ASSESSMENT OF PHARMACOKINETICS AND PHARMACODYNAMICS OF PSYCHOACTIVE DRUGS USING BRAIN MICRODIALYSIS

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

Pooja Sood

Department of Cell Physiology and Pharmacology

University of Leicester

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ABSTRACT

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In order to assess pharmacokinetics and pharmacodynamics (PK/PD) accurately, it is necessary to obtain measurements of the absolute concentrations of compounds in the brain. A major shortcoming of using microdialysis to measure PK/PD is that microdialysis measurements do not give us absolute concentrations of solutes in the brain, since the relationship between dialysate concentrations and true extracellular fluid (ecf) concentrations surrounding the probe is unknown. Several methods have been devised to circumvent this problem. The present study employed a novel method, MetaQuant (MQ) microdialysis, which achieves near 100% recovery, and so enables the measurement of absolute ecf concentrations. I examined the effect of the D_4 receptor agonist, PD168077 on extracellular dopamine levels (that is PD) in medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) of freely moving rats, while simultaneously measuring brain concentrations (that is PK) of the drug. Thus we were able to estimate the PK/PD profile of the drug in the two brain regions. Compared with basal extracellular levels, subcutaneous administration of PD168077 caused significant increase in dopamine in mPFC. Activation of dopamine D_4 receptors in the mPFC may improve cognitive function, which is highly impaired in individuals with schizophrenia. Moreover, it has been consistently shown that phencyclidine (PCP) produces robust cognitive disruption, in a novel object recognition (NOR) test. I studied the efficacy of PD168077 to attenuate sub-chronic PCP induced deficit in the NOR task. Sub-chronic PCP induced a robust cognitive disruption and PD168077 (10 mg/kg, s.c. dose) reversed this disruption. Further MQ dialysis data showed that PD168077 (10 mg/kg, s.c. dose) increased dopamine levels in mPFC that was depleted due to PCP suggesting a mechanism for the observed alleviation of PCP induced cognitive deficits.

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DOPAC	3,4-Dihydroxyphenylacetic acid
5HIAA	5-hydroxyindolacetic acid
5HT	5-Hydroxytryptamine
GABA	γ-Aminobutyric acid
ACSF	Artificial cerebrospinal fluid
BBB	Blood brain barrier
CaMKII	Calmodulin – dependent kinase II
COMT	Catechol-O-methyl transferase
CNS	Central Nervous System
DAT	Dopamine transporter
DI	Discrimination index
E_d	Extraction fraction
ECF	Extracellular fluid
HPLC ECD	High performance liquid chromatography with electrochemical detection
HVA	Homovanillic acid
I.P	Intraperitoneal
IS	internal standard
ITI	Inter Trial Interval
L-Dopa	L-3,4-dihydroxyphenylalanine
LC-MS	Liquid chromatography with mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LSD	Lysergic acid diethylamide
MS	Mass Spectrometry
MAO	Monoamine oxidase
mPFC	medial Prefrontal cortex
MQ	MetaQuant
NAc	Nucleus Accumbens
NMDA	N-methyl-D-aspartic acid
NNF	No net flux
NOR	Novel object recognition

PET	Positron emission tomography
PPI	Pre pulse inhibition
R	Recovery
S.C	Subcutaneous
TIS	Turbo ion spray
VTA	Ventral tegmental area
VMAT	Vesicular monoamine transporter
UHPLC	Ultra high performance liquid chromatography

Chapter 1: General Introduction

1.1 Summary of thesis and thesis introduction

The main objective of the current thesis was to measure absolute concentrations of compounds in the brain using intracerebral microdialysis to investigate the pharmacokinetic (PK) and pharmacodynamic (PD) profile of novel central nervous system (CNS) drugs. Intracerebral microdialysis measures concentrations of compounds in dialysate samples which reflect, but are not identical to, concentrations in the brain extracellular fluid (ecf). The main reason why one needs to measure the true absolute ecf concentrations of drugs is that it is this concentration which provides a critical insight into PK (drug distribution at the target site) and also PD (relationship to its effect). This is important because the extracellular space is the biophase for a multitude of drugs that act on cell-surface bound receptors. The initial experiments of this current research project were carried out to evaluate and validate quantitative methods to measure absolute/true extracellular concentrations of compounds in the brain area of interest.

Two novel approaches were used: dual probe no net flux (NNF) and ultra slow flow using MetaQuant (MQ) microdialysis. *In vitro* calibration experiments showed that dual probe NNF and MQ dialysis yielded absolute concentration of compounds surrounding the dialysis probe tip. Moreover, dual probe NNF and MQ probes gave similar ecf concentrations of amphetamine in the caudate nucleus after 0.5 mg/kg intraperitoneal (i.p.) injection of amphetamine in anaesthetised rats. Furthermore, MQ probes also gave absolute levels of dopamine in the caudate nucleus simultaneously. However, the dual probe NNF method was limited in terms of dopamine levels as it required perfusion of amphetamine through one of the probes which interfered with basal dopamine levels (chapter 3).

The MQ probes were then used to expand the knowledge of D_4 dopamine receptor activation in freely moving rats. Dopamine receptors are the principal targets of drugs used to treat schizophrenia. Among the five mammalian dopamine receptor subtypes, the D_4 subtype is of particular interest because of its high affinity for the atypical antipsychotic drug. I studied the *in vivo* effects of a dopamine D_4 receptor agonist, PD168077. The absolute ecf concentration of PD168077 and its effect on dopamine release were measured in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) simultaneously in the same animals. The results showed that at low doses of 0.1 and 0.5 mg/kg subcutaneous (s.c.) injection, PD168077 induced dopamine release in mPFC and in NAc, with higher increases seen in mPFC (Chapter 4). The behavioural consequence of PD168077 induced release of dopamine was then tested in the novel object recognition (NOR) task. The NOR task is based on the natural tendency of rats to investigate a novel object rather than a familiar object when both are simultaneously present. Thus it tests the integrity of episodic memory: that is the information about events that have happened in the recent past. The results showed that after a 6 hour inter trial interval (ITI), rats treated with the highest dose tested (10 mg/kg) PD168077, explored the novel object more than the familiar object, which was not seen in the control rats. These data suggest that PD168077 improves natural episodic memory. Subsequently, the absolute ecf levels of PD168077 and dopamine release were studied at the high dose of 10 mg/kg dose. The results showed sustained increases in dopamine levels were higher in mPFC compared to NAc in comparison to their respective basal levels. I propose that the episodic memory enhancing property of PD168077 is due to its dopamine releasing action (chapter 5).

A second set of experiments tested the effect of PD168077 on the disrupted NOR, induced by pre-treatment with phencyclidine (PCP), a psycho-pharmacological model for cognitive deficits in schizophrenia. The results showed that PD168077 at 10 mg/kg s.c. dose, reversed PCP induced disruption of episodic memory. Again, absolute ecf levels of PD168077 and dopamine were measured with MQ probes in sub-chronic PCP pre-treated rats, and showed that sub chronic PCP caused a reduction in the basal dopamine levels which was more pronounced in mPFC than in the NAc. Moreover, PD168077 administration to sub-chronic PCP pre-treated rats resulted in an increase in dopamine levels compared to the basal dopamine levels in the sub-chronic PCP pre-treated rats (chapter 6). In conclusion, I propose that PD168077 increases dopamine levels and reverses dopamine depletion predominantly in mPFC, in sub-chronic PCP pre-treated animals.

In addition to the above expansion of knowledge in terms of D_4 receptor function, the results presented in this thesis evaluated the PK and PD of PD168077 in rats. The brain distribution of PD168077 (10 mg/kg) was significantly different in mPFC and NAc. However, the brain distribution of PD168077 did not differ in PCP pretreated rats. By measuring absolute ecf concentrations using MQ probes I can for the first time, attain the PK/PD profile of a novel drug. Thus, the absolute concentration measured by MQ probes can contribute hugely in studies that characterise the PK/PD profile of a novel CNS compound.

1.2 Part 1: Introduction of intracerebral microdialysis

1.2.1 Intracerebral Microdialysis

Intracerebral microdialysis has become a popular tool for monitoring the extracellular concentrations of compounds in different parts of the brain in many disciplines, such as neurobiology, neurochemistry (Smith and Sharp 1994; see review by Bourne, 2003) and neuro-pharmacology (deLange *et al*., 2000; Chefer *et al*., 2003). The technique, albeit invasive, allows neuroscientists to observe chemical events in the brain of anesthetised and of freely moving animals during pharmacological and/or behavioural stimulation. It is mainly utilised in sampling the chemical components of the fluid in the extracellular space of tissues (ecf) (see review by Young 1993 for example). It not only allows measurement of endogenous substances in the brain (e.g., neurotransmitters and their metabolites), but also allows the application of exogenous substances (e.g., neuroactive drugs) to the tissue by retrodialysis. (Ungerstedt and Hallstrom 1987; Benveniste 1989; Bouw and Hammarlund-Udenaes 1998; Fillenz 2005).

The main feature of microdialysis comes from its ability to sample the neurochemical components of the ecf without causing any change to the ecf volume *per se*. The ecf space of the brain plays an important role as a common communication channel for chemical signalling between (1) neurones and other neurones, (2) neurones and glial cells as well as for (3) neurovascular communication. The ecf compartment under normal physiological conditions comprises 20% of the total brain volume (see review by Nicholson and Sykova 1998). In a simplified model defined by Nicholson (2001), the passive diffusion of molecules in the brain ecf is driven by the concentration gradients existing between

the delivery (e.g., release and active transport) sites and the clearance (e.g., uptake, metabolism, and transport) sites for a particular molecule and governs the propagation of chemical signals within the brain microenvironment (Nicholson and Rice 1986). Therefore brain microdialysis provides valuable insight into neurotransmitter levels (Parsons and Justice 1994) and effect of drugs on these levels (Justice 1993; Westerink 1995).

1.2.1 Development of microdialysis

Early efforts to study neurotransmitter levels in the brain were principally carried out by perfusion of the extracellular space: for example methods such as cortical cup perfusion and push-pull perfusion were used (Moroni and Pepeu 1984; Myers 1986). The application of such techniques was often hampered by the challenge of detecting the trace amounts of transmitters in the samples using the relatively insensitive analytical methods available at that time. The first intracerebral microdialysis procedure was carried out by Bito *et al.,* in 1966, who implanted the first dialysis sacs containing 6% dextran in saline into the parenchyma of the cerebral hemispheres of dogs for ten weeks and then analysed the contents of the sac for amino acids *post mortem*. These experiments introduced the idea of sampling a "compartment" surrounded by a dialysis membrane based on the principle that the fluid in the dialysis sac equilibrates with the chemical content of the extracellular compartment. The intention of these early dialysis studies was to reach an average concentration of the substance of interest and not to follow the changes over time.

Taking it one step further, Zetterstrom *et al.,* 1982 developed thin dialysis tubes, so called hollow fibres that were implanted into the brain by drilling holes in each

temporal bone of the rats and then guided the fibre through the brain. Their idea and design of the hollow fibre was to make it function like the blood vessels and that will be devoid of a blood brain barrier when implanted in the brain. They monitored baseline and amphetamine stimulated release of preloaded [^{3}H] dopamine in anaesthetised animals. Ungerstedt and Pycock in 1974 used microdialysis in freely moving animals to study the release of preloaded [^{3}H] dopamine correlated with behavioural activation. On the basis of these early studies, different microdialysis probe types have been designed (horizontal probes, loop shaped probes and concentric probes) and development in analytical techniques have enabled neurotransmitter measurements to be made in localised brain regions (*figure 1.1*).

1.2.3 Principle of microdialysis

The principle of microdialysis is very simple. A cylindrical semi permeable membrane is introduced into the tissue, and is perfused continuously with a fluid of similar composition to the fluid that surrounds the tube outside. By diffusion compounds pass the semi permeable membrane in either direction, driven by the concentration gradient of each compound across the membrane. Therefore, dialysis is the passive movement of molecules across the membrane, down a concentration gradient. A basic microdialysis system consists of a syringe pump for perfusate delivery, a microdialysis probe, the connecting tubes and vials to collect dialysate samples (*figure 1.1*).

