCRs was first explained by the ternary complex model proposed by DeLean and co-workers 25 years ago (De Lean et al. 1980). The model defines the active, high-affinity form of the receptor with a ternary complex involving the agonist, the receptor and the G protein. It also postulates that agonists are able to promote and stabilize the formation of the high-affinity state of the receptor. The reversal of the high-affinity complex to the low-affinity form involves dissociation of the G protein from the ternary complex (De Lean et al. 1980). The freed, active G protein can thereafter interact with effectors (Figure 2, 3).

The discovery of mutant GPCRs with constitutive activity lead to the extension of the model and formulation of the allosteric ternary complex model, also called two-state model (Lefkowitz et al. 1993). According to this extended model, the receptor exists in equilibrium between two states: inactive and active conformation and only the latter can interact with G
proteins. Thus, the process of receptor activation comprises at least two steps: (i) conversions from inactive to active form, and (ii) binding of G protein (Figure 3). The model proposed that ligand binding influences both steps. This model can explain the complex behaviour of various classes of ligands (agonists, partial agonists, inverse agonists, antagonists; Table 2).

It is becoming increasingly clear that the two-state model cannot sufficiently explain the complex behaviour of GPCRs. Experiments on the kinetics of receptor activation have provided strong support for the existence of multiple conformational states of GPCRs (reviewed in Gether, 2000). The ‘sequential binding and conformational selection model’ proposed that the receptor spontaneously alternates between different conformations (active and inactive). Binding of agonist does not occur directly to the active form but is suggested to occur sequentially, resulting in a series of conformational states that are intermediates between the active and the inactive conformations (Gether, 2000). This hypothesis, however, awaits experimental evaluation. The presented models are compared in Figure 3.

<table>
<thead>
<tr>
<th>Ligand class</th>
<th>Mechanism of action</th>
<th>Intrinsic ligand efficacy</th>
<th>Example for D₂/D₃ receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full agonist</td>
<td>Binds with highest affinity to R*, thereby shifts the equilibrium from R to R*.</td>
<td>E ≥100%</td>
<td>DA</td>
</tr>
<tr>
<td></td>
<td>Ternary complex has maximal activity.</td>
<td></td>
<td>NPA Quinpirole</td>
</tr>
<tr>
<td>Partial agonist</td>
<td>Binds with slightly higher affinity to R*, than to R, thereby shifts the equilibrium from R to R*.</td>
<td>Basal ≤ E &lt; 100%</td>
<td>Apomorphine Bromocriptine Ropinirole 7-OH-DPAT PD 128907</td>
</tr>
<tr>
<td></td>
<td>Ternary complex has submaximal activity.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral antagonist</td>
<td>Binds with equal affinity to R and R*, thereby causes no change in the equilibrium.</td>
<td>E ≈ Basal</td>
<td>Raclopride Haloperidol Clozapine Chlorpromazine Spiperol, etc.</td>
</tr>
<tr>
<td>Inverse agonist</td>
<td>Stabilizes the inactive R state over R*, thereby shifts the equilibrium from R* to R.</td>
<td>E &lt; Basal</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Classification of DA receptor ligands according to their efficacy to activate/inhibit intracellular effector systems. Efficacy was measured either by stimulations of [³⁵S]GTPγS binding or by inhibition of forskolin stimulated cAMP accumulation. 100% efficacy is defined as the efficacy of DA. (Based on refs. Gether, 2000; Hall & Strange, 1997; Malmberg et al. 1998; Strange, 1999). Abbreviations: R, inactive receptor conformation; R*, active conformation.

1.2.4.2.3. Interaction of D₂/D₃ receptors with specific G proteins

In vitro studies using mutated or chimeric G proteins or knockout of individual α subunits with antibodies or antisense oligonucleotides showed that D₂ receptors are capable of coupling to multiple G proteins including Gα₁₆, Gα₁₂, Gα₃ (for refs. see Robinson & Caron, 1996).
The earliest observation suggested that D₃ receptors are not coupled to G proteins (Levesque et al. 1992; Sokoloff et al. 1990). Later on it was, however, verified that D₃ receptors can interact with G proteins, and that they influence second messenger systems (Chio et al. 1994; Griffon et al. 1997; Vanhauwe et al. 1999, 2000). Most observations agreed that D₃ receptors activate G proteins less effectively than D₂ receptors (Chio et al. 1994; Newman-Tancredi et al. 1999; Vanhauwe et al. 1999). D₃ receptors are capable of coupling to Go₆ and Go₇ proteins, but in contrast to D₂ receptors, they can also interact with Go₉/11 (Newman-Tancredi et al. 1999).

1.2.4.3. Intracellular signalling pathways of D₂/D₃ receptors

The D₂ receptor was initially identified as the DA receptor subtype that is not coupled to adenylly cyclase (Kebabian & Calne, 1979). Shortly afterwards it was shown that D₂ receptors in fact inhibit adenylly cyclase (Stoof & Kebabian, 1981). Both D₂ and D₃ receptors can decrease intracellular cAMP level (Vanhauwe et al. 1999). In addition D₂ receptors can influence a number of other second messengers through G proteins and also via G protein independent pathways. Intracellular signalling pathways modulated by D₂ receptors are summarized in Figure 4 (reviewed in Missale et al. 1998; Robinson & Caron, 1996; Vallone et al. 2000).

Signal transductions pathways of the D₃ receptor are not described as well as those of the D₂ receptor. In some cell lines, D₃ receptor was able to inhibit Ca²⁺ currents (Seabrook et al. 1994). In CHO cells D₃ receptor was able to stimulate extracellular acidification by the Na⁺/H⁺ exchanger (Coldwell et al. 1999a, 1999b).

Most of the experiments that examined functional coupling of D₂/D₃ receptors were performed using cloned receptors expressed in various cell lines. The drawback of such studies is that cultured cells may not express the same G proteins or second messengers that are

![Figure 3. Different models describing the activation process of GPCRs.](image-url)

A. Classic form of the ternary complex model, where the high-affinity state of the receptor corresponds to the complex of agonist (A), receptor (R) and G protein (G). B. Allosteric ternary complex model, which introduces an isomerization step (R*, active conformation) into the process of formation of the high-affinity state. C. Sequential binding and conformational selection model, in which binding of an agonist occurs sequentially resulting in a number of conformational states of the receptor (R', R'') that are intermediates between R and R*. The G protein may substantially affect the kinetics of the transition from AR' over AR'' to AR*. (Based on refs. De Lean et al. 1980; Gether, 2000; Lefkowitz et al. 1993).
found in neurons expressing D2/D3 receptors. It also might happen that the interaction of D2/D3 receptors with a certain second messenger observed in a cell line is not representative for brain D2/D3 receptors, as it does not normally occur in neurons. The result of experiments with cell cultures should therefore be interpreted with appropriate caution.

1.2.5. Functions of brain D2/D3 receptors

DA receptors mediate a number of different functions in brain, and are also involved in the pathophysiology of several neuropsychiatric disorders. The role of DA receptors under normal conditions and in neurological and psychiatric disorders has been extensively reviewed (e.g. Kaasinen & Rinne, 2002; Missale et al. 1998; Vallone et al. 2000; Wong, 2002). Without aiming for a complete overview of the overwhelmingly huge literature on the function of DA receptors in brain, only a few aspects of DA function relevant for the present work will be mentioned.

The importance of the DA system in controlling locomotion is well known. Degeneration of dopaminergic neurons in the SN causes Parkinson disease, a condition characterised by rigidity, tremor and severe disturbance of voluntary movements and that of coordination. DA is also assumed to mediate a number of higher cognitive functions and is involved in the pathophysiology of schizophrenia, a severe disabling psychiatric disorder with symptoms such as hallucinations, delusions and emotional blunting. In the following paragraphs, some DA related aspects of these two disorders will be discussed, with special emphasis on pieces of knowledge obtained from neuroimaging studies. The major results of in vitro and in vivo DA receptor binding studies are mentioned. We also point out some questions regarding the distribution and function of DA receptors in the brain, which cannot be examined with traditional neuroimaging methods but require instead the application of novel techniques.

The DA hypothesis of schizophrenia postulates that schizophrenia is related to hyperdopaminergic state in brain. Initial PET studies have shown that the density of D2/D3 receptors was indeed increased in the striatum of schizophrenic patients (Wong et al. 1986). Other studies, however, did not verify this observation (Farde et al. 1990). Yet other PET studies showed alterations of pre-synaptic DA function in schizophrenia (Hietala et al. 1995; Laruelle et al. 1996). In vitro studies, on the other hand, have demonstrated changes of intracellular signalling systems in schizophrenia, without explicitly connecting the observations to any neurotransmitter receptor (Jope et al. 1998; Okada et al. 1994; Yang et al. 1998). It is possible that the conflicting results of studies examining D2/D3 receptor density in the brain have been obtained because the function and not the number of receptors is changed in schizophrenia. Traditional radioligand binding techniques, however, cannot be used to examine the function of DA receptors in brain.

Experiments in animal models of Parkinson disease have demonstrated increased numbers of D2 receptors and enhanced D2 receptor/G protein coupling (Geurts et al. 1999; Khan et al. 1999; Newman-Tancredi et al. 2001). Some authors suggested that the augmented DA receptor/G protein coupling could contribute to the development of dopaminergic hypersensitivity, and proposed that similar regulatory mechanisms could take place in pathophysiological conditions (Geurts et al. 1999). To challenge these hypotheses new imaging techniques are necessary, which can be used to localize receptors and measure their function at the same time.

Shortly after the discovery the D3 receptor, the role of this receptor subtype was suggested in the pathophysiology of schizophrenia, due to the abundance of D3 receptors in limbic brain regions. This hypothesis was confirmed by studies that examined the association between schizophrenia and polymorphism of the D3 receptor (Schwartz et al. 2000). The the-
Methodological advances in the examination of the dopamine system in brain

D₃ receptors are probably also involved in the pathophysiology of Parkinson disease (Joyce et al. 2002; Ryoo et al. 1998). This assumption was based on the observation that DA agonists used to treat symptoms of Parkinson disease also have high affinity for D₃ receptors (Joyce et al. 2002). According to the literature, the density of D₃ receptors was also changed in Parkinson disease (reviewed in Joyce, 2001). Examination of D₃ receptors in the human brain was, however, hampered by the lack of selective radioligands with high-affinity for the D₃ receptor.

Postmortem studies described neuronal changes in the mediodorsal nucleus of the thalamus in schizophrenic brains, and suggested that the observed alterations could play a role in the pathophysiology of the disease (Byne et al. 2002; Pakkenberg, 1990, 1992). A PET study in schizophrenic patients reported decreased D₂/D₃ receptor density in the medial thalamus (Talvik et al. 2003). In this study, the mediodorsal nucleus was not examined separately, due to the difficulties arising from the limited spatial resolution of the PET system. Examination of D₂/D₃ receptors in small regions, such as the SN, is also important in disorders such as Parkinson disease. Nevertheless, accurate measurement of D₂/D₃ receptor density in such small structures as the mediodorsal nucleus or the SN necessitates the application of special image analysis techniques, which can compensate for the inaccuracies caused by the low spatial resolution of PET.

**Figure 4.** Intracellular signalling pathways activated by D₂ receptors. D₂ receptors are able to (1) stimulate the Na⁺/H⁺ exchanger, (2) inhibit adenylyl cyclase (AC), (3) potentiate release of arachidonic acid (AA), (4) in some cell lines inhibit, in others stimulate cell growth and differentiation, (5) inhibit Ca²⁺ currents, (6) stimulate phospholipase-C, and thereby increase the production of inositol 1,4,5-trisphosphate (IP₃) from phosphatidylinositol-diphosphate (PIP₂) and subsequently elevate the level of cytoplasmatic Ca²⁺, (7) in some cell lines inhibit, in others stimulate K⁺ currents. Signs beside the arrows indicate the effect of D₂ receptor on the second messenger: +, stimulation; –, inhibition; +/-, stimulation or inhibition depending on the cell line examined.
1.3. IMAGING TECHNIQUES FOR THE EXAMINATION OF D<sub>2</sub>/D<sub>3</sub> RECEPTORS IN BRAIN

1.3.1. Why is the development of new imaging techniques necessary?

*In vitro* and *in vivo* neuroreceptor binding techniques, i.e. autoradiography and PET have substantially contributed to our understanding of the DA system. The history of both methodologies goes back to the mid 1970s (Seeman et al. 1975; Ter-Pogossian et al. 1975). The first experiments using autoradiography to examine DA receptors in the brain were performed by Seeman and colleagues in 1975. The first PET system that used the tomographic principle for image reconstruction was reported in the same year by Ter-Pogossian and co-workers. Both methods have been extensively used to examine the distribution and density of D<sub>2</sub>/D<sub>3</sub> receptors in the human brain under normal conditions and in neuropsychiatric diseases. Traditional radioligand binding autoradiography and PET imaging techniques using D<sub>2</sub>/D<sub>3</sub> selective radioligands, however, cannot answer all the questions that are still pending with regard to the distribution and function of D<sub>2</sub>/D<sub>3</sub> receptors in the brain. In the present work three such questions of D<sub>2</sub>/D<sub>3</sub> receptor imaging are addressed.

1. Some recent discoveries directed the attention on the first step of the intracellular signal transduction pathway activated by D<sub>2</sub>/D<sub>3</sub> receptors, i.e. on the interaction of the receptors and G proteins. It has been demonstrated that D<sub>2</sub> and D<sub>3</sub> receptors activate G proteins with different efficiency in cell lines. The observation that both traditional and atypical antipsychotic drugs behave as inverse agonists at D<sub>2</sub>/D<sub>3</sub> receptors further emphasized the importance of the interaction between receptors and G proteins. Traditional radioligand binding autoradiography and PET, however, provide no information about the function of the visualized receptors. A recently developed technique, agonist stimulated [<sup>35</sup>S]GTPγS binding autoradiography (Sim et al. 1995), in contrast, can be used to localise GPCR in the brain and examine their interactions with G proteins.