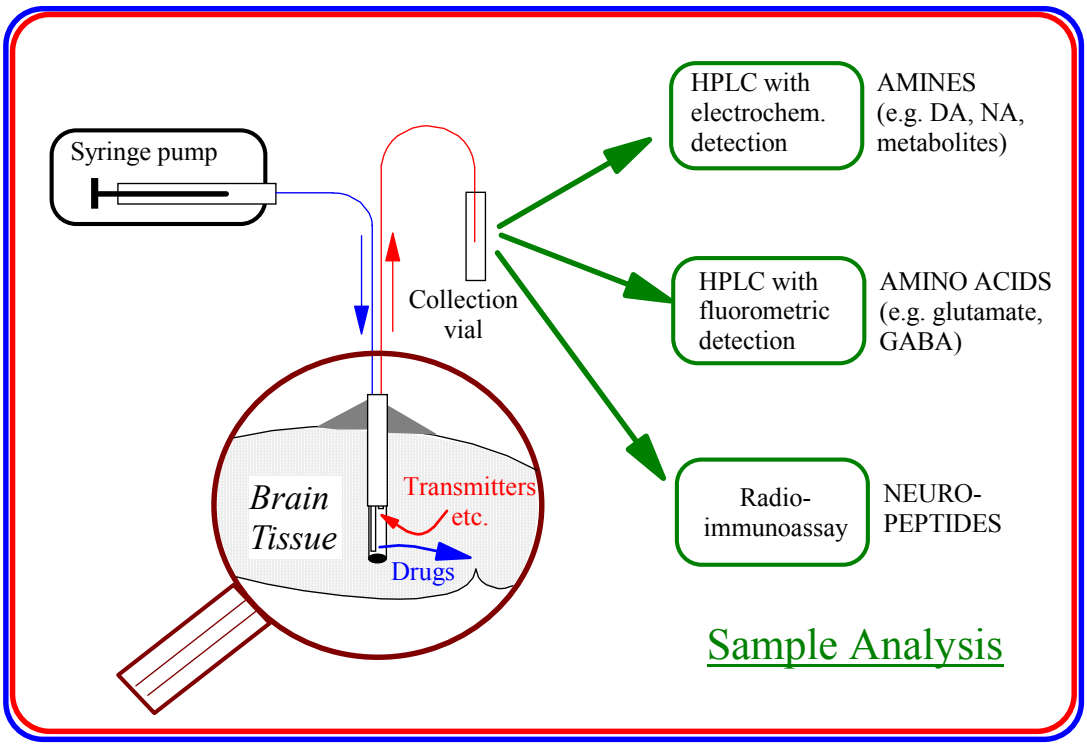

Figure 1.1: A microdialysis experimental set up. The probe can be placed in vitro or implanted in vivo (Young unpublished).

Of the three probe types mentioned (horizontal, loop, concentric), I will focus on concentric type. For the monoprobes used in the experiments reported in this thesis, the probe tip comprises a cylinder of semi-permeable membrane which is sealed at one end and has two tubes (inlet and outlet) inserted in it. The dialysis membranes used in probe construction have different cut off parameters, depending on the molecular weight of the compound to be measured. Typical probe membrane cut off is between 10 and 60 kDa. During the dialysis procedure, a physiologically compatible perfusion fluid (perfusate) such as artificial cerebrospinal fluid (aCSF) is delivered through the probe at a low and constant flow rate (typically ranging between 1 to 10 µl/min). The exchange of compounds occurs in both directions

across the semi-permeable membrane of the probe depending on the direction of the concentration gradients for each compound: diffusion occurs from the region higher concentration to the region of lower down the concentration gradient. For a perfusion fluid that is initially devoid of the compound of interest, the final dialysate concentration obtained is only a fraction of the tissue extracellular diffusible (unbound) level in the ecf surrounding the probe. This fraction is referred to as the relative recovery or extraction fraction (E_d) (Ungerstedt and Hallstrom 1987). The relative recovery is the concentration of a particular substance in the perfusate when it leaves the probe expressed as a percentage value of the concentration in the surrounding medium. When a microdialysis probe is tested *in vitro*, relative recovery decreases as the perfusion flow increases. The recovery reaches a plateau, when the diffusion speed through the surrounding medium reaches its maximum. The relative recovery can be calculated as in equation 1. On this basis, for a perfusion fluid that contains a compound of interest, to deliver exogenous compounds to the tissue, the relative recovery can be calculated using equation 2.

Equation 1: $R = C_{out}/C_{ecf}$, where $C_{in} = 0$;

Equation 2: $R = C_{in}\text{-}C_{out}/C_{in}\text{-}C_{ecf}$

Where, C_{in} = concentration of a compound perfused through the probe,

C_{out} = concentration of a compound that is obtained from the probe,

C_{ecf} = external concentration surrounding the probe tip.

R = relative recovery

In neurochemical studies which examine changes in the levels of endogenous compounds from their baseline values, it may not be necessary to determine the relative recovery, and it is reasonable to assume that recovery remains relatively constant throughout the experiment. These studies typically evaluate percentage changes from a baseline level after pharmacological intervention (e.g., the administration of a drug). However, in PK investigations, where determining true brain concentration of drugs is the prime aim, the knowledge of the relative recovery becomes crucial for the determination of true (absolute) extracellular tissue concentrations (see part 2 of this section). In studies that require the knowledge of true unbound concentrations of endogenous compounds, quantitative microdialysis methods are needed (Morrison *et al*., 1991 a and b).

1.2.4 Considerations in performing and interpreting microdialysis data

In addition to these pre existing factors, changes in dialysis dynamics arise from interactions between the dialysis probe, the perfusion fluid and the living tissue which must also be considered when performing microdialysis.

1.2.4.1 Tissue disturbance, damage and gliosis

There is an initial period of disturbed tissue function after probe implantation characterised by increased glucose metabolism and decreased blood flow (Benveniste *et al*., 1987 and 1989). An initial 60 min stabilisation period before dialysis perfusion is allowed to get a steady baseline before collecting basal samples.

1.2.4.2 Composition of perfusion fluid

The dialysis probe samples the ecf by the principle of diffusion along the concentration gradient into or out of the probe. The direction of this gradient depends on the composition of the perfusion fluid. The composition of the perfusion fluid influences the immediate surroundings of the probe. If, for example, calcium is excluded from the perfusion fluid then the ecf surrounding the probe is depleted of calcium, causing impaired synaptic transmission (Westerink and deVries 1988). On the other hand, including a compound in the perfusion fluid will make it diffuse out from the probe into the tissue along a concentration gradient. This means that the composition of the perfusion fluid should be as close as possible to the normal physiological levels of most of the essential compounds in the ecf (Moghaddam and Bunney 1989).

1.2.5 Advantages and disadvantages of microdialysis

The advantages of microdialysis include that microdialysis sampling does not change the net fluid balance of the surrounding tissue. Because there is no net fluid loss or gain, samples can be collected continuously for hours or days from a single freely moving animal. Most important, each animal can serve as its own control, allowing within subject statistical analysis, so microdialysis requires fewer subjects in comparison to some other methods, such as direct tissue assay. Moreover, microdialysis can be easily coupled with other chemical analyses, such as high-performance liquid chromatography (HPLC), mass spectrometry (MS), and capillary electrophoresis, to allow measurement of a wide range of different chemical types. In addition to using microdialysis for recovering compounds from a tissue, it is also useful for administrating substance to the tissue. In sum, it can be

used for sampling endogenous compounds and for delivery of exogenous compounds to tissue of interest. The major features of microdialysis are:

- Although it is an invasive technique, studies indicate that microdialysis causes minimal damage to the blood brain barrier (Benvensite 1989).
- It allows sampling of the compound (s) of interest from the tissue of interest
- It samples the ecf rather than the whole tissue as in procedures such as biopsies or tissue assays.
- It can be performed in the intact tissue of the living, awake and freely moving animals, as distinct from preparations such as brain slices and synaptosomes.
- It is possible to sample continuously for hours or days in a single animal, and therefore decreases the number of animals needed in an experiment and the variability between subjects.
- It can be used for recovering endogenous compounds and/or delivering exogenous compounds to the tissue. It is possible to estimate the local drug concentration, when the drug given systemically and in addition drugs can be perfused by the probe to the area of interest: to monitor local biochemical effect resulting in physiological, pharmacological and behavioural response (see review by Benvensite and Huttemeier 1990).

As with any technique, there are limitations in the application of intracerebral microdialysis. The implantation of the probe leads to tissue reactions that can interfere with the physiological system under investigation and gliosis around the probe tip compromises measurements. For this reason microdialysis probes only remain viable for neurochemical measurements for 2-3 days after implantation

(Westerink and Tuinte 1986). Most importantly, the diluting effect of the dialysis procedure due to influence of flow rate leads to lower concentrations of compounds in samples compared to that in the surrounding tissue. This means that (1) highly sensitive analytical methods are required to measure compounds in the dialysate, and (2) the true ecf concentration of compounds cannot be calculated without knowing the *in vivo* recovery (chapter 2).

1.3 Dopaminergic neurotransmission

Whilst in principle many different neurotransmitters: for example serotonin, glutamate and dopamine can be measured with microdialysis (see above), the current thesis focused on monitoring dopamine dynamics in response to the psychostimulant drug such as amphetamine, and PD168077 a dopamine D_4 receptor agonist. In this section of the introduction, dopamine and its pathway in the brain will be reviewed.

1.3.1 Dopamine

Dopamine belongs to the catecholamine family of neurotransmitters; this family also includes noradrenaline and adrenaline. Dopamine serves a number of important functions in the mammalian brain, including control of locomotor activity, cognitive function, emotional and appetitive behaviours and regulation of hormones and transmitters. Dopamine is synthesised in two steps: the first step is the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) by enzymatic oxidation, catalysed by the enzyme tyrosine hydroxylase. The second step is the conversion of L-dopa to dopamine by the enzyme dopa decarboxylase (Nagatsu *et al*., 1964 a and b).

Once synthesised in the cytoplasm of the neurone, dopamine is transported into secretory vesicles for storage and subsequent release. When the neurone is stimulated, the dopamine storage vesicles fuse with the plasma membrane in a Ca^{2+}-dependent manner, releasing dopamine into the synaptic cleft. In the synaptic cleft, dopamine can bind to both postsynaptic dopamine receptors and presynaptic dopamine autoreceptors. Several mechanisms exist to clear dopamine from the synapses, to prevent chronic stimulation and eventual desensitisation of dopamine receptors. The main mechanism is transport of most of dopamine back into the presynaptic neurone by an 11-transmembrane domain protein that belongs to the family of catecholamine reuptake pumps. Once taken up into the presynaptic neurone, dopamine can be either recycled into vesicles for further use in neurotransmission or degraded by the action of the enzyme monoamine oxidase (MAO). The other mechanisms for removing extracellular dopamine that is not taken up into the presynaptic neurone are diffusion out of the synaptic cleft or degradation by the action of the enzyme catechol-O-methyl transferase (COMT). In the CNS, COMT is primarily expressed in the postsynaptic neurones, although it is also expressed in certain presynaptic neurones. The sequential action of MAO and COMT degrades dopamine to the metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and then subsequently to homovanillic acid (HVA), which is excreted in the urine (*figure 1.2*).

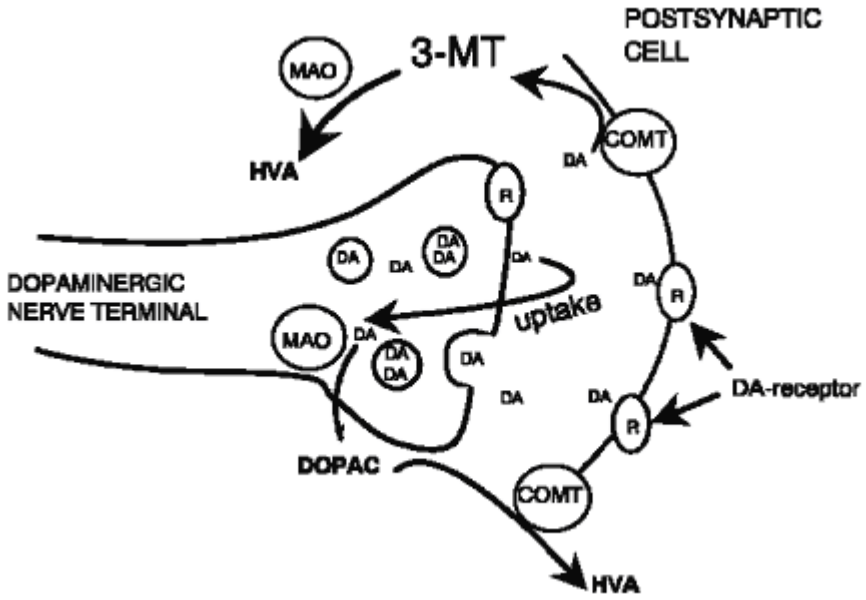

Figure 1.2: Metabolism of dopamine in the dopaminergic nerve terminal and at the synapse. Dopamine (DA), catechol-O-methyl transferase (COMT), monoamine oxidase (*MAO), 3-methoxytyramine (3-MT), homovanillic acid* (*HVA), 3, 4-dihydroxyphenylacetic acid (DOPAC) (figure taken from Feldman et al., 1997).*

1.3.2 Dopamine receptors

Dopamine receptors were first identified in the 1970s, based on their ability to bind to radio-labelled dopamine and other known dopaminergic agonists. Further research revealed two main classes of dopamine receptors, with different affinities for a range of agonists which had different effects on the production of the intracellular second messenger cAMP. The D_1 class of receptors mediate an increase in cAMP while the D_2 class of receptors cause a reduction in cAMP. The two classes can be further divided into five subtypes: the D_1 class of receptors consists of D_1 and D_5 subtypes and D_2 class of receptors consists of D_2, D_3 and D_4

subtypes. Subsequently many molecular biological and pharmacological studies have investigated the localisation and mechanism of action of different dopamine receptor types, which are reviewed in Oak *et al.,* 2000. Briefly, autoradiography studies using radiolabelled agonists and antagonists have helped to elucidate the distribution of dopamine receptor subtypes in the CNS. Although low levels of D_1 and D_2 receptor classes can be found throughout the brain. Both D_1 and D_2 receptors are expressed at high levels in the striatum (caudate nucleus and putamen), where they play a role in motor control by the basal ganglia, as well as in NAc, involved in the pharmacology of chemical dependence and addiction and the olfactory tubercle. D_2 receptors are also highly expressed on the anterior pituitary gland lactotrophs, where they regulate prolactin secretion.