2. The presumed different physiological role of D<sub>2</sub> and D<sub>3</sub> receptors, together with their suggested distinct role in the pathophysiology of schizophrenia and Parkinson’s disease gave strong support for attempts to selectively visualize D<sub>2</sub> and D<sub>3</sub> receptors. *In vitro* studies using receptor binding autoradiography with D<sub>3</sub>-preferring radioligands have been performed already for the examination of D<sub>3</sub> receptors (e.g. Bancroft et al. 1998; Hall et al. 1996b; Murray et al. 1994; Ricci et al. 1995). However, no successful *in vivo* visualization of the D<sub>3</sub> receptor has been carried out yet, due to the lack of selective radioligands with high-affinity for the D<sub>3</sub> receptor.

3. PET with D<sub>2</sub>/D<sub>3</sub> selective radioligands has been used extensively to examine density of D<sub>2</sub>/D<sub>3</sub> receptors in rather large regions of the human brain, such as cortical areas, striatum and thalamus. *In vitro* studies, in contrast, pointed out the importance of examination of small regions in the brain, such as thalamic nuclei or functional subdivisions of the striatum. The accuracy of PET measurements in small brain regions is compromised by the limited spatial resolution of the PET system. Special image analysis techniques are necessary for quantification of D<sub>2</sub>/D<sub>3</sub> receptor binding in these regions, which can compensate for the phenomena arising from the limited spatial resolution of PET.

1.3.2. [<sup>35</sup>S]GTPγS binding autoradiography

1.3.2.1. General description of the method

[<sup>35</sup>S]GTPγS binding autoradiography is a novel *in vitro* imaging method to study the distribution and function of GPCRs in tissue sections. It unifies the advantages of receptor binding autoradiography and that of [<sup>35</sup>S]GTPγS binding by providing information about the
distribution of receptors and the interaction of receptors and G proteins at the same time (reviewed in Sovago et al. 2001). $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding has initially been used to study the activation of GPCRs in cell membranes (Lazareno, 1999). Agonist stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ was modified so that it can also be applied to tissue sections (Sim et al. 1995).

The assay is based on guanine nucleotide exchange at G proteins upon agonist stimulation of the GPCR (Figure 2). Binding of an agonist to the receptor promotes the formation of the high-affinity, G protein coupled state of the receptor. Activation of the receptor induces activation of the G protein. The activated G protein releases GDP and binds $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ instead. As $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ is resistant to hydrolysis the radioligand stays bound to the G protein, and the activated receptor/G protein complex can be localized. One receptor can activate several surrounding G proteins, depending on the efficiency of the ternary complex.

The limitation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding autoradiography is that not all GPCRs can be visualized. Presently only $G_{i/o}$ coupled receptors can be detected with this technique. Labelling of receptors with small density or low G protein coupling efficiency can also be difficult.

1.3.2. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding autoradiography for the examination of $D_2/D_3$ receptors in brain

$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding autoradiography has previously been successfully applied in the examination of $D_2/D_3$ receptors in the rat brain (Culm et al. 2003; Febo et al. 2003; He et al. 2000; Newman-Tancredi et al. 2001). No studies, other than the one reported in the present thesis, have been performed to examine DA receptor stimulated G protein activation in human brain sections, although this assay could provide valuable information on DA receptor function under physiological conditions and in neuropsychiatric diseases.

1.3.3. Positron emission tomography

1.3.3.1. Basic principles

Positron emission tomography (PET) is a non-invasive technique to study the distribution of tracer molecules labelled with positron emitting isotopes in the body. The data obtained by detection of tracer molecules is used to reconstruct images describing the distribution of the tracer in the body (Eriksson et al. 1989).

![Figure 5. Physical principles of PET. The emitted positron ($p^+$ or $\beta^+$) travels a short distance before annihilating with an electron ($e^-$). In the annihilation process two $\gamma$-photons (511 keV each) are formed, which propagate in nearly opposite directions. These two $\gamma$-photons can be detected with a pair of opposed $\gamma$-ray detectors. To ensure that the detected $\gamma$-rays originate from the same annihilation only those signals are accepted which are detected within a short (~10 nsec) timing window. This technique is called coincidence detection.](image-url)
At the time of the radioactive decay, the emitted positron ($\beta^+$ or $p^+$) has relatively high energy so it travels through the body, interacts with electrons ($e^-$) along the way, and loses energy with each interaction. When its momentum is nearly zero, it annihilates with an $e^-$ to produce two $\gamma$-photons (511 keV), which propagate along almost collinear paths (Figure 5). The degree of non-collinearity depends on the momentum of the $\beta^+$ and $e^-$ at the time of the annihilation. The divergence of the angle of the $\gamma$-rays from 180° is in the order of 1° or less, and is usually ignored. The distance the $\beta^+$ travels before annihilation is termed positron range. The magnitude of this range depends on the $\beta^+$ energy, which varies widely among isotopes, and on the tissue where the annihilation occurred (Ollinger & Fessler, 1997). The degree of non-collinearity and the positron range set the lower limit to the spatial resolution of PET systems to about 2 mm.

Two crucial characteristics of positron cameras are sensitivity and spatial resolution, which largely determine accuracy of the image data (Hoffman et al. 1986). Spatial resolution of a PET system is defined by the degree to which the representation of an object is blurred in the image (reviewed in Eberl & Zimmermann, 2004). The representation of a perfect point source in a PET image (the point spread function, PSF) can be approximated by a Gaussian function. The resolution of the system is commonly defined in terms of its full width at half maximum (FWHM), the distance where the intensity in the image drops to half of the maximal value (Figure 6). The best obtainable resolution of a PET system is called intrinsic resolution (Ollinger & Fessler, 1997). It is dependent on non-collinearity of the $\gamma$-photons, positron range and size of the detectors (Eriksson et al. 1989; Links & Wagner, 1982; Phelps et al. 1975). This resolution is rarely achieved in practice. The final resolution of the image is called reconstructed resolution (Ollinger & Fessler, 1997).

PET images are most commonly reconstructed using filtered backprojection, a method for tomographic image reconstruction (reviewed in Bailey & Parker, 2004; Eriksson et al. 1989; Ollinger & Fessler, 1997). In PET, the projection data measured by the detectors represent the line integrals through the radio-isotope distribution. By the mathematical process of backprojection the line-projection data are re-projected onto a 2-dimensional image matrix. If no filtering is used the reconstructed images are blurred; the phenomenon is sometimes referred to as 1/r smearing (Eriksson et al. 1989). The blurring can be removed by a filtering step before backprojection, using a ramp filter. This filter, however, accentuates noise in the data. This can be limited by the use of a noise reduction filter, such as Butterworth, Hamming or Hann filters. Thereby the reduction in noise is traded-off for some decrease in the image resolution (Bailey & Parker, 2004; Ollinger & Fessler, 1997).

1.3.3.2. Partial volume effect

The limited spatial resolution of PET systems influences quantitative PET measurements (Hoffman et al. 1979; Kessler et al. 1984; Mintun et al. 1984). The direct consequence of limited resolution is underestimation of signal from objects smaller than 2-3 times the FWHM of the PET scanner (Hoffman et al. 1979; Kessler et al. 1984). A related effect of the rather low spatial resolution of PET devices implies that activity from surrounding tissue compartments contribute to the signal measured in a volume element, i.e. activity in a region.
is contaminated by activity from neighbouring regions (spill-over effect). These phenomena are commonly known as partial volume effects (PVEs; Henze et al. 1983; Herrero et al. 1989; Kessler et al. 1984; Figure 7). PVE can obscure accurate quantification of radioligand uptake in the brain, as the observed regional time-activity curves (TACs) are influenced by TACs for surrounding tissue components. Due to the complexity of models used to quantify radioligand binding, it is not possible in the individual case to predict the effect of PVE on quantitative receptor ligand binding parameters, and PVE correction has to be performed. Although several different algorithms were developed, validated and tested for PVE correction during the past fifteen years (Aston et al. 2002; Labbe et al. 1996; Meltzer et al. 1990; Muller-Gartner et al. 1992; Rousset et al. 1998a) very few studies have been performed so far to explore the effect of PVE on quantitative receptor ligand binding studies. A possible reason for the small number of such studies is that the implementation of these methods was complicated and the computational costs were high. Recently, however a number of PVE correction methods were implemented in a common software framework (Quarantelli et al. 2004), which enables relatively easy and fast application of PVE correction.

![Illustration of image blurring and the resulting PVEs](image.png)

**Figure 7.** Illustration of image blurring and the resulting PVEs. All circles originally have a diameter of 12 mm and homogenous intensity (blackness) within the entire circle. The circles are not overlapping. The second and third pairs of circles have been filtered with a Gaussian kernel (2 mm and 4 mm FWHM) for demonstration of blurring, virtual loss of intensity within the circles and spill-over. The dashed circles show the original size of the filled circles (12 mm diameter).

The picture was not made from a PET image, but it illustrates the inherent blurring of PET images.

### 1.3.3.3. Selective visualization of $D_2/D_3$ receptors

PET using $^{11}$C or $^{18}$F labelled radioligands is a suitable tool for the examination of neurotransmitter receptors and transporters in the brain. The development of PET radioligands is a challenging task, as the molecules have to satisfy a number of requirements regarding toxicity, lipophilicity, penetration of the blood-brain barrier, affinity and selectivity (reviewed in Halldin et al. 2001). At present there are several different radioligands available for $D_1$ and $D_2$-like receptors.

All currently known $D_2$-selective radioligands possess some affinity to $D_3$ receptors, and vice versa. Therefore, they cannot completely discriminate between $D_2$ and $D_3$ receptors. Based on their relative affinity for $D_2$ and $D_3$ receptors $D_2/D_3$-selective radioligands can be classified as $D_2$-preferring (higher affinity for $D_2$ than for $D_3$), $D_3$-preferring (higher affinity for $D_3$ than for $D_2$) or non-selective (about equal affinity for both subtypes; Table 3).

Selective labelling of $D_3$ receptors is challenged by some properties of the $D_1$ receptor itself. According to previous observations, $D_3$ receptors are expressed in a limited number of
regions in the brain, and their distribution is partially overlapping with that of the more abundant D₂ receptor, e.g. in ventral striatum, hippocampus, SN and thalamus (see details above). In these regions, signal from D₂ receptors can, therefore, obscure or confound the signal originating from D₃ receptors, unless a completely D₃-selective radioligand is used. Some authors have suggested that D₃ receptors are occupied by endogenous DA to a high degree under physiological conditions (Levant, 1995; Schotte et al. 1992, 1996). Therefore, competition with endogenous DA could prevent binding of radioligands to D₃ receptors, especially in in vivo studies.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>DA receptor</th>
<th>Tissue/ cell line</th>
<th>Affinity (nM)</th>
<th>D₂/D₃ selectivity index</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiperone</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>0.1⁵</td>
<td>0.1</td>
<td>(Vanhauwe et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>1.0³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>0.09⁷</td>
<td>0.4</td>
<td>(Seeman et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CCL1-3-hD₃</td>
<td>0.25⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBZM</td>
<td>D₂</td>
<td>LiK-hD₂</td>
<td>1.6³</td>
<td>0.7</td>
<td>(Videbaek et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>HEK-293-hD₃</td>
<td>2.2³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raclopride</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>7.5⁵</td>
<td>1</td>
<td>(Vanhauwe et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>8.5³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLB 457</td>
<td>D₂</td>
<td>rat striatum</td>
<td>0.02⁸</td>
<td>1</td>
<td>(Halldin et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>0.02⁸</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>22³</td>
<td>5.6</td>
<td>(Sokoloff et al. 1992)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>3.9³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinpirole</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>1000³</td>
<td>10</td>
<td>(Coldwell et al. 1999a)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>100³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD 128907</td>
<td>D₂</td>
<td>CHO-hD₂</td>
<td>20⁵</td>
<td>14</td>
<td>(Pugsley et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-hD₃</td>
<td>1.4⁵</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>D₂</td>
<td>CHO-rD₂</td>
<td>61³</td>
<td>78</td>
<td>(Levesque et al. 1992)</td>
</tr>
<tr>
<td></td>
<td>D₃</td>
<td>CHO-rD₃</td>
<td>0.78³</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 3. Affinity of most commonly used ligands and that of DA for D₂ and D₃ receptors. Affinity of the ligands (Kᵢ) was measured in competitive binding studies with the following radioligands: A, [¹²⁵I]iodosulpride; B, [¹²⁵I]NCQ 298; C, [³H]spiroperidol; D, [³H]7-OH-DPAT; E, [³H]spiperone; F, [³H]domperidone. The D₂/D₃ selectivity index was calculated as the ratio of each ligand’s affinity for D₂ and for D₃ receptors. Values <1 indicate D₂ preferring ligands, whereas values >1 indicate D₃-prefering ligands. Kᵢ values of agonists are representative for the high-affinity binding sites. hD₂/hD₃, recombinant human receptors; rD₂/rD₃, recombinant rat receptors.
2. AIMS

The overall objective of the study was to apply and evaluate new postmortem and in vivo imaging methods for the examination of dopamine-D₂/D₃ receptors in the brain. The methodological developments and improvements targeted three different aspects of D₂/D₃ receptor imaging.

1. Regional examination of the interaction between D₂/D₃ receptors and G proteins. For this purpose a new method, agonist stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding autoradiography was established for the examination of D₂/D₃ receptors in human brain sections (Paper I).