D_2 receptors are thought to play a role in schizophrenia because many antipsychotic medications a have high affinity for these receptors, although the localization of D_2 receptors involved remains to be elucidated. D_3 and D_4 receptors are structurally and functionally related to D_2 receptors and may also be involved in the pathogenesis of schizophrenia. High levels of D_3 receptors are found in the limbic system, including the NAc and olfactory tubercle, while D_4 receptors are mainly localised in the frontal cortex, diencephalon and brainstem. D_5 receptors are distributed sparsely and expressed at low levels, mainly in the hippocampus and hypothalamus. Interestingly, the affinity of D_5 receptors for dopamine is 10-fold greater than that of D_1 receptors however the physiological significance of this finding is still unknown. Dopamine receptors can be found presynaptically on the dopaminergic neurones as well as postsynaptically on the target cells. The activation of presynaptic dopamine autoreceptors reduces dopaminergic tone, both by decreasing dopamine synthesis in the presynaptic neurone and by reducing the rate of neuronal firing. These

autoreceptors which belong to the D_2 class of receptors inhibit dopamine synthesis through down-regulation of cAMP –dependent tyrosine hydroxylase (TH) activity (see Missale *et al*., 1998 for a review).

1.3.3 Dopamine pathways and function

The majority of dopamine containing neurones originate in the midbrain, specifically in the substantia nigra pars compacta (A9 cell group) and the ventral tegmental area (VTA) (A10 cell group); (Gardner and Ashby 2000). These dopaminergic neurones form three main projections:

1) The *nigrostriatal pathway*, which contains 80% of the brain's dopamine, projects rostrally from the cell bodies in the substantia nigra to terminals in caudate nucleus and putamen (collectively termed the striatum).

2) The *mesolimbic pathway* originates from the VTA and projects to NAc, amygdala and hippocampus.

3) The *mesocortical pathway* originates from the VTA and projects to the medial frontal and cingulated cortices.

4) The *tuberoinfundibular* pathway projecting from the hypothalamic arcuate and periventricular nuclei to the posterior pituitary, where dopamine is released into the portal circulation and regulates synthesis and release of prolactin and gonadotropin in the anterior pituitary (reviewed in Ben-Jonathan and Hnasko 2001).

This present thesis concentrates on the terminals of the nigrostriatal pathway, mesolimbic pathway and mesocortical pathway.

A derangement in any of these dopaminergic systems can result in disease. The nigrostriatal pathway is primarily involved in regulation of motor function and is implicated in the pathophysiology of Parkinson's disease and in the extrapyramidal side effects of many antipsychotic medications (see review by Strange 2001). The mesolimbic pathway is believed to play a critical role in reward by both natural reinforcers (e.g. sexual behaviour or sucrose) and drugs of abuse, as it appears that both types of reinforcement activate mesolimbic dopamine neurones (Hajnal and Norgren 2001; Balfour 2004). The mesocortical pathway is involved in cognitive processes, including sensorimotor gating, working memory and attention (Daffner *et al*., 2000; Dias and Honey 2002). Dysregulation of dopaminergic signalling in this pathway is believed to underlie some of the sensorimotor gating and cognitive deficits that are characteristic of both schizophrenia and of psychostimulant-induced psychosis (e.g., Swerdlow *et al.*, 1990; Dolan *et al.*, 1995).

1.3.4 Schizophrenia

Schizophrenia is a severe mental disorder characterised by one or more episodes of psychosis, delusions, hallucinations, incoherence and physical agitation; it is classified as a "thought" disorder (Kraepelin 1971). Patients may exhibit disorders of perception, thinking, speech, emotion and or physical activity. Schizophrenic symptoms have been divided into three broad categories (for greater detail see McKenna 1997; Cowan *et al.,* 2000).

1) **Positive symptoms:** The positive symptoms are psychotic behaviours, not seen in healthy people. Patients with positive symptoms often have impaired relationship with reality. These symptoms can be generally described as following (see reviews by Lewis and Lieberman 2000):

- Hallucinations- The most prominent hallucinations in schizophrenia are auditory as the majority of positively symptomatic schizophrenia patients report hearing voices (Hoffman 1999). The voices may talk to the person about their behaviour, order the person to do things, or warn the person of danger. Sometimes the voices talk to each other. People with schizophrenia may hear voices for a long time before family and friends notice the problem. Other types of hallucinations include seeing people or objects that are not there, smelling odours that no one else detects, and feeling things like invisible fingers touching their bodies when no one is near (Copolov *et al.,* 2003).
- Delusions- described as false beliefs that are not part of the person's culture and these delusions do not change (e.g., Hemsley 1975; Gray *et al.,* 1991). The person believes in the delusions even after other people prove that the beliefs are not true or logical. People with schizophrenia can have delusions that seem bizarre, such as believing that neighbours can control their behaviour with magnetic waves. They may also believe that people on television are directing special messages to them, or that radio stations are broadcasting their thoughts aloud to others. Sometimes they believe they are someone else, such as a famous historical figure. They may have paranoid delusions and believe that others are trying to harm them, such as by cheating, harassing, poisoning, spying on, or plotting against them or the people they care about. These beliefs are called "delusions of persecution" (McCarley *et al.,* 1999).
- Thought disorders- described as unusual or dysfunctional ways of thinking. One form of thought disorder is called disorganised thinking. This is when a person has trouble organising his or her thoughts or connecting them logically. They may talk in a garbled way that is hard to understand. Another form is called thought

blocking. This is when a person stops speaking abruptly in the middle of a thought. When asked why he or she stopped talking, the person may say that it felt as if the thought had been taken out of his or her head. Finally, a person with a thought disorder might make up meaningless words (e.g., Nicholson and Neufeld 1993).

- Movement disorders- may appear as agitated body movements. A person with a movement disorder may repeat certain motions over and over. In the other extreme, a person may become catatonic. Catatonia is a state in which a person does not move and does not respond to others (e.g., Goldman *et al.,* 1999). Catatonia is rare today, but it was more common when treatment for schizophrenia was not available.

2) **Negative symptoms:** Negative symptoms are associated with disruptions to normal emotions and behaviours. These symptoms are harder to recognise as part of the disorder and can be mistaken for depression or other conditions (for review see Cools *et al.,* 1990). These symptoms include the following:

- Flat affect- a person's face does not move or show expressions or he/she talks in a dull or monotonous voice
- Anhedonia- a lack of pleasure in everyday life
- Lack of ability to begin and sustain planned activities
- Speaking little, even when forced to interact

People with negative symptoms need help with everyday tasks. They often neglect basic personal hygiene. This may make them seem lazy or unwilling to help themselves, but the problems are symptoms caused by the schizophrenia.

3) **Cognitive symptoms:** Cognitive symptoms often make it hard to lead a normal life and earn a living. They can cause great emotional distress. Cognitive symptoms are subtle. Like negative symptoms, cognitive symptoms may be difficult to recognise as part of the disorder. Often, they are detected only when other additional tests are performed (see review by Emilien 1991; Wong and Van Tol 2003 b). The cognitive symptoms include the following:

- Poor executive functioning- the ability to understand information and use it to make decisions
- Difficulty focusing or paying attention
- Problems with working memory- the ability to use information immediately after learning it

The cause of schizophrenia remains unclear but involves a combination of genetic (as there is a strong hereditary factor in its aetiology) and environmental factors (Swerdlow 1998; Tandon 1999). Some environment influences in the early development have been identified as possible predisposing factors, particularly maternal viral infections. Evidence suggests that schizophrenia is associated with a neuro-developmental disorder affecting mainly the cerebral cortex that occurs during first few months of prenatal development (see Harrison 1997). This view is supported by brain imaging studies showing cortical atrophy, with enlargement of the cerebral ventricles. These structural changes are only present in the early stage of schizophrenic brains and are probably not progressive, suggesting that the changes represent an early irreversible aberration in brain development rather than a gradual neurodegeneration. The studies of post mortem schizophrenic brains show evidence of misplaced cortical neurons with abnormal morphology (Driesen *et al*

2008). It appears to be through a combination of such genetic and development factors with social and environment factors that schizophrenia manifests in the patients.

1.3.4.1 Neurochemical theories underlying schizophrenia

Although many theories exist, the pathogenesis and pathophysiology of schizophrenia are not understood at a fundamental level (see review by Vallone *et al*., 2000 for example). Indeed, an intensive search for neurochemical abnormalities in schizophrenia proved frustrating for many years, as no biochemical markers were found either in *post mortem* brain or in other samples from living patients. More recently, imaging studies have succeeded in detecting abnormalities in neurotransmitter systems. The current ideas about the neurotransmitter systems involved in schizophrenia came mainly from pharmacology, by analysing the effects of antipsychotic drugs. The main neurochemical theories centre on dopamine; glutamate and serotonin (see Mortimer 2004).

1.3.4.1.1 Dopamine theory

The dopamine theory was proposed by Carlsson, who was awarded a Nobel Prize in Physiology and Medicine in 2000. The theory states that the illness is caused by increased and dysregulated levels of dopamine neurotransmission in the brain (Carlsson *et al.,* 2000). Although the dopamine hypothesis is based primarily on the therapeutic effectiveness of dopamine receptor antagonists in treating schizophrenia, it is supported by several additional clinical observations. Human subjects taking drugs such as amphetamine, cocaine and apomorphine that increase dopamine levels or activate dopamine receptors in the CNS produce behavioural syndromes which are very similar to an acute schizophrenic episode. Moreover, hallucinations,

delusion and thought disorder are a known side effect of L-Dopa therapy for Parkinson's disease. Finally, treatment with dopamine receptor-blocking antipsychotics and drugs that block neuronal dopamine storage (e.g. reserpine) are effective in controlling positive symptoms of schizophrenia and in preventing amphetamine-induced behavioural changes. There is a strong correlation between clinical antipsychotic potency and activity in blocking D_2 receptors (Seeman and Lee 1975; Seeman and Van Tol 1994). The studies with receptor imaging have shown that clinical efficacy of antipsychotic drugs is consistently achieved when D_2 receptor occupancy reaches 80% (Seeman and Van Tol 1994; Kapur and Remington 2001). However an exception to this reveals that when the D_2 receptor blockade exceeds 90%, up to one-third of schizophrenic patients fail to respond whereas in most people, atypical antipsychotic drug such as clozapine can be effective at a much lower level of blockade (Seeman *et al*., 1975; Seeman and Van Tol 1995). The interpretation of these studies is complicated by the fact that antipsychotic drug treatment is known to increase dopamine receptor expression. Among all the dopamine receptors, the increase in the dopamine D_4 receptors has attracted attention on the basis of the high degree of genetic polymorphism that it shows in human subjects. Converging evidence suggest that some of the newer antipsychotic drugs that act at dopamine D_2 receptors (e.g., clozapine) turn out to have a higher affinity for the D_4 receptor subtype than for other D_2 receptors (Van Tol *et al*., 1991 and 1992; Earnst *et al.,* 1999). However, genetic studies have failed to show any relationship between schizophrenia and D_4 receptor polymorphism (see Seeman 1994 a and b).

Another variant of the dopamine theory suggests that the dysregulation of dopamine in schizophrenia occurs at specific anatomical locations in the brain. The

mesolimbic pathway is involved in the development of emotions and memory and some studies hypothesise that mesolimbic hyperactivity is responsible for the positive symptoms of schizophrenia (Emilien *et al*., 1999 a and b). This hypothesis is supported by positron emission tomography (PET) scans of brains of patients displaying earliest signs of the disease. These PET images show changes in blood flow to the mesolimbic system that represents changes in the level of functioning of this system (see review by Carlsson 2000). On the other hand, mesocortical dopaminergic neurones that originate from VTA and project to the regions of cerebral cortex, particularly PFC, involved in attention, planning, and motivated behaviour are implicated in the symptomology of schizophrenia. Some studies specifically state that schizophrenia reflects an imbalance between excessive activation of D_2 receptors in the subcortical regions, causing positive symptoms and deficient activation of cortical D_1 receptors, causing negative symptoms. Although these hypotheses agree that dopamine is undoubtedly involved in the pathogenesis of schizophrenia, the details remain far from clear (see review by Wong and Van Tol 2003 b).

1.3.4.1.2 Glutamate theory

Another neurotransmitter implicated in the pathophysiology of schizophrenia is glutamate (Goff and Coyle 2001; Moghaddam and Jackson 2003). NMDA (N-methyl D-aspartate) receptor antagonists such as PCP, ketamine and dizocilpine (MK-801) produce psychotic symptoms such as thought disorder and hallucinations in humans. Further support for a role of glutamate comes from studies showing reduced glutamate concentrations and reduced glutamate receptor densities in post-mortem schizophrenic brains (Goff *et al*., 2001). Other evidence in support of the glutamate theory comes from transgenic mice studies, in which NMDA receptor

expression is reduced. These mice show stereotypic behaviours and reduced social interaction that are suggestive of schizophrenia and which are reduced by antipsychotic drugs (see review by Carlsson *et al.,* 2000).

According to this theory, glutamate and dopamine exert excitatory and inhibitory effects, respectively, on GABAergic striatal neurons, which project to the thalamus and constitute a sensory 'gate'. This sensory gate is disabled with too little glutamate or too much dopamine, allowing uninhibited sensory input to reach the cortex. It is also suggested that abnormal glutamate function, particularly reduced NMDA receptor activation accounts for the cognitive deficit that is increasingly recognised as a central feature of schizophrenia, responsible in part for the negative symptoms of the disease (see review by Wong and Van Tol 2003 b).

1.3.4.1.3 Other theories

Other neurotransmitters such as serotonin (5-hydroxytryptamine; 5HT) and noradrenaline may also be important. Involvement of 5HT dysfunction in schizophrenia is based on the fact that lysergic acid diethylamide (LSD) produces schizophrenic like symptoms (Busatto and Kerwin 1997). In addition, many effective antipsychotic drugs that block dopamine receptors also act as 5HT receptor antagonists. Many antipsychotics that are dopamine selective compounds that also exhibit activity at $5HT_{2A}$ receptors produce fewer extra-pyramidal side effects, although whether the combination with $5HT_{2A}$ receptors accounts directly for the therapeutic effect or merely reduces undesirable side effects associated with D_2 receptor antagonists, remains controversial. Moreover, 5HT modulates dopamine pathways so the two theories are not incompatible (Glennon *et al*., 1984; Gleeson 1989; Geyer 2001).

Amongst all these existing theories of schizophrenia, the dopamine hyperactivity theory remains most attractive. Although it is over simplified and only relates to the positive symptoms, it undoubtedly provides the best framework for understanding the action of antipsychotics. However, effects on 5HT and other receptors may contribute significantly to the clinical profile of some of the newer drugs (Schmidt *et al.,* 1997; Geyer 2001). Together, these theories that excess dopamine receptor activation is mainly responsible for positive symptoms, and deficient NMDA receptor activation for the negative and cognitive symptoms, are driving current efforts to develop novel antipsychotic drugs that increase NMDA receptor activation (see review by Wong and Van Tol 2003 a and b).

1.4 Animal models of schizophrenia

In psychiatric conditions, as with many human disorders, use of animal models in the scientific exploration has become a valuable research tool. The prevailing modern view of neuroscience, based on extensive experimental evidence (Cowan *et al*., 2000), shown that clinically relevant psychiatric conditions had their source as a primary dysfunction of neuronal systems. Also given that the disruption in neuronal activity can affect both animal and human behaviour, various predictive and causative theories can be tested using animal models. Accordingly, it should be kept in mind that the current animal models of schizophrenia are intended to serve as the animal equivalent of the human disorder, and they models are designed to test specific causative or mechanistic hypotheses regarding aspects of schizophrenia (Goldstein and Deutch 1992). Schizophrenic patients typically experience a wide range of symptoms, (often divided into positive symptoms, negative symptoms and cognitive symptoms; see section 1.3.4) and these symptoms are manifested in various combinations across the subtypes of schizophrenia. Also, there are

remarkable variations in the course and outcome of schizophrenia, with only a minority of patients following a chronic, deteriorating course, despite of enduring symptoms or functional deficits in most patients. Finally, a variety of environmental and genetic susceptibility factors have been proposed as the potential causative agents. The animal models of schizophrenia therefore provide a simplified version of the real condition in order to study the mechanisms of the disease and to develop effective therapies. On this basis one can define an "animal model" as an experimental manipulation that elicits behavioural or neurochemical changes that can be related to schizophrenia using the criteria for predictive, construct and face validity (see review by Lewis and Lieberman 2000).

Predictive validity

- A model in which drugs that are effective on the model, will be effective therapeutically

Face validity

- The behaviour in the model is similar to symptoms of the clinical condition

Construct validity

- The model relies on similar mechanisms (constructs) to the clinical condition

The common models show appropriate face validity, but they are evaluated scientifically in terms of their construct and predictive validity with respect to both clinical phenomena and responsiveness to antipsychotic drugs. Here, the objective of the models is the manipulations used to mimic the clinical phenomena. Animal models can be used to study schizophrenia include both models of the full syndrome and models of specific signs or symptoms (see Swerdlow *et al.,* 1998; Vallone 2000 for example). Typically, models are animal preparations which attempt to mimic a

human condition, the human psychopathology associated with the group of schizophrenia disorders. Thus models aim to provide specific observables (behavioural outcomes) that have been identified or associated in schizophrenic patients provide a focus for study in experimental animals. The particular observable behaviour being studied is a symptomatic of schizophrenia, and it must be defined objectively and observed reliably. This approach generally leads to pragmatic advantages in the conduct of mechanistic studies addressing the neurobiological substrates (for example, the brain regions that are involved) of the behaviour in question (Green and Braff 2001). The following section provides some examples of animals models used to study schizophrenia, mainly focussing on models of cognitive deficits in animals, which is relevant to the experiments carried out in this thesis (see chapter 5).

1.4.1 Animal models exhibiting cognitive measures

In the original description of schizophrenia by Kraepelin the cognitive deficiencies played a prominent role in the diagnosis of schizophrenia and distinguishing it from mania, depression and other forms of psychosis. The cognitive dysfunction in schizophrenia is an important target for novel therapies and the effective measurement of the cognitive effects of novel compounds in clinical trials of schizophrenia is critical in the drug development. The clinical trial programmes such as The Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) and Treatment Units for Research on Neuro-cognition and Schizophrenia (TURNS) produced a consensus cognitive battery which is now widely used (Castner 2004; Castner and Goldman-Rakic 2004). These programmes provide an infrastructure for clinical studies of pharmacological agents for enhancing neuro-cognition in patients with schizophrenia. Another network, The

Cambridge Neuropsychological Test Automated Battery (CANTAB) is a computerised assessment programme developed from animal behaviour paradigms and human neuro-psychology. According to the clinical studies reported by MATRICS cognitive deficits are reported across all subtypes of schizophrenia (Green *et al.,* 2004) and severe cognitive deficits, including impairments of attention, working memory, visual memory and learning, appear to be a major factor contributing to impaired social and vocational functioning and also impaired treatment outcome (Gray *et al.,* 1991). The current available therapeutics for schizophrenia such as dopamine D_2 (haloperidol, chlorpromazine) or dopamine D_2/serotonin $5HT_2$ antagonists, are effective in reducing the positive symptoms in the majority of schizophrenic patients but have a minimal beneficial effects on cognitive dysfunction. Other reports suggest that the new generation of antipsychotics (olanzapine and risperidone) targets of subtypes of dopamine receptors may improve cognitive function (Seeman 1992; 1994 a and b). Thus, an important future direction of preclinical research in schizophrenia is the design of animal models and novel treatments that target cognitive dysfunctions relating to this disorder. The validity of animal models of cognitive deficits of schizophrenia depends on appropriate behavioural paradigms for laboratory animals. These models must mimic as closely as possible the empirical measures on which human schizophrenia patients are impaired. The animal analogue of the cognitive deficiencies and the behavioural tests relevant to the deficiencies that I am focussing on here include mainly:

- ✓ attention (Wisconsin Card Sorting Test and Continuous Performance Test)
- ✓ working memory (working memory test),
- ✓ visual memory and learning (NOR task)

The most common animal models of schizophrenia mainly focussing on the cognitive deficits are behavioural models, lesion models, genetical models and pharmacological models. These models can be broadly differentiated in models which create a disease-like state (lesion models, genetical models, and pharmacological models) and models which assess the behaviour (behavioural models). Also these models can be used in combination, for example, a disease-like state can be created by giving PCP, but assess its impact on behaviour with NOR (chapter 5 and 6).

1.4.1.1 Behavioural models

A few examples of behavioural tasks that are modelled in laboratory animals are:

- Wisconsin Card Sorting Test and Continuous Performance Test: Schizophrenic patients fail in tasks including the Wisconsin Card Sorting Test and Category Test, both of which rely on an ability to utilise knowledge or feedback to change or shape behaviour. Although similar cognitive tasks have been conducted in monkeys, they are not possible in rodents. However, it is possible to design behavioural paradigms in rodents that can evaluate cognitive constructs that are comparable with those measured in many human experimental paradigms (Luby *et al.,* 1959; Perry *et al.,* 2001). Among the most common of animal cognitive tasks are those with an attention component such as delayed matching or non-matching to sample and discrete trial delayed alternation, all of which measure important elements of the human experimental paradigms, which may be defined as the ability to guide behaviour by forming internal representations of stimuli that are no longer present in the environment (Robinson 1980). Human psychological tests of attention, on which patients with schizophrenia exhibit impairment, provide a

measure of delay dependent retention of mental representation (for more details see Ellenbroek and Cools 1990).

- Working memory test: Several rodent tasks of working memory are used routinely to understand the cellular basis of working memory. In designing animal models several interesting tasks have been characterised to evaluate behavioural flexibility and strategy-shifting ability in the rat, such as maze-based strategy-shift task (Ragozzino *et al.,* 1999). This task requires rats first to learn either a response strategy or a visually cued place-discrimination strategy (black or white maze arm) to obtain a food reward. This task is particularly useful in assessing task acquisition, behavioural flexibility within and between strategies which appear to be relevant to the cognitive deficits associated with schizophrenia (Seeman 2005). These deficits are in patients with overt frontal lobe damage with a display of profound deficits in tasks that require behavioural flexibility, strategy shifting, and response to environmental feedback (Stefani and Moghaddam 2005).

- NOR test: Previous clinical post-mortem studies reported that cognitive deficits of schizophrenia are manifested within a limited number of critical brain regions, such as the prefrontal or temporo-parietal cortex (Tzschentke and Schmidt 1997; Tandon 1999). Recent clinical studies however, have suggested that patients show a widespread pattern of dysfunction that cannot easily be ascribed to dysfunction within such circumscribed brain regions but also in visual regions and the cerebellum (Swerdlow 1998). Visual or perceptual closure refers to the ability of the brain to form complete object representations on the basis of fragmentary visual information. The investigation of visual/perceptual closure in schizophrenic patients provides an index of their ability to form complete representations to the amount of visual information provided, the level of information require for object recognition

can be manipulated (Geyer *et al*., 1995). Thus, for example, if an image/object is repeated either immediately or after some delay, participants are able to make identification with significantly less visual information i.e., recognition of familiar image or object. NOR is a procedural analogue of standard visual recognition and episodic memory tasks used in primates and rodents. With this task, one can demonstrate that rats have a visual memory profile, which is analogous to that in primates. The NOR task for rodents is a non-spatial, non-aversive memory test which involves the replacement of a familiar object with a novel object in a memory retention trial. The NOR task is ethologically relevant as they rely on the animal's natural exploratory behavioural tendency and avoid the confounding influence of reinforcement (or reward motivated: see review by Ellenbroek and Cools 1990 for example).