2. Selective visualization of D₃ receptors in the brain with PET. In this project the binding of a novel, putatively D₃ selective radioligand, \[^{11}\text{C}]\text{RGH-1756} was evaluated in the monkey brain under physiological conditions and after reserpine pretreatment (Papers II and III).

3. Improving the accuracy of in vivo imaging of dopamine-D₂/D₃ receptors with PET by partial volume effect correction. In this study the effect of PVE is examined on the regional parameters for \[^{11}\text{C}]\text{FLB 457} and \[^{11}\text{C}]\text{raclopride} binding in the human brain. The relevance of PVE correction in pre-clinical and clinical PET studies on D₂/D₃ receptors is also discussed (Papers IV and V).
3. THEORY AND EXPERIMENTAL PROCEDURES

3.1. SUBJECTS

3.1.1. Brain tissue

Human brains were obtained postmortem at clinical autopsy at the National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden, and at the Department of Pathology, University of Oulu, Finland (Paper I). The study was approved by the Ethics Committee at Karolinska Institutet. Tissue was obtained from six male subjects with no documented history of neurological or psychiatric disorders (age: 32-70 y, mean: 51 y, post-mortem time: 5-48 h, mean: 21 h, storage time in brain bank: 2.5-20 y, mean: 9 y).

3.1.2. Cynomolgus monkeys

Altogether four (1 male and 3 females) Cynomolgus monkeys participated in the examinations with $^{11}$C-RGH-1756 (Papers II and III). The studies were approved by the Animal Research Ethics Committee of the Northern Stockholm Region, Sweden. The Cynomolgus monkeys (weight 3.0-8.2 kg) were supplied by the Swedish Institute for Infectious Disease Control, Solna, Sweden.

3.1.3. Human subjects

No new PET examinations involving human subjects were performed within this thesis. The PET and magnetic resonance (MR) image datasets were obtained from the comparison group in an ongoing clinical study, for which the approvals of the Research Ethics and the Radiation Safety Committees of the Karolinska Hospital have been obtained (Papers IV and V). Nine healthy volunteers (age: $57 \pm 7$ y, mean $\pm$ SD) were previously examined with $^{11}$C-FLB 457 and $^{11}$C-raclopride, and also underwent MR examination. All individuals were healthy according to medical history, physical examination, MR imaging of the brain and blood and urine chemistry.

The image datasets of all nine subjects were included in Paper IV on $^{11}$C-FLB 457, whereas one subject was excluded from the analysis in Paper V on $^{11}$C-raclopride, as the appropriate input function was not possible to define.

3.2. COMPOUNDS AND RADIOCHEMISTRY

$[^{35}]S$GTPγS (specific radioactivity, SA: 1080-1160 Ci/mmol) was purchased from Amersham Biosciences AB (Uppsala, Sweden).

Derivative RGH-1756 and its desmethyl precursor, 04512626 were synthesized at Gedeon Richter Ltd., Budapest, Hungary. $[^{11}]$C-RGH-1756 was synthesized as previously described by O-methylation of the corresponding desmethyl precursor using $[^{11}]$Cmethyl triflate (Langer et al. 2000). SA of $[^{11}]$C-RGH-1756 was 4-270 GBq/μmol. According to in vitro competitive radioligand binding studies with RGH-1756, the affinity of the compound was about 100 times higher for D₃ than for D₂ receptors ($K_i$ against $[^{3}]$Hspiperone was 0.12 nM at D₃ receptors, and 12.2 nM at D₂L and 15.2 nM at D₂S receptors (Kiss et al. 2000). RGH-1756 also had rather high affinity for 5-HT₁A receptors ($K_i$ against $[^{3}]$H8-OH-DPAT was 0.96 nM).

Radiosynthesis of $[^{11}]$C-FLB 457 and $[^{11}]$C-raclopride was performed as described earlier (Halldin et al. 1991, 1995). SA was 39-578 GBq/μmol and 49-353 GBq/μmol for $[^{11}]$C-FLB 457 and $[^{11}]$C-raclopride, respectively.
All other compounds and chemicals were obtained from standard commercial sources and were of analytical grade wherever possible.

3.3. AUTORADIOGRAPHY

3.3.1. Cryosectioning

Whole hemispheres were removed, frozen and cryosectioned as described earlier (Hall et al. 1994, 1998, 2001) using a heavy-duty cryomicrotome (Leica Cryomacrotome CM3600, Leica, Nussloch, Germany). Briefly, 100 µm thick horizontal cryosections were cut parallel to the Talairach anterior commissure – posterior commissure plane (Talairach & Tournoux, 1988). The cryosections were transferred to gelatine or poly-L-lysine coated glass plates (10x22 cm), dried at room temperature and then stored with dehydrating agents at –25 °C until the experiments.

Cryosections containing the dorsal parts of caudate and putamen and thalamus (distance from vertex, DFV: 62.0-74.1 mm), ventral parts of caudate and putamen and globus pallidus (DFV: 72.1-85.6 mm), and substantia nigra (DFV: 85.5-103.1 mm), respectively, were used to study the distribution of DA receptor activated G proteins (Paper I).

3.3.2. [35S]GTPγS binding autoradiography

The [35S]GTPγS binding autoradiographic procedure was carried out as described previously (Sim et al. 1995) with slight modifications (Paper I). During the initial series of experiments the optimal assay conditions were defined by varying the incubation time, temperature and buffer composition. The effects of different experimental conditions on DA stimulated [35S]GTPγS binding were measured in duplicates. The conditions, which resulted in the highest signal-to-noise ratio in the striatum, that is the highest stimulation over basal binding, were subsequently used to characterize agonist stimulated [35S]GTPγS binding in human brain section.

The optimal assay conditions were as follows. The sections were preincubated in HEPES buffer (50 mM, pH 7.4, containing 150 mM NaCl, 75 mM KCl, 5 mM MgCl₂, 0.2 mM EGTA, 1 mM DTT and 2 mM GDP) for 30 min at 37 °C. The sections were subsequently incubated with 50 pM [35S]GTPγS in the same buffer for 90 min at 37 °C. Basal binding was determined in the absence of agonists. DA (1 mM, in the presence of 100 µM pargyline, a selective monoamine oxidase-B inhibitor) or quinpirole (1 mM) was used to stimulate [35S]GTPγS binding at DA receptors. Receptor subtype-specificity of DA stimulated [35S]GTPγS binding was examined by the simultaneous addition of raclopride (100 µM) or SCH23390 (100 µM). After incubation, the sections were washed in cold HEPES buffer for 2x10 min, followed by a brief dip in ice-cold distilled water. The sections were dried on a warm plate and subsequently exposed to β-radiation sensitive film (Kodak Biomax MR, Amersham Biosciences AB, Uppsala, Sweden) for 1-2 days.

3.4. POSITRON EMISSION TOMOGRAPHY

3.4.1. PET system

Radioactivity in the brain was measured using an ECAT Exact HR PET system (CTI/Siemens, Knoxville, TN) run in three-dimensional mode. Transmission scans (10 min) were obtained with rotating ⁶⁸Ge/⁶⁸Ga sources prior to the emission scans, and were used for attenuation correction. Images were reconstructed using a Hann filter (2 mm FWHM), after
correction for attenuation, random and scattered events. The reconstructed volume was displayed as 47 image slices with an inter-slice distance of 3.125 mm.

The transaxial resolution of the system decreases from 3.6 mm FWHM at the centre of the field of view to 4.5 mm FWHM tangentially and 7.4 mm FWHM radially at 20 cm from the centre. Axial resolution is 4 mm FWHM at the center of the field of view, and 6.8 mm at 20 cm from the centre (Wienhard et al. 1994). The resolution of the PET images along the x and y axes was estimated from measurements with a $^{68}$Ga/$^{68}$Ge rod source of 2 mm diameter. The image resolution along the z axis was calculated from the resolution of the PET system and the filter used during image reconstruction. The PSF of the PET images was estimated as 6 mm x 6 mm x 6 mm FWHM uniformly throughout the entire field of view (Papers II-V).

3.4.2. Examinations with $[^{11}C]$RGH-1756

The PET examinations of the Cynomolgus monkeys (Papers II and III) were essentially performed as described earlier (Karlsson et al. 1993). Anaesthesia was induced and maintained by repeated intramuscular injections of a mixture of ketamine (3-7 mg/kg/h Ketalar®, Pfizer) and xylazine hydrochloride (0.5-2 mg/kg/h Rompun®, Vet. Bayer). A venous cannula was inserted into a sural vein for administration of $[^{11}C]$RGH-1756. A head fixation system was used to secure a fixed position of the monkey head during the PET measurements. Heart and breath rate and rectal temperature of the monkeys were monitored continuously during the PET examinations. Body temperature was maintained by a heating blanket. The radioligand was dissolved in 4-5 ml physiological phosphate buffer (pH=7.4) and the solution was injected intravenously (iv) in 5 seconds as a single bolus. In each PET experiment 15-54 MBq of $[^{11}C]$RGH-1756 was administered. Radioactivity in the brain was measured according to a pre-programmed sequence of frames for up to 93 minutes after the injection of $[^{11}C]$RGH-1756.

In the initial experiments (Paper II) the saturability of $[^{11}C]$RGH-1756 binding was examined in a monkey by varying the injected mass of unlabelled RGH-1756 (0.14-0.42 µg). Specificity of $[^{11}C]$RGH-1756 binding was examined in pretreatment experiments by the iv administration of one of the following compounds to the monkey 15-20 min before the injection of $[^{11}C]$RGH-1756: unlabelled RGH-1756 (0.02 mg/kg), the dopamine-D$_2$ receptor antagonist raclopride (1 mg/kg), and the 5-HT$_{1A}$ receptor antagonist WAY-100635 (0.5 mg/kg). To obtain anatomical correlates for the PET images an MRI examination of the monkey was performed using a GE Signa scanner (1.5 Tesla) with a T2 weighted sequence. Blood samples of 2.0 ml were obtained from the femoral vein of the monkey 5, 15, 30, 45 and 60 minutes after the injection of $[^{11}C]$RGH-1756 in three experiments. Fractions of radioactivity in the monkey plasma that corresponded to unchanged $[^{11}C]$RGH-1756 and labelled metabolites were determined using a modification of an HPLC-method previously developed for other PET ligands (Halldin et al. 1995). The radioactivity peaks in the HPLC chromatograms corresponding to either RGH-1756 or its labelled metabolites were integrated separately, and the areas were expressed as percentage of the sum of areas of all detected peaks.

In the second study the effect of reserpine induced DA depletion was examined on the binding of $[^{11}C]$RGH-1756 (Paper III). The monkeys were examined with $[^{11}C]$RGH-1756 two times on separate experimental days. On the first experimental day a baseline measurement was performed. Twenty-four hours before the second PET examination, each monkey received an intravenous injection of reserpine (0.5 mg/kg).
3.4.3. Examinations with $^{11}$CFLB 457 and $^{11}$Craclopride

Examination of the nine healthy comparison subjects was performed in the frame of an ongoing clinical study (Papers IV and V). All subjects were examined with $^{11}$CFLB 457 and $^{11}$Craclopride and underwent MR examination. A plastic helmet was made for each subject and used with a head fixation system to prevent head movements during the examinations (Bergstrom et al. 1981). The injected radioactivity was 151-265 MBq and 192-202 MBq in the examinations with $^{11}$CFLB 457 and $^{11}$Craclopride, respectively. Radioactivity in the brain was recorded in a series of consecutive time frames (3x1, 4x3, 12x6 min) over 87 min for $^{11}$CFLB 457, and (3x1, 4x3, 6x6 min) over 51 min for $^{11}$Craclopride.

To obtain high-resolution anatomical correlates for the PET images the subjects were examined using magnetic resonance imaging (1.5T, GE Signa MRI system, Milwaukee, WI). A T1w protocol (3D-SPGR, TR=20 msec, TE=5 msec, flip angle=35°) was used to acquire 156 axial slices with 1 mm slice thickness. The field of view was 26 cm. The image matrix was 256x256, which yielded a voxel size of 1.02x1.02x1.00 mm.

3.5. IMAGE ANALYSIS, CALCULATIONS AND STATISTICS

3.5.1. $^{35}$S GTPγS binding autoradiography

The autoradiograms were digitized using a ScanMaker E6 high-resolution scanner (Mikrotek). Measurements and image processing were performed using Adobe Photoshop 6.0 and Matlab 6.5. To quantify $^{35}$S GTPγS binding the optical density values were transformed into units of nCi/mg tissue using $^{14}$C-calibration scales (American Radiolabeled Chemicals Inc, St Louis, MO, USA).

Agonist stimulated binding in brain sections was expressed as the percentage increase in $^{35}$S GTPγS binding induced by the agonist relative to that observed under basal (agonist-free) conditions. Results of multiple measurements in the same region were averaged for each subject. These values were then used to calculate the mean agonist stimulated $^{35}$S GTPγS binding of the entire group. Standard error of mean (SEM) was calculated from the inter-individual variance and the number of subjects (Paper I).

3.5.2. PET studies with $^{11}$C RGH-1756

3.5.2.1. Image processing

All PET images obtained in the initial evaluation of $^{11}$C RGH-1756 (Paper II) were co-registered to the MR image of the same monkey using the Karolinska Computerized Brain Atlas System (Roland et al. 1994).

Each PET image obtained in the examination after administration of reserpine (Paper III) was co-registered to the baseline PET image of the same monkeys using a manual, image-overlay based technique (MARS, Willendrup et al. 2004).

3.5.2.2. Delineation of regions of interest

The uptake of $^{11}$C RGH-1756 was measured in more regions of the monkey brain during the initial evaluation of the radioligand (Paper II) than in the study that examined the effect of reserpine on the binding of $^{11}$C RGH-1756 (Paper III).