1.4.1.2 Lesion models

These models provide some degree of validity, although further research is required to determine the potential brain region implicated as in schizophrenia. Lesions typically involve using excitotoxic agents, which destroy neuronal tissue via stimulation of excitatory glutamate release or by acting as direct glutamate receptor agonists. Moreover, a number of targeted lesion animal models have been developed; I will mention two of them as examples as below, to address some of the issues surrounding progressive neuro-developmental or neuro-degenerative changes in schizophrenia:

- PFC lesions: These lesions are targeted in the PFC region given the evidence for the involvement of the PFC in schizophrenia. The PFC is well characterised to be involved in higher cognitive functions such as attention, working memory, emotional expression and social interaction. Moreover, this model features the

hypo-function of dopaminergic projections at the level of PFC, in particular, which has been implicated in the metabolic hypo-frontality as observed in schizophrenic patients. In the adult rat, lesion of PFC, results in an enduring hyper-responsiveness to stress, and amphetamine-induced stereotypy, suggesting that striatal dopamine neurotransmission is increased, which again is observed in schizophrenia (for example., Bubser *et al.,* 1997).

- Hippocampal or perirhinal cortex lesions: Most theories are in agreement that the hippocampus and perirhinal cortex are important for learning and memory. In general, agreement exists that hippocampal function is essential for spatial memory, but wide disagreement occurs on the role of the hippocampus in non-spatial, visual recognition memory, or in episodic memory. Studies have found that selective lesions of hippocampus impair delay-dependent (long delay between acquisition trail and retention trail) visual memory with a profile different from that produced by damage to the perirhinal cortex. Therefore provides a differentiated role of behaviour to the relevant brain area under investigation. Furthermore, lesion studies demonstrate that rats have a visual recognition memory system fundamentally similar to primates that depends on the function of the hippocampus (for example., Winocur 1997).

1.4.1.3 Pharmacological models

Most pharmacological models involve the administration of drugs that induce or exacerbate schizophrenic symptoms in humans. The pharmacological animal models of schizophrenia are based on the understanding of the alterations in various neurotransmitter systems. These models generally have some degree of construct validity, providing some understanding of the fundamental basis of cognition. This model is relevant to this current thesis as I tested a psycho-pharmacological

cognitive deficit model, see chapter 6. These models can be divided in terms of the neurotransmitter systems that are affected depending on the pharmacological agent used as described below:

- Dopamine: Perhaps the best-known pharmacological model, which is based on the dopamine hypothesis of schizophrenia, involves amphetamine administration. The dopamine hypothesis of schizophrenia proposes that dysfunction in neurotransmission of dopamine is the underlying cause of the symptoms of the disorder. Specifically, the positive symptoms of schizophrenia are suggested to be connceted with the hyperactivity of mesolimbic dopaminergic neurones. The hypo-dopaminergic state in the frontal-cortical terminal field of mesocortical dopamine neurones has also been proposed to be the basis of negative symptoms. This mesolimbic dopaminergic hyperactivity in schizophrenia may be maintained by interfering with pre- or postsynaptic mechanisms. The evidence indicated presynaptic hyperactivity and excess of dopamine release is critical in schizophrenia (Davis *et al.,* 1991; Duncan *et al.,* 1999). Further, amphetamine and related substances such as 3,4 methylene-dioxy-methamphetamine have been shown to produce psychotic symptoms in healthy subjects. In addition, many patients with schizophrenia experience an exacerbation of psychotic symptoms in response to psychostimulants such as amphetamine and methylphenidate at doses that do not induce psychotic episodes in normal controls. Also, postsynaptic increase in the number of dopamine receptors or associated downstream signal transduction pathways could result in heightened sensitivity to dopamine. Similarly in animal studies, the administration of amphetamine and related psychostimulants reliably stimulates behavioural changes such as hyperlocomotion and stereotypy. Moreover, amphetamine-induced stereotypic behaviour can be attenuated by treatment with

antipsychotics, further asserting the predictive and construct validity of this model (see reviews by Roberts 1991; Roberts *et al.,* 1997).

PPI refers to the reduction of startle reaction to a startle-eliciting stimulus when it is shortly preceded by a weak stimulus (Hoffman and Searle 1965). Theoretical expositions of PPI invariably attribute it to a competition between the prepulse and pulse stimuli for limited processing resources. When the prepulse and pulse stimuli are presented in close proximity, the preceding prepulse triggers a protective or gating mechanism that limits the processing of, and therefore the reaction to, the succeeding pulse stimulus (Braff *et al.*, 1992; Henze *et al.,* 2000). PPI also provides an operational measure of sensorimotor gating, a process by which an organism filters extraneous information from the internal and external sources (Braff and Geyer 1990). In healthy humans, rodents, or nonhuman primates, the prepulse stimulus serves to attenuate the motor response to the pulse. PPI deficits are found in a number of psychiatric populations, most notably patients with schizophrenia, obsessive–compulsive disorder, and Tourette's syndrome. As a model of the gating deficits in schizophrenia, PPI is believed to have face, construct, and predictive validity. In humans, PPI is measured via electro-myographic recordings of the eye blink response at the orbicularis oculi below the eye. In rodents, startle can be measured as the whole-body flinch. Glutamate, dopamine, and serotonin have been implicated both in the aetiology of schizophrenia and the modulation of PPI. Effective antipsychotic agents appear to ameliorate PPI deficits in some patients with schizophrenia and in rodents given psychotomimetic compounds. A significant deficit in PPI has been reported in schizophrenic and presumably psychosis-prone subjects (Braff *et al.*, 2001). Although PPI impairment is not unique to schizophrenia (Castellanos *et al*., 1996; Braff *et al*., 2001) the sensitivity of PPI to

dopaminergic manipulations fits readily to the dopamine hypothesis of schizophrenia (Snyder 1976; Swerdlow *et al.,* 1994). Apomorphine-induced PPI disruption has therefore been commonly employed as a screening test for potential antipsychotic compounds (Swerdlow and Geyer 1998). However, the psychological mechanism whereby apomorphine leads to PPI disruption remains a subject of speculation.

- Glutamate: The actions of glutamate are mediated by both ionotropic (mainly NMDA receptors) and metabotropic receptors (mGlu receptors). Similar to the pharmacological animal models involving dopaminergic system, glutamatergic systems, have been implicated both in the aetiology of schizophrenia and the modulation of PPI. The glutamate hypothesis of schizophrenia is formed from the evidence that PCP and ketamine (non-competitive NMDA antagonists), produce schizophrenia-like symptoms in healthy humans (Allen and Young 1979; Karper *et al.,* 1995). In rodents (Mansbach and Geyer 1989; Dulawa and Geyer 1996) or nonhuman primates (Linn and Javitt 2001) administration of these same compounds reduces PPI. The cognitive symptoms can perhaps be mimicked more reliably by the administration of PCP, which appears to act predominantly on glutamatergic NMDA receptors (Moghaddam *et al.,* 1997). Evidence strongly suggests that PCP and other NMDA receptor antagonists induce schizophrenia-like symptoms in healthy subjects and exacerbate psychosis in patients with schizophrenia. This has led to the speculation that schizophrenia may involve hypo-function of NMDA receptors. Moreover, neurochemical processes such as long-term potentiation are disrupted by NMDA antagonists, in accordance; a decreased release of glutamate has been reported in the PFC of patients (Selemon and Goldman-Rakic 1990; Lewis *et al.,* 2000) with schizophrenia and in rat model of schizophrenia (Moghaddam *et*

al., 1997). Similarly reduced expression of non-NMDA glutamate receptor subtypes in the medial temporal lobe of patients has also been reported. One main proposal is that glutamate may also be involved in schizophrenia through its interactions with dopamine. Repeated exposure to PCP has been reported to reduce basal dopamine utilisation in the PFC in monkey (Goldman-Rakic and Selemon 1997). This neurochemical effect persisted even after PCP treatment was stopped, suggesting an enduring effect. Further, PCP treatment has been shown to disrupt rodent performance in cognitive functional models such as reversal learning and NOR, effects which are attenuated by antipsychotic drugs such as clozapine. Thus these findings clearly support the face and predictive validity for this model. Taken together, these findings implicate altered glutamate neurotransmission and NMDA receptor function, in particular, in the cognitive deficits observed in schizophrenia, which has been tested in the experiments (see chapter 5 and 6) mentioned in this thesis.

- GABA: On the basis of both theory and experimental evidence, alterations in GABA neurotransmission in the PFC of patients have also been proposed. Evidence shows reduced GABA uptake sites in the temporal lobe, increased $GABA_A$ receptor binding in superficial layers of cingulate cortex, parallel with reduced gene expression for glutamic acid decarboxylase in the PFC (Simpson *et al.,* 1992; Karper *et al.,* 1996). This provides direct support for GABAergic involvement in this disorder. Also a mechanistic interaction between dopaminergic and GABAergic systems in schizophrenia is supported by the fact that GABA neurons in the middle layers of PFC receive direct synaptic input from dopamine terminals and exert inhibitory control over excitatory output of pyramidal neurones. These neuronal circuits undergo substantial developmental changes in late

- Serotonin (5HT): The 5HT system has also been implicated in schizophrenia. The two major classes of psychedelic hallucinogenic drugs such as lysergic acid diethylamide (LSD) and mescaline were believed to mediate their effects through $5HT_{2A}$ receptors. In addition, polymorphisms of the $5HT_{2A}$ receptor genes are reported to be a minor risk factor for schizophrenia and also that a loss of $5HT_{2A}$ receptors in the PFC along with an accompanying increase in $5HT_{1A}$ receptors have been reported in schizophrenia (Hitchcock *et al.,* 1997). Moreover, the relatively high affinity of atypical antipsychotics for the $5HT_{2A}$ receptor supports a role of 5HT systems in schizophrenia (Kapur *et al.,* 1999). LSD has been shown to disrupt PPI in humans and rats (Geyer and Braff 1987). Further, this effect is mediated through direct stimulation of 5-HT_{2A} receptors. Indeed, the disruptive effect of PCP on PPI also has been proposed to be mediated through indirect activation of $5HT_{2A}$ receptors (Tyson *et al.,* 2004). Interestingly, both LSD and mescaline have been shown to enhance glutamatergic transmission in rodents. $5HT_3$ receptor antagonists have also been shown to attenuate the behavioural hyperactivity caused by PCP, as well as amphetamine administration, but $5HT_3$ receptor binding sites are not altered in schizophrenia, and the efficacy of $5HT_3$ antagonists in clinical trials of schizophrenia has been variable. Despite the evidence for altered serotonergic markers in schizophrenia, there is comparatively

little evidence of a primary dysfunction of serotonergic systems in this disorder. Moreover, the relevance of LSD administration in animal models is unclear; repeated administration of LSD in humans or animals leads to behavioural tolerance, unlike the situation in schizophrenia. Thus, despite some support for face and predictive validity in the pharmacological model involving serotonergic systems, construct validity remains as difficult to establish as in the dopamine and glutamate animal models of schizophrenia. However, manipulation of 5HT system is not very relevant to the current thesis as it does not efficiently target the cognitive dysfunction in schizophrenia.

1.4.1.4 Genetical models

- Dopaminergic mutants: On the basis of dopaminergic dysfunction in schizophrenia, a clear candidate for a genetic model of this disorder is the dopamine transporter knockout mice (DAT-KO). Mice lacking DAT are unable to re-uptake released dopamine and the neurotransmitter levels in extracellular fluid remain elevated. The genetic removal of DAT leads to persistently elevated dopaminergic tone, which is in fact more relevant to the dopamine hypothesis of schizophrenia. The absence of DAT leads to several physiological changes. There is a 300-fold increase in the amount of time required to clear dopamine from the synapse that results in a five-fold increase in the extracellular levels of dopamine. In response to the elevated dopamine levels, dopamine D_1 and D_2 (but not D_3) dopamine receptor numbers are decreased. Functional coupling of dopamine D_2 autoreceptors is lost in DAT-KO mice. As a result of a lack of recycled dopamine and the diminished autoreceptor function, dopamine synthesis is increased, but the enhanced dopamine synthesis is not able to restore normal levels of intracellular dopamine (Gainetdinov *et al.,* 1999 a).

The DAT-KO mice display characteristics of dwarfism, are hyperactive in a novel environment and display sleep dysregulation. The dopaminergic hypothesis of schizophrenia could suggest that alterations in DAT-mediated functions contribute to this disorder. In fact DAT-KO mice reproduce several features of the amphetamine animal model of schizophrenia. The DAT-KO mice are hyperactive, stereotypic, and show significant deficits in sensorimotor gating – pre-pulse inhibition (PPI) of acoustic startle response and spatial cognitive function. Importantly, the hyper-locomotion of these mice is reversed by inhibition of dopamine synthesis or blockade of dopamine receptors by antagonists (haloperidol and clozapine). These mice also show significant cognitive impairment in spatial cognitive tests (Gainetdinov *et al.,* 1999 b). In particular, in the 8-arm radial maze test, mutant animals make significantly more perseverative errors. However, no deficits in social interaction were found in these animals (Smithies 1993).