In the first PET study the uptake of $^{11}$C RGH-1756 was examined in several regions throughout the entire monkey brain (Paper II). The regions of interest (ROIs) were drawn on the MR image of the monkey, and were thereafter transferred to the PET images to measure the regional radioactivity concentration. ROIs for accumbens, caudate, putamen, hippocam-
Theory and experimental procedures

pus, mesencephalon, thalamus, cingulate gyrus, cerebellar hemispheres, frontal and temporal cortices and the whole brain were delineated using the atlas of Paxinos & Toga (Paxinos, 2000).

In the PET studies with reserpine (Paper III) the uptake of $[^{11}\text{C}]$RGH-1756 was examined in the ventral striatum (ROI corresponding to the accumbens), dorsal striatum (ROI encompassing the putamen) and the cerebellum (lobules 9-10 were not included in the ROI). ROIs were drawn on the summation PET images from the baseline examinations, which represent radioactivity concentration measured between 9-93 min. Delineation of the ROIs was guided by an atlas of a cryosectioned Cynomolgus monkey brain in situ (Karlsson et al. 1993).

3.5.2.3. Quantification of $[^{11}\text{C}]$RGH-1756 binding in the monkey brain

The ROIs were used thereafter to measure total radioactivity concentration ($C_{T(i)}$, nCi/ml) in selected regions of the monkey brain. The time curve for $C_{T(i)}$ was plotted versus time for each ROI. A triple-exponential function was fitted to the TACs to reduce the noise at single time points. The TACs were normalized to the injected radioactivity. Non-specific binding ($C_{N(i)}$) was estimated from the radioactivity concentration measured in the cerebellar hemispheres, as this structure contains negligible amounts of D$_3$ receptors (Bancroft et al. 1998; Levant, 1998; Levesque et al. 1992; Ricci et al. 1995). Specific binding ($C_{B(i)}$) was calculated for each ROI as the difference between the total and non-specific binding. Binding potential (BP) was estimated as the ratio of specific to non-specific binding. In the initial PET study with $[^{11}\text{C}]$RGH-1756 (Paper II), the $C_{B}$ and $C_{N}$ measured at the time of peak equilibrium, i.e when $dC_{b}/dt=0$ (Farde et al. 1989), was used to calculate BP. In the PET study that examined the effect of reserpine on $[^{11}\text{C}]$RGH-1756 binding (Paper III) the TACs for cerebellum and for specific binding in each ROI were integrated for the time period of 9-45 min for the calculation of BP:

$$BP = \frac{\int_{9}^{45} C_{B(i)} dt}{\int_{9}^{45} C_{N(i)} dt}$$

The two approaches for the calculation of BP yielded similar results.

An attempt was made to perform a Scatchard analysis based on the results of the initial PET examinations with $[^{11}\text{C}]$RGH-1756 performed in the same monkey (Paper II). Values of $C_{B(i)}$ and $C_{N(i)}$ obtained at the time of equilibrium were divided by the SA of $[^{11}\text{C}]$RGH-1756 to calculate the concentration of specifically bound (B; fmol/ml) and that of free (F; fmol/ml) radioligand. Scatchard analysis was performed using B and F values obtained from the experiments with varying injected mass of the RGH-1756, as described earlier (Farde et al. 1997; Ginovart et al. 1997). The density of $[^{11}\text{C}]$RGH-1756 binding sites ($B_{\text{max}}$) and apparent affinity of the ligand ($K_d$) were obtained from the Scatchard plot.

3.5.3. PET studies with $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride

3.5.3.1. Image processing

The MR images were segmented using the Bayesian probabilistic approach implemented in SPM2 (Ashburner & Friston, 1997). The original MR and the probability maps of gray matter (GM), white matter (WM), cerebrospinal fluid (CSF) and skull were thereafter co-registered to the PET image of the same subject using mutual information cost function (Maes et al. 1997) in SPM2 (Papers IV and V).
3.5.3.2. **Delineation of regions of interest**

ROIs were defined to obtain regional TACs for $[^{11}C]$FLB 457 and $[^{11}C]$raclopride binding (Papers IV and V). ROIs for striatum, thalamus, hippocampus, amygdala, anterior cingulate and insular cortex were delineated manually on the high-resolution T1w MR images. Segmentation of these regions and delineation of subregions within the thalamus and the striatum followed published anatomical recommendations. For the segmentation of the insular cortex, the methods of Crespo-Facorro et al. (2000) and of Kasai et al. (2003) were adapted. Anterior cingulate was delineated according to Ballamier et al. (2004), whereas hippocampus and amygdala were segmented following the guidelines of Pruessner et al. (2000).

Segmentation of the thalamus and its subregions was slightly modified as compared to the original recommendation of Buchsbaum et al. (1996) and that of Gilbert et al. (2001). Originally the inferior border of the third ventricle was proposed as the inferior boundary of the thalamus. Our approach was, in contrast, to use the rostral end of the superior colliculi as the inferior boundary of the thalamus, in order to reduce inter-rater differences in segmentation. According to the method of Gilbert et al. (2001) the thalamus is subdivided into five small regions (Figure 8A). Some of these thalamic subregions were, however, so small that statistical noise at late time points of the TACs prevented quantitative analysis of $[^{11}C]$FLB 457 binding in these regions. According to previous PET studies with $[^{11}C]$FLB 457 (Yasuno et al. 2004) and in vitro autoradiographic studies (Hall et al. 1996a; Rieck et al. 2004) the density of D$_2$ receptors is similar in the anterior and central medial thalamic subdivisions. The same principle applies to the anterior and central lateral subdivisions. Therefore only three thalamic subregions were defined for the full quantitative analysis: medial, lateral and posterior. The ventral striatum, the dorsal part of caudate and the dorsal part of putamen (Figure 8B) were delineated according to the method of Mawlawi et al. (2001) and Martinez et al. (2003).

Manual segmentation of the regions was performed using MRICro (Rorden & Brett, 2000). The ROIs were then re-sliced using the corresponding transformation matrix obtained during the co-registration of the MR image to the PET image of the same subject. The re-sliced ROIs were transferred to the PET images to obtain the regional TACs of $[^{11}C]$FLB 457 and $[^{11}C]$raclopride before PVE correction.

An automated ROI fitting algorithm was used to define ROIs for frontal, parietal, temporal and occipital cortices, subventricular GM, WM and CSF. The automated method is based on the coordinate system of the Talairach atlas (Talairach & Tournoux, 1988), as described previously in detail (Andreasen et al. 1996; Quarantelli et al. 2002).
3.5.3.3. **Partial volume effect correction**

PVE correction was performed using an ROI based method proposed by Rousset et al (1998a), which accounts for spill-in and spill-out effects between any possible pair of ROIs (Papers IV and V). This method was shown to be as effective and accurate as established, pixel-based methods (Muller-Gartner et al. 1992) in recovering activity loss due to PVE. Moreover, this method provided more consistent estimates when applied to real data, and did not require any *a priori* knowledge of regional tracer levels, as was the case for pixel-based methods (Rousset et al. 1998b).

Briefly, uncorrected TACs were first obtained by applying the complete ROI set (manually and automatically segmented GM ROIs, plus two additional ROIs for the WM and the CSF compartments) to the PET images. Then a matrix of regional transfer coefficients was calculated for the same ROI set, based on the known resolution of the PET images. The transfer coefficient ($\omega_{ij}$) represents the fraction of activity transferred from ROI$_i$ to ROI$_j$ because of PVE, and is derived as follows.

$$\omega_{ij} = \frac{1}{n_{vox}} \int_{ROI_i} RSF_i(r)dr$$

where $n_{vox}$ is the number of voxels in ROI$_j$, $RSF_i(r)$ represents the regional spread function of ROI$_i$, and is calculated by integration of the system’s PSF over ROI$_i$, and $r$ is a three dimensional vector in the image space. The original PET values and the transfer coefficients compose a system of linear equations, the unknowns being the true mean ROI values. In general form for $N$ number of ROIs:

$$t_j = \sum_{i=1}^{N} \omega_{ij}T_i$$

where $t$ represents the observed and $T$ the PVE corrected (True) activity concentration in the ROIs. The system of linear equations is solved inverting the matrix of regional transfer coefficients by Single Value Decomposition, thus providing the PVE corrected ROI values. In the matrix inversion process, activity in CSF is forced to zero to increase accuracy by taking advantage of the *a priori* knowledge of the lack of appreciable activity in CSF (Quarantelli et al. 2004). Regional recovery coefficients (RC), that are for each ROI the ratio of observed to true activity in the absence of surrounding activity (Hoffman et al. 1979, 1982) represent the diagonal of the transfer matrix. The algorithm for PVE correction was implemented in a common software framework (Quarantelli et al. 2004; Rask et al. 2004).

3.5.3.4. **Quantification of $^{11}$CFLB 457 and $^{11}$Craclopride binding in brain**

The same approach was used for the quantification of radioligand binding for $^{11}$CFLB 457 and $^{11}$Craclopride (Papers IV and V). The standard two tissue compartment model (2-TCM, Figure 9) with estimated arterial input functions was used for kinetic analysis of radioligand binding before and after PVE correction. This model was proven to be useful for the quantification of both $^{11}$CFLB 457 and $^{11}$Craclopride binding to D$_2$/D$_3$ receptors (Endres et al. 1997; Farde et al. 1986, 1989; Lammertsma et al. 1996; Olsson et al. 1999). The four rate constants ($K_1$, $k_2$, $k_3$ and $k_4$) were estimated by standard nonlinear least–squares analysis using the Marquardt algorithm (Marquardt, 1963). Cerebral blood volume was set as 5% for all ROIs (Yamaguchi et al. 1986). Binding potential (BP, Mintun et al. 1984) and total distribution volume ($DV_{tot}$) were calculated in the following way: $BP=k_3/k_4$, $DV_{tot}=K_1/k_2(1+k_3/k_4)$.

As no arterial blood samples were taken from this set of comparison subjects during the PET examinations, the input functions for $^{11}$CFLB 457 and $^{11}$Craclopride were estimated according to previously published experimental data (Endres et al. 1997; Farde et
Methodological advances in the examination of the dopamine system in brain


To allow for the comparison of radioligand binding before and following PVE correction, the arterial plasma and blood curves were estimated as follows. The overall strategy for the generation of the simulated arterial curves was to find an arterial curve for each subject that provided binding parameters consistent with the results of the simplified reference tissue model (SRTM, Lammertsma & Hume, 1996). It has previously been demonstrated that BP values of $^{11}$CFLB 457 and that of $^{11}$Craclopride obtained with SRTM and 2-TCM are in good agreement (Lammertsma et al. 1996; Olsson et al. 1999, 2004).

In the first phase of the analysis SRTM was used to calculate BP and R1 ($R1 = K_{1}/K_{1}^*$, where $K_{1}^*$ is the influx rate constant of the reference region) before PVE correction for $^{11}$CFLB 457 and $^{11}$Craclopride. Cerebellum was used as reference region, as it contains negligible amounts of $D_{2}/D_{3}$ receptors (Camps et al. 1989; Camus et al. 1986; De Keyser et al. 1988; Hall et al. 1994, 1996a; Olsson et al. 1999).

In the following step of the analysis the 2-TCM was fit to the non-PVE corrected (henceforth observed) TACs of $^{11}$CFLB 457 and $^{11}$Craclopride in each subject using several different simulated plasma and whole blood curves. For $^{11}$Craclopride the single-tissue compartment model was used for the cerebellum to obtain $K_{1}^*$, as the 2-TCM did not reach convergence for this region. BP and R1 were then also calculated using the rate constants obtained from the 2-TCM.

The plasma curve and the corresponding blood curve, which resulted in parameters that were in best agreement with results of the SRTM, were selected for further analysis for each subject. The agreement between BP and R1 values calculated with 2-TCM and SRTM was assessed using intra-class correlation coefficient (ICC$_{3,1}$) according to the definition of Schrout & Fleiss (1979). For $^{11}$CFLB 457 ICC$_{3,1} \geq 0.72$ for R1 and ICC$_{3,1} \geq 0.73$ for BP, whereas for $^{11}$Craclopride ICC$_{3,1} \geq 0.72$ for R1 and ICC$_{3,1} \geq 0.40$ for BP in each subject (Table 4). Furthermore, the simulated plasma curves were adjusted so that the rate constants were within the previously published, physiological range (Endres et al. 1997; Farde et al. 1986, 1989; Lammertsma et al. 1996; Olsson et al. 1999, 2004).

3.5.3.5. Statistical analysis

Analysis of PVE correction induced changes in rate constants, BP and DV$_{tot}$ of $^{11}$CFLB 457 (Paper IV) was performed using analysis of variance for repeated-measures (ANOVA, general linear model module, Statistica 7.1, StatSoft, Tulsa, OK). For $^{11}$Craclopride the multivariate approach was used (Paper V). Each parameter was tested for two between subject effects. The first main effect reflected the overall change of parameters after PVE correction, whereas the other main effect referred to the difference of parameters between
distinct brain regions. The interaction of these two main effects was also examined. Significance level was set at \( \alpha < 0.05 \). For the ANOVA approach ([\(^{11}\)C]FLB 457, Paper IV) Mauchly’s test of sphericity was used to examine whether the sphericity assumption was fulfilled, and the correction method of Huynh & Feldt was used whenever it was violated (Huynh & Feldt, 1976). The Cook’s distance was used to detect outliers.

If the ANOVA indicated significant interaction effect, planned comparisons were performed with Bonferroni correction to test the difference of observed and PVE corrected values in each ROI.

Pearson’s R was calculated to examine the relationship of ROI volumes to regional RC values and to changes of binding parameters (BP and \( DV_{\text{tot}} \)) of both radioligands following PVE correction. Bonferroni correction was applied to detect significant correlations. Significance level was set at \( \alpha < 0.05 \).

<table>
<thead>
<tr>
<th></th>
<th>BP</th>
<th>R1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-TCM</td>
<td>SRTM</td>
</tr>
<tr>
<td>Ventral striatum</td>
<td>1.6 ( \pm ) 0.4</td>
<td>2.0 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Dorsal caudate</td>
<td>2.1 ( \pm ) 0.3</td>
<td>2.1 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Dorsal putamen</td>
<td>2.3 ( \pm ) 0.3</td>
<td>2.9 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Med. thalamus</td>
<td>2.2 ( \pm ) 0.4</td>
<td>2.8 ( \pm ) 0.6</td>
</tr>
<tr>
<td>Post. thalamus</td>
<td>1.9 ( \pm ) 0.4</td>
<td>2.0 ( \pm ) 0.6</td>
</tr>
<tr>
<td>Lat. thalamus</td>
<td>1.5 ( \pm ) 0.3</td>
<td>1.6 ( \pm ) 0.4</td>
</tr>
<tr>
<td>Hippocampal compl.</td>
<td>1.3 ( \pm ) 0.4</td>
<td>0.9 ( \pm ) 0.3</td>
</tr>
<tr>
<td>Insula</td>
<td>1.2 ( \pm ) 0.2</td>
<td>1.0 ( \pm ) 0.3</td>
</tr>
<tr>
<td>Ant. cingulate</td>
<td>0.8 ( \pm ) 0.2</td>
<td>0.5 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Temporal cx.</td>
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<td>0.7 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Frontal cx.</td>
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<td>0.3 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Parietal cx.</td>
<td>0.6 ( \pm ) 0.2</td>
<td>0.3 ( \pm ) 0.2</td>
</tr>
<tr>
<td>Occipital cx.</td>
<td>0.5 ( \pm ) 0.2</td>
<td>0.3 ( \pm ) 0.2</td>
</tr>
</tbody>
</table>

Table 4. BP and R1 values calculated using 2-TCM and SRTM. [\(^{11}\)C]raclopride was used to examine the striatum and [\(^{11}\)C]FLB 457 was used for all other regions. The data shows the mean \( \pm \) SD of results obtained in nine and eight subject for [\(^{11}\)C]FLB 457 and for [\(^{11}\)C]raclopride, respectively. The 2-TCM columns show the average of those parameters for each subject that was obtained using the individually optimized plasma curves.
4. RESULTS AND COMMENTS

4.1. AUTORADIOGRAPHIC MAPPING OF DOPAMINE-D<sub>2</sub>/D<sub>3</sub> RECEPTOR STIMULATED [<sup>35</sup>S]GTP<sub>γγγγ</sub>S BINDING IN THE HUMAN BRAIN (PAPER I)

Agonist stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding autoradiography had earlier been successfully applied for the examination of D<sub>2</sub>/D<sub>3</sub> receptors in the rat brain (Culm et al. 2003; Febo et al. 2003; He et al. 2000; Newman-Tancredi et al. 2001). DA, quinelorane and quinpirole, but not SKF-238393, a D<sub>1</sub> agonist stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding in the striatum and the cingulate cortex of the rat brain. Previously agonist stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding autoradiography has not been applied for the examination of DA receptors in human brain sections.

The objectives of the present study were to establish agonist stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding autoradiography for the examination of DA receptors in whole human hemisphere sections, and to describe the distribution of dopamine-D<sub>2</sub>/D<sub>3</sub> receptor activated G proteins.

4.1.1. Optimisation studies

In previous experiments examining agonist stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding at DA receptors in rat brain sections, the assay conditions were almost the same as originally proposed by Sim et al. (1995). The procedure involved two preincubations without agonist (15 min each, in 50mM Tris-HCl buffer, pH 7.4, supplemented with 100 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA and 2 mM GDP for the second preincubation only), which were followed by an incubation of 1-2 h in the same buffer with the addition of [<sup>35</sup>S]GTP<sub>γγγγ</sub>S and an agonist. The assay was usually performed at room temperature.

No stimulation of [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding was observed on DA receptors in human brain sections by application of the assay condition used to examine rat brain. Therefore, the optimisation of assay conditions was first necessary for the measurement of DA stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding in human brain sections. The optimisation procedure generally involves varying the incubation time and temperature, modifying the concentration of GDP, magnesium, sodium and changing the buffering agent. In the present optimisation studies, however, no DA stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding was observed in human brain sections by changing the incubation temperature, lengthening the incubation time or by modifying the concentration of GDP, magnesium or the type of buffer used. Changes to the concentration of sodium in the incubation buffer needed careful consideration. As it has been shown that high Na<sup>+</sup> concentration shifts DA receptors to low affinity state (Grigoriadis & Seeman, 1985; Kapur & Seeman, 2001; Watanabe et al. 1985), increasing the concentration of Na<sup>+</sup> in the incubation solution might inhibit DA receptor activation. On the other hand increasing the concentration of monovalent salts in the incubation buffer could be beneficial for the reduction of basal binding (Happe et al. 2001; Sim et al. 1995), which was rather high in the 100 µm thick human brain sections. Considering the advantageous and disadvantageous effects of increasing the concentration of monovalent salts in the incubation buffer, we

<table>
<thead>
<tr>
<th>Na&lt;sup&gt;+&lt;/sup&gt; (mM)</th>
<th>K&lt;sup&gt;+&lt;/sup&gt; (mM)</th>
<th>Basal binding (nCi/mg tissue)</th>
<th>Stimulation by DA (% above basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>1.4 ± 0.4</td>
<td>24.1 ± 5.6</td>
</tr>
<tr>
<td>150</td>
<td>75</td>
<td>1.2 ± 0.0</td>
<td>26.7 ± 12.7</td>
</tr>
<tr>
<td>200</td>
<td>100</td>
<td>0.6 ± 0.0</td>
<td>21.6 ± 12.7</td>
</tr>
</tbody>
</table>

Table 5. The effect of different concentrations of monovalent salts on basal and DA (1 mM) stimulated [<sup>35</sup>S]GTP<sub>γγγγ</sub>S binding in the striatum in human brain section. Mean ± SEM is presented, number of subjects examined: n =2.
tested the effect of keeping the original sodium concentration (100 mM) and adding potassium (50 mM) to the incubation buffer. We also hypothesized that the mixture of sodium and potassium in the incubation buffer would mimic the composition of both the intra- and the extracellular compartment, and thereby promote that conformation of DA receptors and G proteins, which is present in the brain under physiological conditions. Indeed, after modifying the concentration of monovalent salts in the incubation buffer DA stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ was \ detected \ in \ the \ striatum \ in \ human \ brain \ sections \ at \ room \ temperature \ (9.5 \pm 2.1 \ %, \ mean \pm \ SEM, \ number \ of \ subjects = 2 \ for \ all \ results \ obtained \ in \ the \ optimisation \ procedure). \ DA \ stimulated \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ was \ even \ higher \ when \ the \ incubation \ was \ performed \ at \ 37 ^\circ \text{C} \ (24.1 \pm 5.6 \ %; \ Table \ 5). \ Thereafter, \ the \ effect \ of \ different \ concentrations \ of \ sodium \ and \ potassium \ was \ examined \ on \ basal \ and \ DA \ stimulated \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ (Table \ 5). \ Similar \ to \ previous \ results \ (Happe \ et \ al. \ 2001; \ Hilf \ et \ al. \ 1989; \ Sim \ et \ al. \ 1995), \ a \ progressive \ decrease \ in \ basal \ binding \ was \ detected \ by \ increasing \ the \ concentration \ of \ monovalent \ salts. \ At \ very \ high \ salt \ concentrations \ (300 \ mM) \ a \ tendency \ for \ lower \ DA \ stimulated \ binding \ was \ observed, \ probably \ due \ to \ the \ aforementioned \ effect \ of \ sodium \ on \ the \ affinity-state \ of \ dopamine \ receptors \ (Grigoriadis \ & \ Seeman, \ 1985; \ Kapur \ & \ Seeman, \ 2001; \ Watanabe \ et \ al. \ 1985). \ Therefore \ the \ mixture \ of \ 150 \ mM \ NaCl \ and \ 75 \ mM \ KCl \ was \ used \ afterwards. \ The \ incubation \ time \ was \ set \ to \ 90 \ min \ in \ order \ to \ avoid \ substantial \ damage \ to \ the \ tissue \ sections.

4.1.2. Characterisation of agonist stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ at \ D_2/D_3 \ receptors \ in \ the \ human \ brain

Basal \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ was \ heterogeneous \ in \ the \ brain, \ and \ was \ highest \ in \ striatum, \ frontal \ and \ insular \ cortices \ and \ the \ medial \ part \ of \ thalamus. \ In \ comparison \ with \ the \ basal \ condition, \ addition \ of \ DA \ resulted \ in \ increased \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ in \ several \ brain \ areas, \ including \ striatum, \ SN, \ thalamus \ and \ hippocampus \ (Table \ 6).

Raclopride, a D_2/D_3-selective antagonist potently inhibited the stimulatory effect of DA on \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ in \ the \ striatum \ and \ the \ hippocampus, \ whereas \ SCH23390 \ did \ not \ diminish \ DA \ stimulated \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ in \ any \ regions. \ This \ observation \ indicates \ that \ DA \ induced \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ is \ mediated \ primarily \ by \ D_2/D_3 \ receptors, \ and \ the \ involvement \ of \ D_1 \ receptors \ in \ the \ present \ assay \ is \ unlikely. \ This \ is \ in \ line \ with \ the \ generally \ accepted \ suggestion \ that \ agonist \ stimulated \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ autoradiography \ is \ suitable \ for \ the \ examination \ of \ G_i/G_o, \ but \ not \ G_s \ coupled \ receptors \ (Happe \ et \ al. \ 2001; \ Sim \ et \ al. \ 1995). \ However, \ in \ some \ regions, \ including \ the \ ventral \ part \ of \ caudate \ and \ putamen, \ hippocampus, \ SN, \ and \ some \ cortical \ regions, \ raclopride \ did \ not \ completely \ abolish \ DA \ stimulated \ \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding \ at \ D_4 \ receptors, \ as \ this \ receptor \ subtype \ is \ present \ in \ these \ brain \ areas \ (Lahti \ et \ al. \ 1995;
Methodological advances in the examination of the dopamine system in brain

Primus et al. 1997). However, to verify the possible role of D4 receptors in stimulating \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding in human brain sections, it is necessary to use a selective and highly potent agonists at the D4 receptor.

<table>
<thead>
<tr>
<th></th>
<th>DA (1 mM)</th>
<th>DA (1 mM) and Raclopride (0.1 mM)</th>
<th>Quinpirole (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean SEM</td>
<td>n  N</td>
<td>mean SEM  n  N</td>
</tr>
<tr>
<td>Putamen</td>
<td>23.0 2.3 3 13</td>
<td>3.0* 0.8 3 7</td>
<td>16.3 6.0 3 6</td>
</tr>
<tr>
<td>dorsal put.</td>
<td>31.0 1.9 3 6</td>
<td>-4.5 5.8 2 2</td>
<td>20.9 8.8 3 3</td>
</tr>
<tr>
<td>ventral put.</td>
<td>15.7 4.5 3 7</td>
<td>6.0* 1.4 3 5</td>
<td>11.7 3.5 3 3</td>
</tr>
<tr>
<td>Caudate</td>
<td>20.2 0.3 3 13</td>
<td>0.8* 1.1 3 7</td>
<td>16.6 3.3 3 6</td>
</tr>
<tr>
<td>dorsal caud.</td>
<td>24.4 1.6 3 6</td>
<td>-11.7 6.5 2 2</td>
<td>23.8 4.9 3 3</td>
</tr>
<tr>
<td>ventral caud.</td>
<td>15.2 3.1 3 7</td>
<td>6.8 3.9 3 5</td>
<td>9.4 3.0 3 3</td>
</tr>
<tr>
<td>Subst. nigra</td>
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<td>6.6 0.6 2 3</td>
<td>12.8 5.8 3 3</td>
</tr>
<tr>
<td>Amygdala</td>
<td>17.3 7.5 2 3</td>
<td>9.6 8.6 2 3</td>
<td>4.3 1.4 2 2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>15.7 6.0 3 6</td>
<td>4.3* 0.9 3 6</td>
<td>8.5 2.4 4 6</td>
</tr>
<tr>
<td>Ant. cingulate</td>
<td>12.7 1.8 3 11</td>
<td>4.7 5.4 3 4</td>
<td>8.9 5.1 3 6</td>
</tr>
<tr>
<td>Thalamus</td>
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<td>1.1 0.9 2 2</td>
<td>9.6 6.3 3 5</td>
</tr>
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<td>Insular cx.</td>
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<td>-0.2 2.3 3 6</td>
<td>5.5 2.2 3 5</td>
</tr>
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<td>4.4 5.1 3 8</td>
<td>1.7 2.1 4 8</td>
</tr>
<tr>
<td>Occipital cx.</td>
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<td>1.7 1.1 3 6</td>
<td>1.3 1.5 4 8</td>
</tr>
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<td>2.3 0.5 4 9</td>
</tr>
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<td>Pallidum</td>
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<td>5.0 6.7 3 5</td>
<td>4.3 5.1 3 3</td>
</tr>
</tbody>
</table>

Table 6. DA and quinpirole stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding in whole human hemisphere sections, and the effect of raclopride on DA stimulated binding. Data are presented as % stimulation above basal. *, significant difference compared to DA stimulated binding, Mann-Whitney U test (p<0.05); n, number of subjects; N, total number of sections studied. From Paper I.