Accordingly, the behaviours of DAT-KO mice might have the same level of validity as a model of schizophrenia as with amphetamine-induced behaviours, and the main criticism of these models is that they do not correlate with those of individuals with schizophrenia, most notably the lack of deficit in social interactions. In addition, psychostimulants, known to exacerbate psychotic reactions in patients produce paradoxical 'calming' effects in these DAT-KO mice. In addition in clinical studies no major DAT alterations or association between markers in the DAT gene and schizophrenia have been found. However, a possibility that disordered DAT function can amplify disturbances in other neuronal components resulting in some symptoms of schizophrenia cannot be totally excluded. Thus, DAT-KO mice might be useful to study certain features of schizophrenia, but might not reproduce the full spectrum of schizophrenia-related behaviours (Fauchey *et al.,* 2000 a and b).

- Glutamatergic mutants: NMDA receptors are composed by assembly of the NR1 subunit with any one of four (NR_{2A}–D) subunits. Mice deficient in NMDA glutamate receptors (NMDA receptor number reduced to 10%) have been generated by targeted mutation of the crucial NR1 subunit gene (NR1-KO mice). NR1-KO mice display hyperactivity similar to those observed in the PCP or MK-801 treated animals. Both antipsychotic drugs, haloperidol and clozapine, could attenuate this hyperactivity, but only clozapine was effective at doses that did not affect wild-type activity. Additionally, the NR1-KO mice showed deficiencies in social interaction and sexual behaviours that were improved with clozapine treatment. These preliminary observations suggest that NR1-KD mice might serve as a genetic counterpart to PCP or MK-801 models of schizophrenia (Darrah *et al.,* 2008). However, the other crucial characteristics, such as sensorimotor gating- PPI of acoustic startle response and cognitive functions, still needed to be tested to strengthen this assertion (see review by Mohn *et al.,* 1999).

The neurochemical characterisation of these mice showed that striatal dopamine content was normal. It should be emphasised that attempts to decipher the role of dopamine in psychostimulant action of NMDA antagonists and the question of whether NMDA antagonists elevate dopamine release to exert their behavioural effects has remained a matter of debate. The data on these mice support a model of reciprocal functional interaction that occurs at the level of postsynaptic dopamine receptors and NMDA receptors located on striatal medium spiny GABA neurones, rather than at the level of presynaptic dopaminergic activity (Deutch 1992).

Among the other glutamatergic mutants, mice lacking the NR_{2A} subunit of NMDA receptor are of particular interest. NR_{2A} mutant mice, which represent another

example of malfunction of NMDA receptor function, exhibited an increased locomotor activity in a novel environment and cognitive deficits manifested in various learning tasks. Hyperactivity in these NR_{2A} mutant mice was attenuated by treatment with the antipsychotics haloperidol and risperidone. In a preliminary neurochemical investigation, both dopamine and 5HT metabolism were found to be modestly affected in the frontal cortex and striatum of these mutant mice. Further investigation of the behavioural phenotype in addition to the status of both dopamine-mediated and 5HT-mediated systems is required in these NMDA receptor deficient mice (Kishimoto *et al.,* 2001; Miyamoto *et al.,* 2001 c). Also investigations on mice with mutations in other glutamate receptors such as AMPA and metabotropic glutamate receptors that modulate their activity in the synthesis, metabolism and uptake of glutamate, and co-agonists glycine can provide crucial insights, which are currently lacking (Miyamoto *et al.,* 2001 a and b).

- Other mutants: 5HT represents another neurotransmitter that is thought to be involved in psychotic behaviours but its role is less well-understood. Several 5HT mutants are currently available but have yet to be fully characterised in behavioural paradigms relevant to schizophrenia. Similarly, mutations in cholinergic, particularly, muscarinic, and noradrenergic systems might result in phenotypes relevant to schizophrenia, but currently it is unclear which of the components of these systems would be the most probable candidates (Murphy *et al.,* 1999; Tandon 1999).

CHAPTER 2: Application of intracerebral microdialysis in PK/PD studies: *In vitro* assessment of recovery obtained by various microdialysis methods

2.1 Introduction

A principle objective of the work presented in this thesis is to develop and validate a microdialysis method for assessing PK/PD profile of CNS active drugs. The introduction of this chapter covers the background of the application of intracerebral microdialysis in assessing PK/PD profile of drugs and the concluding part focuses on the importance of assessing *in vivo* recovery and calibration methods in quantitative microdialysis. This section also discusses some difficulties and limitations encountered where the goal is to characterise concentration-time profile by quantifying (measuring absolute) drug concentrations in order to extract PK parameters.

2.1.1 Microdialysis versus other in vivo techniques in PK studies

Ideally, an *in vivo* method to be used for determination of the local PK of a drug in the brain should exhibit the following characteristics (Parsons and Justice 1994):

- It should measure drug concentrations with high selectivity without interference from metabolites.
- It should allow the measurement of free unbound concentrations of the drug.
- It should allow the determination of local drug concentration and local concentration differences with in the brain.

- It should be possible to determine concentration versus time profiles within individual animals.

Several techniques that have been developed over the years, which fulfil some but not all of the above mentioned criteria. In the following sections some of these techniques will be discussed and compared with microdialysis in monitoring drug concentration in the brain regions.

2.1.1.2 Quantitative autoradiography (QAR)

QAR involves injection of a radioactive isotope (deLange *et al.,* 1997; Jiao *et al.,* 2006) and then the auto-radiographic images are produced by exposing the isotope containing brain sections to X-ray films. These images are then analysed by computer assisted scanning micro-densitrometry. Using this technique the total concentration of radio-labelled drugs in small regions of the brain of about 100-200 cells can be measured. QAR permits quantification of regional differences in drug entry into the brain. Although it is a sensitive approach to determine the spatial distribution of drugs in the brain, only a single measurement can be made in each animal such that the time course of a drug's distribution can not be achieved within one animal. Moreover, no distinction can be made between bound and free drug concentrations and also often no differentiation can be made between parent drug and metabolites.

2.1.1.3 Positron emission tomography (PET)

Non-invasive studies of the living brain have been revolutionized by the development of PET. PET scanning is achieved with radioisotope imaging and enables the study of function as well as structure. The image obtained depends on the distribution of an injected or inhaled isotope and its emitted radiation. Positron

emitting isotopes (^{11}C, ^{13}N, ^{15}O or ^{18}F) binding to the drug of interest, make it possible to map early phenomena of the distribution of drugs with in the brain (Goethals *et al.,* 1991; Ponto and Ponto 1992). An important disadvantage of PET scanning, however, is the instability of the isotopes often resulting in a very short half life. Therefore, synthesis of the radioisotope should take place immediately before the *in vivo* experiment, and correction for the decay of the isotope is essential to obtain reliable results. Additionally, no free drug concentrations can be measured and possible interference of signals originating from metabolites may be problematic.

2.1.1.4 Serial sampling of cerebrospinal fluid (CSF)

The concentration-time profiles of free drugs in the brain of individual animals can be obtained by means of serial sampling of CSF via cannulae inserted in the *cisterna magna*. The use of cerebrospinal fluid concentrations is attractive, since it has been demonstrated that at least for certain anaesthetic and convulsant drugs, CSF is pharmacokinetically indistinguishable from the target site of drug action (Danhof and Levy 1984). However, the concentrations in CSF do not reflect regional concentration differences with in the brain. After intracerebroventricular (i.c.v.) administration, processes like efflux of the drug out of the ventricular compartment and the transport of the drug into the brain parenchyma may result in spatial differences in drug concentration (Covell *et al.,* 1985). It has also been demonstrated that regional differences in cerebral blood flow exist (Hertz and Paulson 1980). So in the cases where drugs distribution is dependant on blood flow, no information in the difference in regional distribution can be obtained. Another disadvantage is that with CSF sampling is that the volume of the CSF is thoroughly

changed, which consequently affects the concentration gradient between brain and CSF.

2.1.1.4 *In vivo* voltammetry

In vivo voltammetry is based on a miniaturized version of electrochemical oxidation *in vitro*. In this approach, a three electrode system consisting of a working, a reference and an auxiliary electrode is permanently implanted in a discrete area of a brain. By applying a potential difference between the working electrode (carbon) and reference electrode (Ag/AgCl), the drug of interest is oxidised, resulting in a concentration-dependent flow of electrons which can be detected with the auxilliary (Pt) electrode. This technique in principle enables the determination of drug concentration versus time profiles with high resolution in individual animals (see review by Stamford in 1986). Also, some degree of selectivity can be acquired by varying the potential difference. Unfortunately, only a limited number of drugs can be oxidized at reasonable oxidation potentials, and this limits the applicability severely. Furthermore, the properties of the electrodes may undergo profound changes after implantation into the brain; the sensitivity of the electrodes change in an unpredictable way, the resolution between adjacent peaks may change and the position of the oxidation potential of a compound may shift. This means that there is a constant doubt about the identity and the concentration of the compound of interest (Pantano and Kuhr 1991).

2.1.1.5 Intracerebral microdialysis

Intracerebral microdialysis is based on measurements of drug concentrations in the dialysate which reflect, but are not identical to the concentrations in brain ecf. There are several advantages of this technique: the concentrations of compounds can be monitored in individual animals in a selected area of the brain; dialysate concentrations reflect the free unbound concentrations in the brain; good temporal and spatial resolution, high selectivity in determination the compound of interest without interference from metabolites, serial continuous samplings in a single animal (Ungerstedt 1984; Brunner and Langer 2006). However, there is also a disadvantage in the use of this technique; the difficulty of estimating *in vivo* recovery of the compound of interest leading to difficulty in quantifying brain concentration of drugs.

2.1.2 Pros and cons of application of intracerebral microdialysis in PK/PD studies

Prior to the introduction of microdialysis sampling, virtually all PK studies performed discrete-point sampling in the blood or peripheral tissues, to measure drug concentrations. As mentioned before, blood was the default site of PK measurements in drug research studies. In studies where tissue concentrations are required whole tissue was generally sampled, which is not ideal as both these samples contain both unbound and bound drugs *(figure 2.1)*. However, to construct the tissue concentration-time profile, different groups of animals were sacrificed at each time point leading to large variability in the data. In contrast, microdialysis does not involve disturbance of biological matrices, and allows more frequent sampling to characterise rapid changes in concentrations (Tsai 2003). Moreover this

sampling reduces the total number of animals required by allowing numerous measurements from each animal, thus reducing inter-subject variability. However, one should consider this carefully as microdialysis does not measure intracellular concentrations of compounds and it is therefore not useful for studies involving drugs with intracellular targets e.g. second messenger system. It can, however, be used to study drug concentrations close to the site of action (Scot *et al.,* 1991; Langer *et al.,* 2005).

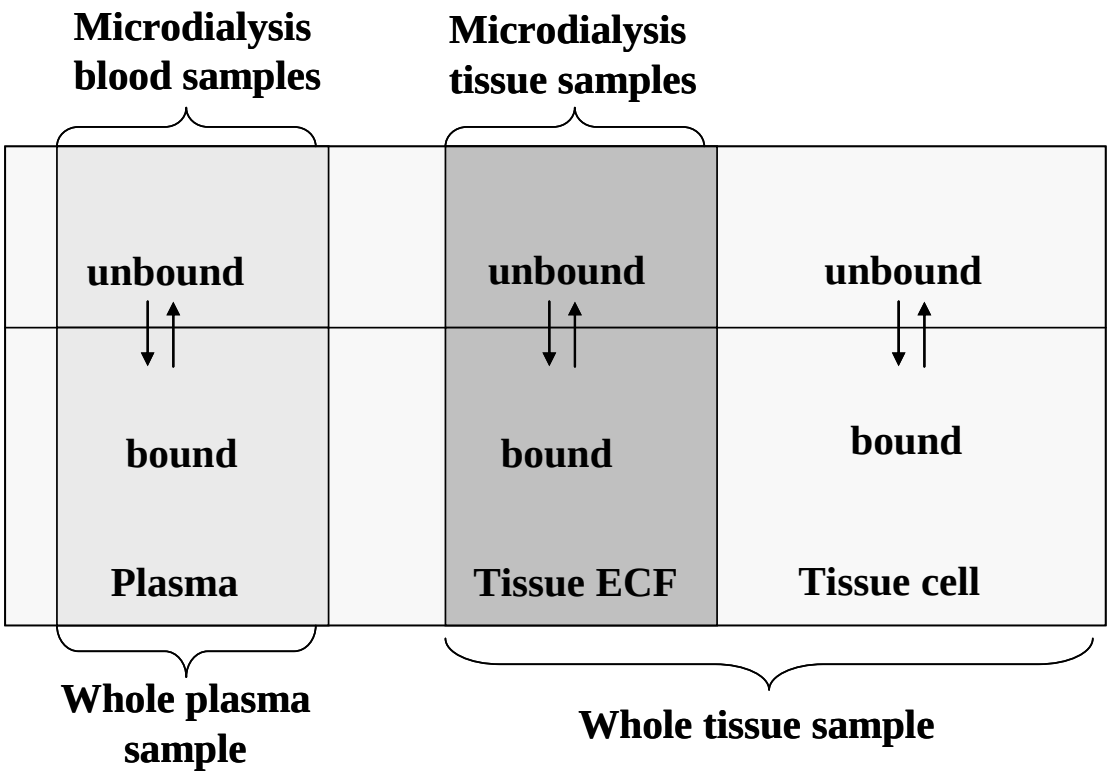

Figure 2.1: Distribution of bound and unbound drug concentration in plasma, and ecf of the tissue (adapted from Hammarlund-Udenaes 2000).