The highest DA stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding was measured in the striatum (Figure 10). Within the striatum the level of DA stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding was heterogeneous. Statistical analysis (two-way ANOVA) revealed that DA stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding was significantly higher in the dorsal than in the ventral parts of caudate and putamen. Previous studies using traditional radioligand autoradiography demonstrated no dorsoventral gradient in the density of D2 receptors within in the striatum (Camus et al. 1986; Hall et al. 1994; Piggott et al. 1999). In contrast, the density of D3 receptors is much higher in the ventral striatum, including the nucleus accumbens than in the dorsal part (Hall et al. 1996b; Murray et al. 1994). In this study, DA stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding was not examined in the nucleus accumbens. However, the lower stimulation in the ventral part of the striatum could be due to low or no stimulation at D3 receptors, as it is known that dopamine-D3 receptors are only weakly coupled to G proteins (Chio et al. 1994; Newman-Tancredi et al. 1999; Vanhauwe et
The dorsal and the ventral parts of the striatum are viewed as two functionally distinct compartments, with different input and output connections (Kunzle, 1975, 1977; Selemon & Goldman-Rakic, 1985) and different characteristics of dopaminergic neurotransmission (Cragg et al. 2000, 2002). The dorsal part is involved in the regulation of motor function, whereas the ventral part mediates emotional and motivational processes. The observed difference of D\(_2\) receptor induced G protein activation between ventral and dorsal parts of caudate and putamen might reflect the distinct functional role and DA transmission pattern of these receptors.

The level of DA stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding detected in the SN (22%, Figure 11) was almost as high as levels detected in the striatum, despite that the density of D\(_2\) receptors in the SN is less than one-fourth of the striatal density (Hall et al. 1994). The D\(_2\)-like receptors in the SN are somatodendritic autoreceptors, whereas in other regions D\(_2\) receptors are located both postsynaptically and presynaptically (for more reviews see Elsworth & Roth, 1997). It has been suggested that DA autoreceptors are more sensitive to the effects of DA than heteroreceptors (Carlsson et al. 2004; Elsworth & Roth, 1997). The more efficient coupling of DA receptors and G proteins in the SN could contribute to the higher responsiveness of autoreceptors.

Relatively high levels of DA stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding were detected in thalamus and in some limbic brain regions, including amygdala, hippocampus and anterior cingulate, although these regions contain low or very low densities of D\(_2\)-like receptors. The higher stimulation could therefore reflect regional differences in the efficiency of DA receptor/G protein coupling.

The distribution of quinpirole stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding was similar to that of DA stimulated. The highest quinpirole stimulated binding was measured in striatum, followed by SN, whereas moderate levels of stimulation were detected in hippocampus, thalamus, anterior cingulate and amygdala. Quinpirole stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding was numerically lower than DA stimulated binding. However, no statistically significant differences have been observed between the level of DA and quinpirole stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding (Table 6). Quinpirole, like DA, stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding to a somewhat higher level in the dorsal than in the ventral parts of caudate and putamen. Statistical analysis revealed a pronounced tendency for higher stimulation in the dorsal parts.

In the present study agonist stimulated \(^{35}\text{S}\)GTP\(\gamma\)S binding autoradiography was established to examine DA receptors in the human brain. The results demonstrate that this novel method could be a suitable technique for the examination of dopamine-D\(_2\)/D\(_3\) receptors in the postmortem human brain, by providing useful new information about DA receptor/G protein coupling.
4.2. EVALUATION OF THE PUTATIVE DOPAMINE-D₃ SELECTIVE LIGAND, [¹¹C]RGH-1756 IN THE MONKEY BRAIN WITH PET (PAPERS II AND III)

In vitro examination of the D₃ receptors has been performed already using D₃-preferring radioligands, like [³H]PD 128907 and [³H]7-OH-DPAT, which also possess some affinity for D₂ receptors (Table 3). No successful in vivo labelling of D₃ receptors has yet been performed, and currently there are no selective radioligands available for the examination of D₃ receptors with PET.

The novel phenoxyalkylpiperazine derivative, RGH-1756, has about one-hundred times higher affinity for D₃ than for D₂ receptors (Laszlovszky et al. 2000). This indicates a higher selectivity of RGH-1756 for D₃ receptor than that of known D₃-preferring ligands, such as PD 128907 and 7-OH-DPAT. These properties of RGH-1756 render the molecule a promising candidate for in vitro and in vivo imaging of the dopamine-D₃ receptors in the brain. Recent successful radiolabelling of the molecule with carbon-11 (Langer et al. 2000) makes it possible to examine the potential of [¹¹C]RGH-1756 as a radioligand for PET.

<table>
<thead>
<tr>
<th></th>
<th>Low mass</th>
<th>High mass</th>
<th>unlabelled RGH-1756</th>
<th>Raclopride</th>
<th>WAY-100635</th>
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</thead>
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<td>SA (GBq/µmol)</td>
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<td>56.3</td>
<td>58.2</td>
<td>57.4</td>
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<td>0.11</td>
<td>0.22</td>
<td>0.21</td>
</tr>
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<td>0.05</td>
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<td>0.38</td>
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<tr>
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<td>0.33</td>
<td>0.17</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 7. BP of [¹¹C]RGH-1756 in selected regions of the monkey brain. The results were obtained in a baseline examination (low mass column), in an examination with high injected mass of unlabelled RGH-1756 (high mass column) and in pretreatment examinations with one of the following compounds: unlabelled RGH-1756 (0.02 mg/kg), raclopride (1 mg/kg) or WAY-100635 (0.5 mg/kg). BP was calculated as the ratio of specific to nonspecific binding at the time of peak equilibrium.

4.2.1. Characterisation of [¹¹C]RGH-1756 binding in the monkey brain with PET (Paper II)

One cynomolgus monkey was repeatedly examined with [¹¹C]RGH-1756 in order to describe the distribution and specificity of [¹¹C]RGH-1756 binding in the monkey brain.

After iv injection of [¹¹C]RGH-1756, 1.0-1.1% of the total injected radioactivity was in the brain 30 seconds following the injection of the radioligand, which is rather low when compared to established radioligands, such as [¹¹C]raclopride (Halldin et al. 1991) or [¹¹C]FLB 457 (Halldin et al. 1995).
Results and comments

[11C]RGH-1756 was homogenously distributed within the monkey brain, and no conspicuous accumulation of radioactivity could be seen by visual inspection of the PET images. BP of [11C]RGH-1756 was low in all regions examined (Table 7). The highest values were measured in striatum, thalamus and temporal cortex. The inter-regional differences of BP were, however, small. The in vitro density of D₃ receptor is highest in the islands of Calleja and the nucleus accumbens (Bancroft et al. 1998; Levesque et al. 1992). The islands of Calleja are too small to be detected with PET, so this region was not analyzed in the present study. Nucleus accumbens did not stand out as having particularly high [11C]RGH-1756 binding. The relative regional distribution of [11C]RGH-1756 binding was thus not fully consistent with the rank order of regional D₃ receptor densities predicted by in vitro studies (Bancroft et al. 1998; Levesque et al. 1992; Stanwood et al. 2000a).

Pretreatment experiments were used to characterize the specificity of [11C]RGH-1756 binding. Pretreatment with unlabeled RGH-1756 resulted in decreased binding of the radioligand in all brain regions, except temporal cortex and cingulate gyrus. The effect of unlabelled RGH-1756 on the binding of [11C]RGH-1756 indicates saturable binding in the monkey brain. Pretreatment with raclopride (1 mg/kg) diminished binding of [11C]RGH-1756 in many areas, including accumbens, caudate, putamen, mesencephalon, and frontal cortex, suggesting that binding of [11C]RGH-1756 may represent specific binding to D₂/D₃ receptors. Pretreatment with WAY-100635 (0.5 mg/kg) had no effect on the radioactivity concentration of [11C]RGH-1756 in any brain areas. Thus, it can be rule out that [11C]RGH-1756 binds to 5-HT₁₅ receptors in vivo.

Although BP of [11C]RGH-1756 was generally low, the pretreatment experiments with unlabeled RGH-1756 and raclopride showed some specific binding to binding sites that are presumably D₂/D₃ receptors. Based on this assumption we made an attempt to measure the density of D₃ receptors in the monkey brain by varying the injected mass of RGH-1756. After injection of the radioligand with higher mass of the unlabelled compound BP of [11C]RGH-1756 was somewhat lower in striatum, thalamus and temporal cortex. In these regions the injected mass of RGH-1756 was inversely related to the BP of [11C]RGH-1756 (Figure 12). No effect of the injected mass of RGH-1756 on the BP of [11C]RGH-1756 was observed in the other brain regions. Scatchard analysis was performed for the regions in which the inverse relationship between the BP of [11C]RGH-1756 and the injected mass of RGH-1756 was observed. As BP values of [11C]RGH-1756 obtained in the experiments with low and high injected mass were hardly different in any regions, data points of the Scatchard plots were situated close together. Therefore the results did not provide reliable measures of Bₐ₃₃ of D₃ receptors. The most likely reasons for the unsuccessful Scatchard analysis were the low signal from [11C]RGH-1756 binding and the small difference in the injected mass of RGH-1756 between the examinations.

The results of the present study do not demonstrate that [11C]RGH-1756 is suitable for in vivo imaging of D₃ receptors. This

Figure 12. Relationship between the injected mass of RGH-1756 and the BP of [11C]RGH-1756.
Correlation coefficient (R) in putamen (Pu) -0.43; accumbens (Acc) -0.99; thalamus (Tha) -0.98; caudate (Cd) -0.70 (from Paper II).
was somewhat unexpected, since RGH-1756 has promising *in vitro* binding characteristics. Common reasons for failures *in vivo* are low affinity under physiological conditions, high non-specific binding, and rapid degradation of the radiolabelled molecule. In the present study there was no support for high non-specific binding or unusually rapid metabolism, whereas it cannot be excluded that the affinity at *in vivo* conditions is not sufficient to visualize the minute densities of D\textsubscript{3} receptors in the brain.

With regard to imaging of D\textsubscript{3} receptors there is, however, another biological condition which has to be taken into account. *In vitro* radioligand binding studies suggested that D\textsubscript{3} receptors are occupied by DA to a high degree (Levesque et al. 1992; Schotte et al. 1992, 1996). This assumption gained further support by the observation that depletion of endogenous DA with reserpine and α-methyltyrosine increased the binding of [\textsuperscript{3}H]7-OH-DPAT in the rat brain in *ex vivo* autoradiographic experiments (Levant, 1995).

4.2.2. **Examination of the effect of dopamine depletion on the binding of [\textsuperscript{11}C]RGH-1756 (Paper III)**

Based on previous experimental data, it is possible that endogenous DA prevents *in vivo* binding of [\textsuperscript{11}C]RGH-1756 in the monkey brain. To challenge this assumption the effect of reserpine induced DA depletion on the binding of [\textsuperscript{11}C]RGH-1756 was examined in three monkeys.

Reserpine effectively decreases synaptic concentration of DA (Guo et al. 2003; Kuczenski, 1977; Stanwood et al. 2000b) by depletion of available stores of catecholamines. It inhibits catecholamine uptake into storage vesicles by the blockade of vesicular monoamine transporter proteins (Alvaro et al. 2001). Reserpine had frequently been used in PET studies to examine the effect of endogenous neurotransmitters on the binding of several radioligands, including [\textsuperscript{11}C]raclopride (Ginovart et al. 1997), [\textsuperscript{11}C]SCH23390 (Chou et al. 1999), [\textsuperscript{11}C]NPA (Cumming et al. 2002), [\textsuperscript{11}C]NNC 112 (Guo et al. 2003), [\textsuperscript{11}C]WAY-100635 (Maeda et al. 2001) and [\textsuperscript{11}C]PE2I (Poyot et al. 2001). The binding of the aforementioned radioligands was increased after the administration of reserpine, except for [\textsuperscript{11}C]PE2I, which showed lower binding following reserpine treatment and [\textsuperscript{11}C]WAY-100635, which was not influenced by reserpine.

In the present study the binding of [\textsuperscript{11}C]RGH-1756 was examined 24 hours after the administration of 0.5 mg/kg reserpine in the striatum in three monkeys, and the results were compared to baseline measurements of the same monkeys.

Results of the baseline measurements were in good agreement with results of the previous study with [\textsuperscript{11}C]RGH-1756. In the baseline measurements 1.0-1.5\% of the injected radioactivity was in brain one minute after the administration of the [\textsuperscript{11}C]RGH-1756. Within the brain the regional distribution of [\textsuperscript{11}C]RGH-1756 was homogenous. Specific binding of [\textsuperscript{11}C]RGH-1756 was low and not different between the ventral and the dorsal striatum. Baseline BP values were low, and there were no evident regional differences of BP in any monkey (Table 8).

After administration of reserpine 1.0-1.2\% of the injected radioactivity was in the brain. The peak value of whole brain uptake of [\textsuperscript{11}C]RGH-1756 was observed during the first minute of the examination, as for the baseline measurements. Reserpine caused no evident changes in the distribution of [\textsuperscript{11}C]RGH-1756 within the monkey brain, and no obvious regional accumulation of radioactivity could be seen by visual inspection of the PET images. Reserpine had no consistent effect on specific [\textsuperscript{11}C]RGH-1756 binding in the monkey brain. In the first and second monkey, specific [\textsuperscript{11}C]RGH-1756 binding decreased after the administration of reserpine both in ventral and dorsal striatum. The decrease was most pronounced during the first half of the PET measurements in both monkeys. In the third monkey, specific [\textsuperscript{11}C]RGH-1756 binding was slightly increased after the administration of
Results and comments

Reserpine. The increase of specific $[^{11}C]RGH-1756$ binding was most evident during the first 20 minutes of the PET measurement. BP values of $[^{11}C]RGH-1756$ were low in all monkeys after the administration of reserpine, and no consistent changes of the regional BP values were observed when compared to the baseline values (Table 8).

After reserpine treatment there was no consistent increase in specific binding and BP of $[^{11}C]RGH-1756$ in the striatum of the monkeys. On the contrary, BP of $[^{11}C]RGH-1756$ was decreased in the striatum of two monkeys after administration of reserpine. This observation does not support the hypothesis that binding of $[^{11}C]RGH-1756$ to $D_3$ receptors in the monkey brain is inhibited by high occupancy of $D_3$ receptors by endogenous DA. Nevertheless, the results of the present study confirm the view from previous experiments that $[^{11}C]RGH-1756$ is not suitable for in vivo examination of $D_3$ receptors.

The signal obtained for possible specific binding of $[^{11}C]RGH-1756$ in the Cynomolgus monkeys’ brains was low both in the baseline and the pretreatment measurements. Such low signal, as obtained for specific binding of $[^{11}C]RGH-1756$, can be confounded by noise to a high degree. In our case, this low signal to noise ratio may explain the inconsistency in reserpine induced changes of specific $[^{11}C]RGH-1756$ binding in the three monkeys.

Results of the present examinations, together with results of the previous study (Paper II) demonstrate that the cause of low specific binding of $[^{11}C]RGH-1756$ is the insufficient in vivo affinity of the ligand for $D_3$ receptors, and not high occupancy of $D_3$ receptors by endogenous DA. Despite the promising in vitro results $[^{11}C]RGH-1756$ was not suitable for PET imaging of $D_3$ receptors.

<table>
<thead>
<tr>
<th></th>
<th>Monkey 1</th>
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<th>Monkey 2</th>
<th></th>
<th>Monkey 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Reserpine</td>
<td>Baseline</td>
<td>Reserpine</td>
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<td>Reserpine</td>
</tr>
<tr>
<td>Ventral striatum</td>
<td>0.2</td>
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<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Dorsal striatum</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 8. BP of $[^{11}C]RGH-1756$ in the ventral and the dorsal striatum in three monkeys measured in baseline examinations and after administration of reserpine. BP was calculated as the ratio of specific to non-specific binding after the integration of the TACs between 9-45 minutes of the examination as described in ‘Experimental procedures’.
4.3. INCREASING THE ACCURACY OF IN VIVO QUANTIFICATION OF DOPAMINE-D₂/D₃ RECEPTORS IN THE HUMAN BRAIN BY PARTIAL VOLUME EFFECT CORRECTION (PAPERS IV AND V)

Despite of the well-known influence of PVE on quantitative PET measurements, systematic investigation of the effect of PVE on the most commonly used receptor binding parameters, such as BP, $DV_{tot}$ and kinetic rate constants, has not yet been performed. A few studies examined the influence of PVE on observed $[^{18}F]$DOPA (Rousset et al. 2000) and $[^{11}C]$raclopride (Yokoi et al. 1998) binding. The first study demonstrated that apparent net blood-brain clearance and the equilibrium distribution volume of $[^{18}F]$DOPA, and the relative activity of dopa decarboxylase were significantly underestimated in the human striatum due to PVEs (Rousset et al. 2000). The latter study showed that BP ($k_3/k_4$) of $[^{11}C]$raclopride is underestimated without PVE correction by 71% and 44% in caudate and putamen, respectively (Yokoi et al. 1998).

The PET studies reported in the following sections had a two-fold aim: (i) to estimate the influence of PVE on quantitative PET studies with $[^{11}C]$FLB 457 and $[^{11}C]$raclopride in the human brain and (ii) to evaluate the relevance of a ROI based PVE correction method (Rousset et al. 1998a) with the purpose to formulate recommendations on the application of PVE correction in PET studies with these two D₂/D₃ selective radioligands.

The standard 2-TCM with simulated arterial input function was used to calculate kinetic rate constants ($K_1-k_4$), BP and $DV_{tot}$ of $[^{11}C]$FLB 457 and $[^{11}C]$raclopride before and after PVE correction, as described in detail above.

4.3.1. Volumetry and calculation of recovery coefficient

PVE correction, according to Roussset et al. (1998a), relies on geometric and volumetric information obtained from high-resolution MR images. To perform PVE correction a number of regions were segmented manually and their volumes were measured (Table 9). Results of the volumetric measurements were generally in good agreement with previously published data, considering that the subjects examined in the present study were older than in previous ones (for comparison see the following refs. on striatum (Gunning-Dixon et al. 1998; Krishnan et al. 1990; Raz et al. 2003), thalamus (Gilbert et al. 2001), insula (Crespo-Facorro et al. 2000; Kasai et al. 2003), anterior cingulate (Ballmaier et al. 2004), hippocampus and amygdala (Bonilha et al. 2004; Niemann et al. 2000; Pruessner et al. 2000).

The RC of each region was derived from the matrix of transfer coefficients used for PVE correction. The variability of RC between different brain regions was small (0.42-0.66). The lowest RC value was calculated in the smallest and the thinnest structures, ventral striatum and anterior cingulate. The highest values were obtained in the largest structures, WM and cerebellum. The inter-individual variability of regional RC values was small (Table 9). Despite of the small differences in regional RC between subjects a highly significant correlation was found between the volumes of the structure and the corresponding RC value for all striatal and thalamic subregions and hippocampus.

The low average regional RC values suggest that uptake of $[^{11}C]$FLB 457 and $[^{11}C]$raclopride could be severely underestimated throughout the entire brain. The difference of RC between brain regions was small, considering the substantial difference in shape and size of the regions. For some regions, such as caudate, putamen, hippocampus and thalamic subregions, the low RC could largely be explained by the small size of the regions. For neocortical regions the thinness of these structures renders them prone to PVE. The highly significant correlation found between the volume and the RC of some brain regions confirms the results of studies with geometric phantoms performed early during the history of PET imaging (Hoffman et al. 1979; Kessler et al. 1984). These studies demonstrated that the
relationship between the size of a simple geometrical object (cylinder, sphere) and its RC can be described by a sigmoid curve. For objects of a size 0.5-2 times the FHWM of the PET system the relationship between the volume and the RC of the object is linear. No such relationship was found for cortical regions, probably due to the complicated shape and the arising multiple self-interactions within these regions.

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>RC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral striatum</td>
<td>1.2 ± 0.2</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Dorsal caudate</td>
<td>3.1 ± 0.6</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Dorsal putamen</td>
<td>4.0 ± 0.4</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>Thalamus</td>
<td>6.4 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td>Lateral thalamus</td>
<td>2.7 ± 0.6</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>Medial thalamus</td>
<td>2.3 ± 0.3</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>Posterior thalamus</td>
<td>1.4 ± 0.3</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Hippocampal complex</td>
<td>2.8 ± 0.4</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Insular cortex</td>
<td>5.8 ± 0.6</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Anterior cingulate</td>
<td>7.6 ± 1.7</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>84.8 ± 10.6</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>90.7 ± 13.3</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>50.4 ± 6.2</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>30.1 ± 5.1</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>142.8 ± 17.7</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>White matter</td>
<td>475.5 ± 58.8</td>
<td>0.66 ± 0.01</td>
</tr>
<tr>
<td>CSF</td>
<td>559.6 ± 71.8</td>
<td>1.00</td>
</tr>
<tr>
<td>Whole brain</td>
<td>1215.3 ± 115.4</td>
<td>-</td>
</tr>
<tr>
<td>Intracranial volume</td>
<td>1774.9 ± 132.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 9. Average volume of brain regions (mean ± SD), and the average recovery coefficient (RC). Volume of ventral striatum, dorsal part of caudate and that of putamen was measured in eight subjects, all other volumes were measured in nine subjects. For bilateral structures the measurements were performed on both sides and the results were averaged for each subject.

4.3.2. Time activity curves for $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride

The effect of PVE correction on TACs for $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride binding varied between the regions examined. With the exception of WM all PVE corrected TACs were higher than the corresponding observed ones. The increase of TACs for GM regions after PVE correction varied between 5-70% of the observed values for $[^{11}\text{C}]$FLB 457 and between 40-60% of the observed values for $[^{11}\text{C}]$raclopride. The effect of PVE correction on regional TACs varied with time for both radioligands, thus not only the amplitude but also the shape of regional TACs was changed after PVE correction. Analysis of the transfer coefficient matrices revealed that changes of the TACs after PVE correction can partly be explained by the varying degree of self-interaction (regional RC values), and partly by the
spill-in of activity from neighbouring regions. Former factor (RC) was responsible for the changes of the amplitude of the TACs, whereas spill-in was important for the alterations in the shape of the curves.

4.3.3. Kinetic rate constants

Observed and PVE corrected TACs for $^{11}$C]FLB 457 and $^{11}$C]raclopride were entered into kinetic analysis of radioligand binding to calculate the rate constants. PVE correction had different effect on rate constants of $^{11}$C]FLB 457 and $^{11}$C]raclopride. The

Figure 13. Rate constants, BP and $DV_{tot}$ of $^{11}$C]FLB 457 before and after PVE correction. Observed values are denoted by empty signs (mean-SD), whereas PVE corrected values are represented by filled signs (mean+SD). The brain regions examined are: 1. med. thalamus, 2. post. thalamus, 3. lat. thalamus, 4. hippocampal complex, 5. insular cortex, 6. temporal cortex, 7. ant. cingulate, 8. frontal cortex, 9. parietal cortex, 10. occipital cortex, 11. cerebellum. *, Significant difference between observed and PVE corrected values, ($p<0.05$, Bonferroni corrected).
results demonstrated that rate constants of $[^{11}\text{C}]$FLB 457 are more susceptible to PVE than those of $[^{11}\text{C}]$raclopride.

PVE correction had a significant overall effect on all four rate constants ($K_1$-$k_4$) of $[^{11}\text{C}]$FLB 457 (Figure 13). Planned comparisons were used to identify the regions, where the individual $k$ values were changed after PVE correction. For $[^{11}\text{C}]$FLB 457 PVE correction had the largest effect on $K_1$, as this rate constant was increased in all regions examined. $k_2$ was increased in most cortical regions, $k_3$ was increased in temporal and insular cortices and medial thalamus, whereas $k_4$ was hardly changed after PVE correction. SD of all rate constants was increased after PVE correction for both radioligands.

PVE correction induced a significant increase in $K_1$ and $k_3$ of $[^{11}\text{C}]$raclopride, whereas $k_2$ and $k_4$ were not changed (Figure 14). $K_1$ was significantly increased in the dorsal caudate and putamen but not in the ventral striatum, whereas $k_3$ was increased in the entire striatum.

Changes of amplitude and shape of the TACs translated into changes of the rate constants of $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride. $K_1$ was significantly increased in almost all regions after PVE correction, indicating that the transport rate of $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride from plasma to brain is significantly underestimated throughout the whole brain. The simultaneous increase of $K_1$ and $k_2$ in cortical regions indicates that the rate of ligand exchange between plasma and tissue is significantly underestimated without PVE correction. $k_3$ was increased after PVE correction in brain regions, which are known to contain moderate to high densities of $D_2/D_3$ receptors. As $k_3$ is the most closely related to $B_{\text{max}}$, it can be assumed that the density of $D_2/D_3$ receptors would be underestimated in these regions if PVE correction was not applied.

### 4.3.4. Binding potential

Binding potential was calculated as $k_3/k_4$ for both radioligands. The effect of PVE correction on BP of $[^{11}\text{C}]$FLB 457 varied between regions to a great extent. Statistical analysis revealed that PVE correction induced a significant increase in BP in temporal and insular cortices, medial and posterior thalamus. The rank order of regional BP values was slightly changed after PVE correction.

PVE correction had a significant effect on BP of $[^{11}\text{C}]$raclopride in all subregions of the striatum. BP was increased by 30-50% after PVE correction, and the highest increase was observed in the ventral striatum. SD of BP was substantially increased in all regions after PVE correction for $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride.

Changes in BP of $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride after PVE correction were in accordance with changes of the rate constants. Increase of BP also indicated that density of $D_2/D_3$ receptors was underestimated without PVE correction in a few brain regions. No correlation was found between the volume of brain regions and the increase of BP after PVE correction. It is therefore not possible to predict in advance the exact degree of underestimation of BP due to PVE, the correction has to be performed for each individual.

### 4.3.5. Total volume of distribution

The total volume of distribution ($DV_{\text{tot}}$) was calculated as a complex of rate constants, as defined above. PVE correction had a substantial influence on $DV_{\text{tot}}$ of $[^{11}\text{C}]$FLB 457 and $[^{11}\text{C}]$raclopride.

For $[^{11}\text{C}]$FLB 457 the smallest increase of $DV_{\text{tot}}$ was observed in the lateral thalamus (8%), whereas the highest increase was measured in the posterior thalamus and the parietal cortex (54% and 57%, respectively). SD of $DV_{\text{tot}}$ was also increased after PVE correction for $[^{11}\text{C}]$FLB 457. No correlation was found between regional volumes and rate of increase of $DV_{\text{tot}}$ after PVE correction in any extrastriatal brain areas.
DV<sub>tot</sub> of [<sup>11</sup>C]raclopride was significantly increased in all subregions of the striatum after PVE correction. The relationship between volume of brain structures and PVE correction induced increase of DV<sub>tot</sub> was examined by linear regression. In the ventral striatum no correlation was found between the two parameters. In the dorsal part of putamen and that of caudate, however, there was a tendency towards significant negative correlation between volume of the structures and the change of DV<sub>tot</sub> after PVE correction.