A state of equilibrium exists between unbound and bound compounds across the plasma and the tissue. Microdialysis can be employed to measure the unbound concentrations from the plasma and the tissue ecf space. This concentration reflects the unbound/bound levels of the compound in the plasma and the tissue.

Several microdialysis methods have been employed to make measurements in blood and brain samples of conscious animals simultaneously (see chapter 3). These studies compared the concentrations of two compartments, blood and brain PK parameters, to elucidate the BBB penetration of CNS drugs. This can be a crucial factor in CNS drug discovery, as many novel candidate drugs fail at clinical trial due to failure in BBB penetration. Moreover both drug and neurotransmitter levels in brain ecf can be determined from dialysates (Boschi and Scherrmann 2000; Plock and Kloft 2005; Li *et al.,* 2006). Thus using microdialysis the concentration-effect profile of a drug can be determined. However, these studies only present drug time-course profile or concentration-effect (e.g., transmitter change) profile, respectively. Therefore, it is necessary to use intracerebral microdialysis for brain PK and PD (simultaneously) to investigate the drug effect along with the concentration changes in time course *in vivo* (deLange *et al.,* 1997; Sood *et al.,* 2008 and 2009). Previously, this has been difficult to achieve due to drawbacks in traditional quantitative microdialysis methods that did not allow PK and PD profiling of a drug simultaneously (see below). This is changed recently due to the development of MQ probes and has moreover made PK and PD measurements possible from the same animal at the same time (Sood *et al.,* 2009). The experiments described in this thesis (chapter 3, 4, and 6) have successfully employed MQ probes to do so.

2.1.3 Methods in microdialysis to assess PK/PD parameters:

Quantitative microdialysis

Microdialysis samples do not exactly represent the concentration surrounding the probe, as the microdialysis is not typically carried out at equilibrium conditions. The dialysate concentration reflects only a fraction of the surrounding ecf concentrations

of compounds. The ratio between the analyte concentration in the dialysate and that in the ecf is called relative recovery. However, relative recovery does not quantify the true/absolute unbound tissue concentration, which is required for PK/PD assessment of drugs (Gardner and Hammarlund-Udenaes 1998; Gardner *et al.,* 1998). Quantitative microdialysis is generally used in PK studies, while PD studies generally use relative dialysate concentrations. Quantitative microdialysis i.e., "microdialysis to estimate concentrations of analytes that is found in the monitoring medium in the absence of the probe" (deLange *et al.,* 2000) is critical in neuro-PK studies, where knowledge of relative recovery becomes crucial for determination of absolute (true) extracellular tissue concentrations of drugs. In determining the relative recovery of a substance and thus assessing its true concentrations outside the probe, a number of factors need to be considered (deLange *et al.,* 1999):

- It is important to assess recovery of drugs *in vivo* as *in vitro* conditions do not adequately model *in vivo* conditions.
- Recovery may be region specific and therefore recovery determined in one region will not necessarily be applicable to other region, particularly as brain location are not homogenous.
- Recovery may vary over the duration of an experiment.

The quantitative assessment of ecf concentrations on the basis of dialysate concentrations/recovery is a problem (deLange *et al.,* 1997; Van der zeyden *et al.,* 2008). The recovery of a probe can be determined by *in vitro* calibration that is by performing dialysis in a beaker containing a compound at a known concentration. However a number of additional factors affect diffusion when the probe is implanted in the brain tissue, including tortuosity of the tissue (affecting diffusion

of the substance) and active processes occurring in the tissue (including intra-/extra-cellular exchange and metabolism), and can affect recovery of compounds *in vivo* (e.g., see review by Stenken 1997). Hence, *in vitro* recovery is not a good measure of *in vivo* recovery. In order to determine the true concentrations *in vivo*, several approaches that have been proposed, but all have problems. The main methods are NNF, extrapolation to zero flow rate and calibration with retrodialysis.

2.1.3.1 NNF method

The NNF principle was described by Lonnroth *et al* (1987) as a means of determining the true external concentration in the medium surrounding the probe tip. According to the laws of diffusion, compounds will move from areas of higher concentration to areas of lower concentration. Thus if the perfusate concentration (C_{in}) is lower than the external concentration, there will be a net influx into the probe (dialysis), and if C_{in} is greater than external concentration there will be a net outward flux from the dialysis probe. If, however, the dialysate concentration is the same as the external concentration there will be NNF and $C_{in} = C_{out}$. By applying known concentrations of a compound (C_{in}) and measuring the resultant dialysate concentration (C_{out}), a plot of $C_{out} - C_{in}$, against C_{in}, and the point at which the regression line crosses the x-axis is the point of NNF, which represents the external concentration *(figure 2.2).*

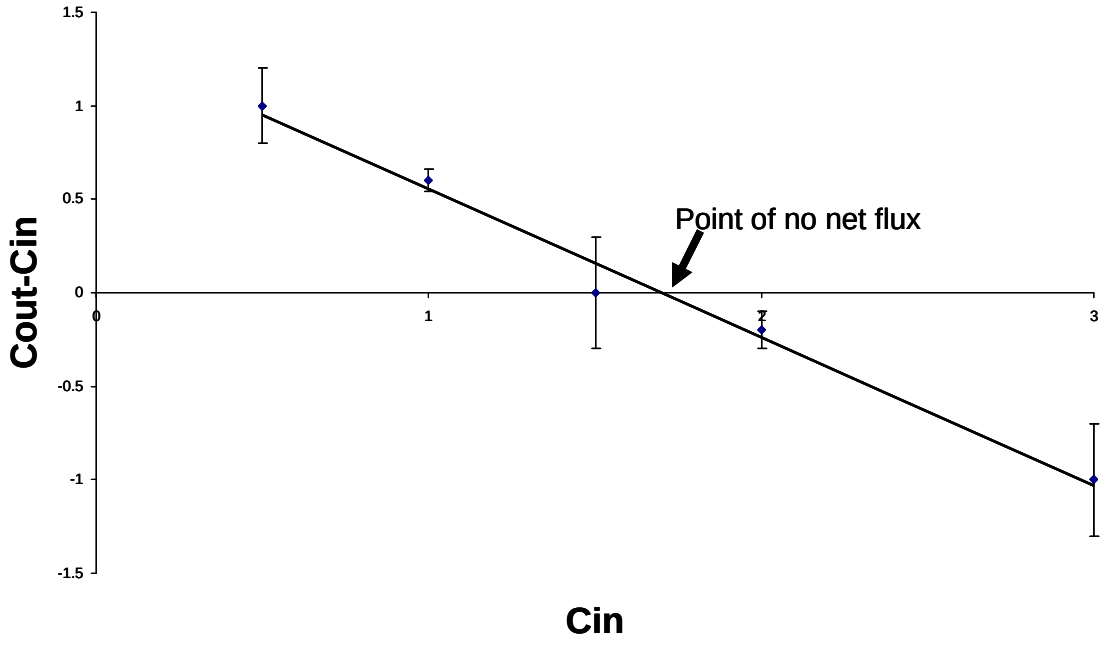

Figure 2.2: NNF graph showing point of no net flux i.e., Cext (Cecf; Lonnroth et al., 1987).

This calibration method requires a steady state tissue concentration over the duration of the experiment, during which consecutive perfusion of the microdialysis probe with different concentrations (C_{in}) takes place (Justice 1993). In vivo probe recovery is determined by the slope of the regression line. The main disadvantage of this approach is the length of time it takes to collect all the required data samples since at least two hours of collection at each C_{in} is required. This means that the compound(s) under scrutiny must be in steady state, over a period of many hours (Stenken 1997), thus precluding measurements of dynamic processes, such as drug distribution following bolus administration.

2.1.3.2 Extrapolation to zero flow rate

The relative recovery is inversely proportional to flow rate, so, as the flow rate decreases, the recovery increases, to the point at which there is no flow, and equilibrium is achieved, such that the dialysate concentration is the same as the external concentration. However, as flow rate decreases, so sample size also decreases, making sample handling and analysis difficult. Clearly at zero flow rate

there will be no sample to collect, thus precluding measurement in this way. However, if successive dialysis perfusions are carried out at different flow rates, and the dialysate concentration (C_{out}) at each flow rate is plotted against the flow rate, and then it is possible to extrapolate back to a theoretical concentration occurring at zero flow rate (Jacobson *et al.,* 1985).

As with NNF, this method has the disadvantage that it has poor temporal resolution as long sampling time needed for small flow rates. Similarly, the need for repeated measurements over several hours means that it is only applicable to steady-state situations. Also in extrapolating to zero flow with a presumed exponential function the compound concentration obtained only estimates the actual recovery without being able to determine its true value. This uncertainty can be reduced to an extent by including measurements at very low flow rates thus increasing the precision of estimated value at zero flow to a minimum within the constraints mentioned above (deLange *et al.,* 2000).

2.1.3.3 Retrodialysis

Larsson in 1991 developed a method to attain an *in vivo* calibration that allowed determination of recovery for every dialysate sample during the experiment, where a calibrator was added to the perfusate and *in vivo* recovery of this compound was determined. However, a limitation of this method is that it assumes that the diffusional behaviour of the calibrator is similar to that of the drug under investigation, an assumption which is not always realistic. Also, based on the principle that dialysis is a two way process, the probe recovery can be measured by adding the drug itself to the perfusate and measuring its loss from the perfusate *in vivo* by retrodialysis (Bouw and Hammarlund-Udenaes 1998). This provides

dialysate recovery, since recovery $_{(loss)}$ = recovery $_{(gain)}$. Before starting *in vivo* retrodialysis experiments with a drug of interest, an *in vitro* preparation is used to check if the movement of the drug molecules across the membrane is fast and symmetrical in both directions (*figure 2.3*), since these are prequisites for this method for the determination of the transient drug concentrations *in vivo* (deLange *et al.,* 1997).

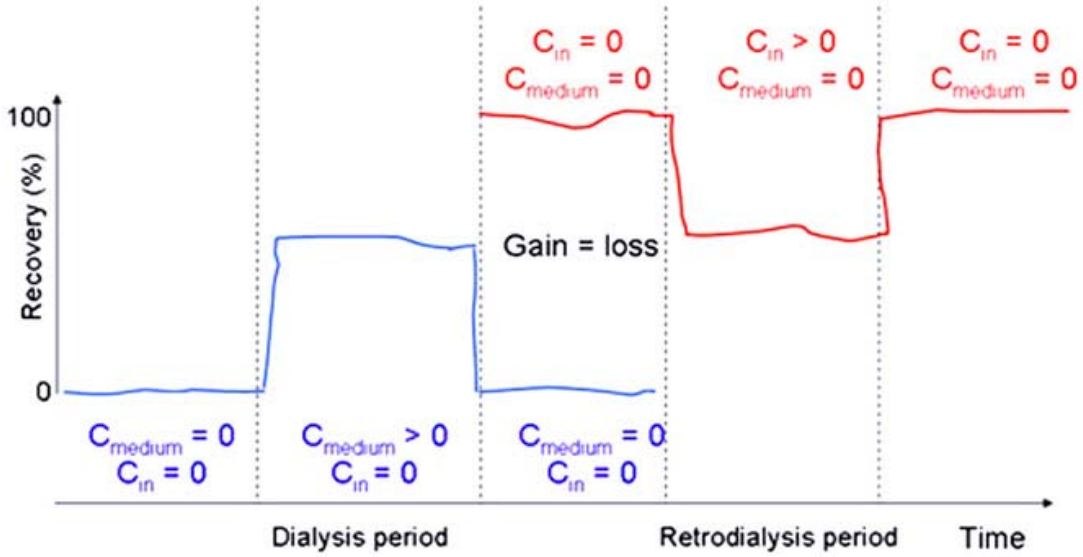

Figure 2.3: In vitro preparation for calibration using retrodialysis.