Increase of BP after PVE correction partly explains the increase of total distribution volume of [<sup>11</sup>C]FLB 457 and [<sup>11</sup>C]raclopride. For most regions, higher K<sub>1</sub> values also contributed to the measured substantial increase of DV<sub>tot</sub>. An explanation to the large increase of DV<sub>tot</sub> after PVE correction could be deduced from the pharmacological interpretation of the parameter, which states that DV<sub>tot</sub> is equal to the ratio of the radioligand concentration in

Figure 14. Rate constants, BP and DV<sub>tot</sub> of [<sup>11</sup>C]raclopride before and after PVE correction. Observed values are denoted by empty signs (mean-SD), whereas PVE corrected values are represented by filled signs (mean+SD). The brain regions examined are: 1. ventral striatum, 2. dorsal caudate, 3. dorsal putamen. *, Significant difference between observed and PVE corrected values, (p<0.05, Bonferroni corrected).
tissue to plasma at equilibrium. As radioactivity concentration is compromised by PVE in tissue, but not in plasma, their ratio would be underestimated without correcting tissue radioactivity concentration for PVE. The degree to which tissue radioactivity concentration is compromised by PVE depends largely on the regional RC, which is, in turn, determined by the volume of the region, provided that the shape of the region is rather simple. This explains the tendency for significant negative correlation between volume of a region and the rate of increase in \(DV_{tot}\) for striatal subregions.

4.3.6. Application of PVE correction in clinical PET studies

Results of the present study demonstrated that PVE has sizeable effect on the observed TACs and binding parameters of \([^{11}C]\)FLB 457 and \([^{11}C]\)raclopride. Therefore the application of PVE correction is highly recommended in all PET studies when volumetric differences are present between the subjects or the groups compared, i.e. for the examination of \(D_2/D_3\) receptors in neurodegenerative disorders or during aging. In such PET studies, PVE correction can compensate for the decrease in observed radioactivity concentration caused by regional brain atrophy in comparisons with healthy subjects.

Application of PVE correction could also be useful in studies where the true values of receptor-binding parameters are of interest, for example in calculation of receptor density (\(B_{max}\)) and ligand affinity (\(K_d\)) in Scatchard analysis.

In routine clinical studies with \([^{11}C]\)FLB 457 and \([^{11}C]\)raclopride, where no between-group differences are detected in the volume of the brain regions, PVE correction is not essential since the same degree of underestimation of the binding parameters can be expected in both groups. Nevertheless, PVE correction could be useful for the detection of small changes in radioligand binding by improving the contrast between different regions of the brain. Previous studies (Kessler et al. 1984) have demonstrated that the true increase in radioactivity concentration is higher than the observed increase, and thereby small changes in radioactivity concentration might be undetectable without PVE correction. PVE correction can therefore help to detect small changes in radioligand binding in patients compared to control subjects, which would otherwise be overlooked. In the present study only healthy comparison subjects were examined; and thus, further studies are needed to clarify this question.

Some aspects of PVE correction, however, might limit its application in clinical PET studies. One such aspect is the inherent depreciation of precision. The higher SD of receptor binding parameters after PVE correction could hinder the detection of between group differences. The other limitation of PVE correction is that its results are influenced by segmentation errors. Such errors propagate through the entire process of analysis and can finally lead to incorrect estimates for receptor binding parameters. This phenomenon can be observed in the present study for hippocampus, ventral striatum and cerebellum. PVE corrected \(K_1\) of hippocampus and ventral striatum is lower than \(K_1\) in other regions, probably because of segmentation errors, i.e. WM was included in the ROI of these structures. It was not possible to completely separate GM and WM in the cerebellar hemispheres. Therefore, cerebellum was considered as a large GM region by the correction algorithm, which resulted in incomplete PVE correction. PVE corrected TAC of the cerebellum does not give a reliable estimate of nonspecific binding, which prohibits the application of reference tissue methods for the calculation of BP for \([^{11}C]\)FLB 457 and \([^{11}C]\)raclopride. Arterial input function is necessary for the calculation of receptor binding parameters after PVE correction, which might not always be accomplishable in clinical PET studies. Thus, in clinical studies the benefits of PVE correction (higher accuracy of parameters and improved contrast between brain regions) and its drawbacks (loss of precision and necessity of arterial blood sampling) must be considered at the same time.
5. SUMMARY AND FUTURE PROSPECTS

The present thesis focused on examining three thus far unexplored aspects of dopamine-D_2/D_3 receptors by the application of newly established techniques.

In Paper I, the first application of agonist stimulated [³⁵S]GTPγS binding autoradiography is reported for studies on D_2/D_3 receptors in the human brain. The present study has shown that this technique is suitable for the examination of dopamine-D_2/D_3 receptors in the postmortem human brain. The functional response to DA, the physiological agonist, and to quinpirole, a prototype D_2/D_3 agonist, was described in human whole hemisphere cryosections. The results indicated that this method could be a suitable tool for the examination of coupling between D_2/D_3 receptors and G proteins in neuropsychiatric diseases. Alterations of the interaction between D_2/D_3 receptors and G proteins were suggested in disorders such as schizophrenia or Parkinson disease. DA stimulated [³⁵S]GTPγS binding autoradiography could thus be a potential method to examine the function of D_2/D_3 receptors and G proteins in these conditions.

A drawback of agonist stimulated [³⁵S]GTPγS binding autoradiography is that it is limited to the examination of receptors coupled to pertussis toxin-sensitive G proteins (Happe et al. 2001; Sim et al. 1995). The inability to detect receptor stimulation through G_s is probably due to the slower dissociation of GDP from G_s compared to G_o and G_i (Carty & Iyengar, 1994; Weiland & Jakobs, 1994). Therefore D_1 and D_3 receptors cannot be studied today using this technique. For the functional examination of D_1/D_3 receptors, the development of new techniques is necessary or, if possible, the modification of the present assay is needed.

It is becoming increasingly evident that molecules involved in signal transduction are not randomly scattered in the cell membrane and within the cytosol, but are organised in functional compartments of the cell, termed caveolae. Plasmalemmal caveolae are specialized microdomains in the cell, enriched in a large number of signaling molecules, including GPCR, and G proteins and their effectors, such as protein-kinases, adenylyl cyclase and molecules involved in Ca^{2+} dependent signaling (reviewed in Shaul & Anderson, 1998). Caveolae are rather stable structures, for example they are resistant to solubilisation by Triton X-100. This raises the possibility that signalling pathways of GPCR could be studied by autoradiographic methods even beyond the interaction with G proteins. Naturally, examination of further steps of the signalling pathway involves a number of difficulties, such as synthesis of suitable radioligands and the establishment of the appropriate assay conditions. The radioligands, such as [³⁵S]GTPγS, should be analogues of the endogenous substrates but must be resistant to metabolism. Establishment of the appropriate assay conditions might be difficult, since in vitro interaction of several molecules would be necessary for the activation of effectors. It is questionable whether all these molecules can be preserved in tissue sections in such a state that ensures possible interaction with other signalling molecules. On the other hand, establishment of an assay capable of examining the activation of second messenger systems and effectors in situ in tissue sections, could clarify many so far unknown aspects of intracellular signalling of GPCRs.

Paper II and III report an attempt on the selective in vivo labelling of dopamine-D_3 receptors in the brain with PET. The binding characteristics of the putative D_3-selective radioligand, [¹¹C]RGH-1756 were described in the monkey brain. Previous in vitro radioligand binding studies have demonstrated high-affinity and high selectivity of RGH-1756 binding to D_3 receptors (Kiss et al. 2000; Laszlovszky et al. 2000). Despite the promising in vitro characteristics of the molecule, [¹¹C]RGH-1756 yielded very low signal for specific D_3 binding in the monkey brain. Pretreatment with unlabelled RGH-1756 inhibited
binding of the radioligand, suggesting saturable binding of $^{[11]}\text{C}]\text{RGH-1756}$. After administration of raclopride, the binding of $^{[11]}\text{C}]\text{RGH-1756}$ was slightly lower, which could indicate that $^{[11]}\text{C}]\text{RGH-1756}$ binds to $D_2/D_3$ receptors in the monkey brain. The signal for specific binding of $^{[11]}\text{C}]\text{RGH-1756}$ was, however, so small that observed effects of the pretreatment drugs could be confounded by noise of the measurements. The low specific binding of $^{[11]}\text{C}]\text{RGH-1756}$ was somewhat unexpected, as RGH-1756 showed favourable binding characteristics in previous in vitro experiments. It has been suggested that $D_3$ receptors are occupied by endogenous DA to a high degree (Levant, 1995; Schotte et al. 1992, 1996). As DA has very high affinity for the $D_3$ receptor (Sokoloff et al. 1990), it is possible that the endogenous transmitter could prevent the binding of $^{[11]}\text{C}]\text{RGH-1756}$ to $D_3$ receptors. To test this hypothesis, the effect of reserpine induced DA depletion was examined on the binding of $^{[11]}\text{C}]\text{RGH-1756}$ in the monkey brain. After reserpine treatment, there was no consistent increase in specific binding of $^{[11]}\text{C}]\text{RGH-1756}$. This observation does not support the assumption that binding of $^{[11]}\text{C}]\text{RGH-1756}$ to $D_3$ receptors is inhibited by high occupancy of $D_3$ receptors by endogenous DA. The possible reason for low specific binding of $^{[11]}\text{C}]\text{RGH-1756}$ is, therefore, the low in vivo affinity of the compound. Despite the promising in vitro results, $^{[11]}\text{C}]\text{RGH-1756}$ is not suitable for in vivo examination of $D_3$ receptors. The results of the studies with $^{[11]}\text{C}]\text{RGH-1756}$ point out that labelling of receptors with such low densities as the $D_3$ receptors requires ligands with very high affinity and selectivity.

Several radioligands have previously been considered for in vitro imaging of the $D_3$ receptor. $^{[3]}\text{H}]7$-OH-DPAT and $^{[3]}\text{H}]\text{PD 128907}$, two partial agonists, have been most extensively used in studies on the $D_3$ receptor. $7$-OH-DPAT has about 78 times higher affinity for $D_3$ than for $D_2$ receptors (Table 3, Levesque et al. 1992). PD 128907 is somewhat less selective, as it has about 14 times higher affinity for $D_3$ than for $D_2$ receptors (Pugsley et al. 1995). The selectivity of pramipexole, another $D_3$ preferring agonist used in patients with Parkinson disease, is similar to that of PD 128907 (Camacho-Ochoa et al. 1995). None of these $D_3$ preferring compounds has been used as a radioligand in in vivo imaging studies. However, their affinity and selectivity profiles in comparison to that of RGH-1756 suggest that they are probably not ideal for in vivo imaging of the $D_3$ receptor. Therefore, the development of novel compounds is probably necessary for selective in vivo labelling of $D_3$ receptors.

In papers IV and V, the influence of PVE was estimated on the quantification of $^{[11]}\text{C}]\text{FLB 457}$ and $^{[11]}\text{C}]\text{raclopride}$ binding in the human brain. Changes of the kinetic rate constants, $BP$ and $DV_{\text{tot}}$ of the two radioligands, as derived from the standard 2-TCM, were evaluated in the brain after PVE correction. PVE correction altered the amplitude and the shape of TACs of $^{[11]}\text{C}]\text{FLB 457}$ and $^{[11]}\text{C}]\text{raclopride}$ in most brain regions, which was reflected in the kinetic rate constants and the derived receptor binding parameters. The effect of PVE correction was different for the two $D_2/D_3$ specific radioligands due to the different levels of radioligand uptake in distinct regions of the brain. Observed binding of $^{[11]}\text{C}]\text{FLB 457}$ was extremely high in the striatum and moderate or low, yet measurable in extrastriatal regions. The effect of PVE correction on parameters of $^{[11]}\text{C}]\text{FLB 457}$ binding varied to a large extent between the extrastriatal regions examined, depending on the size, shape and the $D_2/D_3$ receptor density of the regions. $^{[11]}\text{C}]\text{raclopride}$ uptake reached high level in the striatum, but radioligand concentration was very low in all other brain areas. The influence of PVE correction on binding parameters of $^{[11]}\text{C}]\text{raclopride}$ was similar for the three examined subdivisions of the striatum.
could be an important factor for the degree of underestimation of observed radioactivity concentration as compared to the true values. However, spill-in of radioactivity from surrounding tissue compartments also contributed to the observed regional radioactivity concentration. This implies that knowing the volume of a region within the brain is insufficient to draw conclusions about the resulting PVEs. Interactions between the different regions have to be considered, as well. In line with the previous statement, no significant correlation was found between regional volume and regional increase of either BP or $D_{V_{tot}}$ after PVE correction. Although a tendency was observed towards a negative correlation between regional volume and increase of $D_{V_{tot}}$ for $[^{11}C]$raclopride after PVE correction in the dorsal putamen. Volume of brain regions, however, is a poor predictor of changes in binding parameters after PVE correction. It is therefore not possible to predict the effect of PVE on regional binding parameters, and PVE correction has to be performed in each individual case.

In the present work some recommendations were also made on the application of PVE correction in clinical PET studies. Both the advantages and drawbacks of PVE correction have to be considered when PVE correction is applied in PET studies. The advantages are increased accuracy of measurements and improved inter-regional contrast of radioligand uptake within the brain. The disadvantages are the loss of precision of the measurements and the susceptibility of the PVE correction algorithm to segmentation errors.

For the future, the development of such PVE correction algorithms would be beneficial, which are free from drawbacks of the presently used method. Amplification of noise is a huge problem of the currently used PVE correction method, as it can hinder quantification of radioligand uptake in small regions. Some errors in PVE corrected TACs of $[^{11}C]$raclopride and $[^{11}C]$FLB 457 were also observed using the present algorithm, most likely due to inaccurate segmentation of gray and white matter. To solve this problem the development of a new method would be necessary, which could handle tissue fraction effects better than the present method.
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