In the first where the drug is present in the extracellular medium (dialysis period i.e. gain of solute into dialysate) and then in the second stage, the drug is present in the perfusate (retrodialysis period i.e., loss of drug to extracellular medium), with time in between with no drug. The dashed lines represent changes of concentration (deLange *et al.,* 1997).

After this *in vitro* step to confirm symmetrical movement of the drug molecules in both directions, *in vivo* retrodialysis of the drug is performed prior to or after the experiment drug administration to obtain the recovery (loss) values, this value is then used to adjust the dialysate concentrations to obtain tissue concentration,

assuming Recovery $_{(loss)}$= Recovery $_{(gain)}$. The recovery is calculated by the formula: Recovery (%) = $[(C_{out} - C_{in})/C_{in}] * 100$.

The tissue drug concentration measured using this method does not take into account changes in recovery over time since it assumes that the recovery remains constant over the experimental period. This method is not applicable to endogenous compounds as calibration period requires a C_{ecf} of zero, a condition that cannot be met for endogenous compounds.

As mentioned above, all these methods have major drawbacks that limit their use in measuring accurate absolute concentrations of compounds in a dynamic system to yield PK/PD parameters simultaneously. To address this problem I developed a new method: dual probe NNF and also validated the use of a novel method; MQ microdialysis to achieve my goals (see below).

2.1.4 Development of novel methods in quantitative microdialysis

2.1.4.1 Dual probe NNF

I developed a method based NNF method using dual probes. The principle aim was to develop a suitable procedure to determine absolute brain concentrations of neurotransmitters and drugs in dynamic system. The method adopted is a modification of the traditional NNF principle (Lonnroth *et al.,* 1987) and the method comprised essentially of implanting two probes into the brain with a distance 1mm apart. A different concentration of the compound of interest was perfused continuously in each probe *(figure 2.4),* and at each time point a two point

NNF plot (i.e., C_{in} vs $C_{out} - C_{in}$) was constructed. The absolute external concentration at each time point was then attained by the intercept of each plot on the x-axis; giving a measure of the absolute tissue concentration (C_{ecf}) at each time point. Following a systemic injection of a drug, the time course of its distribution in ecf was followed (Sood *et al.,* 2008), thus providing a means for monitoring dynamic processes. It was utilised to study the fate of the drug at the tissue site in a dynamic system.

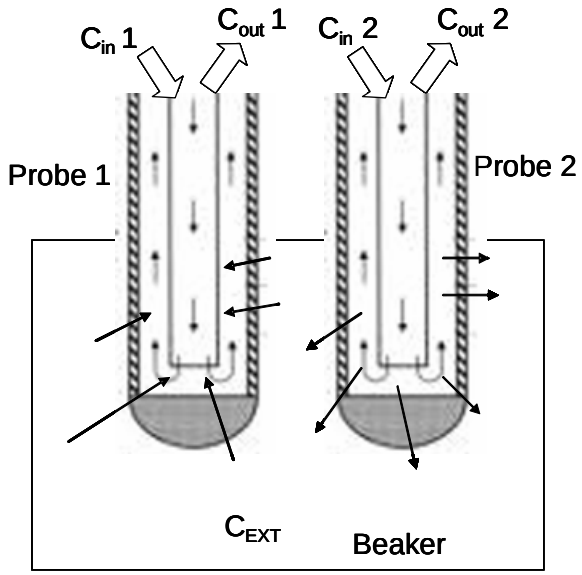

Figure 2.4: Diagram of dual probe dialysis tips in vitro set up.

In the dual monoprobes assembly, one of the two probes was perfused with a zero concentration of a given compound (aCSF) and the other was perfused with higher concentration of for example 10 nM concentration of compound of interest. A dynamic system was achieved by varying the beaker concentrations at 0, 5, 3 6 and 10 nM of compounds of interest within an individual experiment (figure 2.4). The C_{out}-C_{in} values of each probe were plotted against each C_{in} values respectively for each time point, and NNF point was obtained by linear regression (as shown in *figure 2.2*).

2.1.4.2 Novel ultra slow-flow method using MQ probes

MQ probes developed by Brains On Line, Groningen, Netherlands *(figure 2.5)* essentially have a very low flow rate (around 0.15 µl/min) through the dialysis tip, and a secondary input flow run at a high flow rate (0.80 µl/min), to provide an overall output of flow rate of 0.95 µl/min, such that manageable sample sizes are achieved. Since the flow in the dialysis tip is so low, the recovery approaches 100% - that is the dialysate concentration is close to equal to the ecf concentration. These probes were in the very early stages of development, and our objective was to validate their performance.

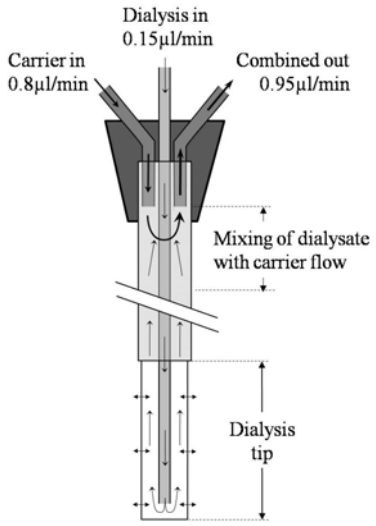

Figure 2.5: Diagram of the MQ dialysis probe (Sood et al., 2009). The dialysis tip is perfused at ultra-slow flow rate through the 'dialysis in' tube, which projects to the dialysis tip of the probe. The outflow from the dialysis tip mixes with the carrier flow in the body of the probe, and the mixture leaves the probe through the 'combined out' tube.

The main focus of this current research project was to validate and assess the use of these novels methods in obtaining PK and PD parameters simultaneously. MQ ultra-slow flow method was tested and validated in evaluating the PK/PD profile of novel CNS drug in freely moving rodents (see chapters 2, 3, 4, and 6).

2.1.4 Aims

Experiment 1: Assessment of recovery *in vitro* using various microdialysis calibrating methods:

- The recovery of conventional microdialysis probes were assessed *in vitro*.
- Quantitative microdialysis methods: MQ probes, extrapolation to zero flow method, retrodialysis, dual probe NNF were assessed as a measure of absolute external concentration around the probe *in vitro*.

2.2 Materials and methods

2.2.1 Construction of conventional microdialysis monoprobe

Monoprobes were constructed as described in Young and Bradford 1991. Two lengths of fused silica capillary tubing (150 μm: SGE Ltd; UK) were inserted into a 10mm length of cuprophan dialysis tube (320 μm: COBE Laboratories; UK), which had previously been sealed at one end using epoxy resin. This dialysis tip assembly was glued into a 12 mm length of stainless steel tube (340 μm) using cyanoacrylate gel, leaving a 2 mm length of dialysis membrane protruding from the end. The whole assembly was then glued into a purpose built perspex Y-piece such that the

stainless steel shaft extended 10.5 mm from the block. A sleeve of stainless steel tube of 6 mm length (340 μm) with a silicon rubber connector was glued, using epoxy resin, into the remaining holes in the perspex block, covering the exposed ends of the fused silica capillaries. All stainless steel tubes and rods were supplied precut by Coopers Needle Works; Birmingham, UK. Microdialysis probes were made in batches of 10 and stored in dry conditions until use.

2.2.2 Conventional microdialysis procedure *in vitro*

Microdialysis was performed *in vitro* where probes were connected to a perfusion system. The perfusion was made using 2.5 ml syringes (Supelco SGE; UAS) of aCSF: (mM: sodium chloride, 125; potassium chloride, 3.3; magnesium sulphate, 2.4; potassium dihydrogen phosphate, 1.2; calcium chloride, 1.8, pH 7.4), using an Instech 2000 perfusion pump. The syringes were connected to the probes inlet using PEEK tubings (0.1 mm i.d., 0.5 mm o.d; Upchurch Scientific Ltd). The delivery tube (PEEK tubing) length was as such that the dead time between the probe outlet and the collection vial was 30 min. This dead time was taken into account when analysing the data. The probes were perfused continuously with aCSF at flow rate of 1 μl/min. The monoprobes were immersed in 10 ml solution containing either of 100 nM of mixture dopamine, and DOPAC, or 100 μM of amphetamine, or 100 μM of PD168077 in a beaker. The beaker solution was maintained at 37ºC over a heated stirrer. The perfusion was started immediately and a 30 min sample was collected to clear the dead space and followed by a 30 min equilibration sample, both the samples were discarded. Then consecutive 30 min dialysate samples (C_{out}) were collected for 180 min *(figure 2.6)* and analysed for the

contents. The recovery of each probe was then calculated as percentage by using the formula: Recovery (%) = (C_{out}/C_{medium}) * 100, for each time point.

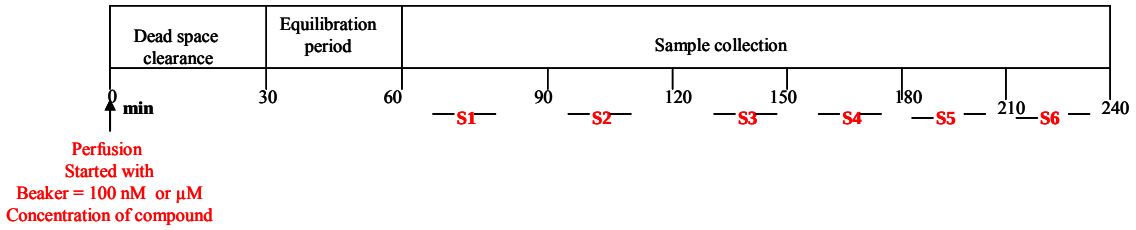

Figure 2.6: A diagram of the timeline of the experimental procedure of using conventional homemade probes to determine recovery of compounds in vitro.

2.2.3 Assessment of quantitative methods in vitro

2.2.3.1 MQ probes description

MQ microdialysis probes, comprising a cellulose dialysis tip (length 4 mm; diameter 216 µm: cut off of 18 kDa: Cremers and Ebert 2007) were supplied by BrainsOnLine (BrainLink, Netherlands). MQ probes consisted of a very slow flow rate (around 0.15 µl/min) through the dialysis tip, and the flow is facilitated by a secondary perfusion at a high flow rate (0.80 µl/min), to provide an overall output of flow rate of 0.95 µl/min, yielding a dilution factor of 6.3 in the final volume of the samples.

2.2.3.2 MQ probes recovery procedure *in vitro*

The MQ probe was connected and perfused continuously with aCSF (as described above) using a CMA402 (CMA / Microdialysis, Stockholm, Sweden) a two channel

perfusion pump. The delivery tube (PEEK tubing, supplied by Upchurch Scientifics, Sweden) length was as such that the dead time between the probe outlet and the collection vial was 30 min which was taken into account when analysing the data. MQ probes were immersed in a 10 ml solution containing either of 100 nM of mixture of dopamine, and DOPAC or of 100 µM of amphetamine or 100 µM of PD168077 in a beaker. The beaker solution was maintained at 37ºC over a heated stirrer. The perfusion was started immediately and a 30 min sample was collected to clear the dead space and followed by a 30 min equilibration sample, both the samples were discarded. Then consecutive 30 min dialysate samples (C_{out}) were collected for 120 min then the beaker concentration was changed to distilled water and two 30 min samples were collected *(figure 2.7)* and analysed for the contents. The recovery of each probe was then calculated as percentage by using the formula: Recovery (%) = (C_{out}/C_{medium}) * 100, for each time point and corrected for the dilution factor, which was 6.3. the dilution factor was calculated by: (Total outlet volume/slow flow volume) = 0.98 (µl/min)/ 0.15 (µl/min) = 6.3.

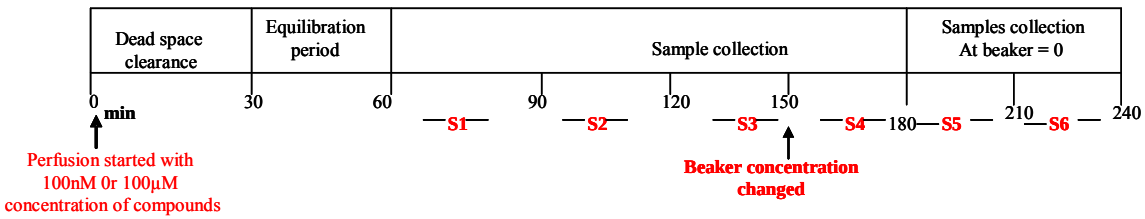

Figure 2.7: A diagram of the timeline of the experimental procedure of using MQ probes to determine recovery of compounds in vitro.

2.2.3.3 Extrapolation to zero flow method

The monoprobes were connected to the perfusion system as mentioned before. The monoprobes were immersing in beaker of 100 nM concentration (C_{medium}) of dopamine. The beaker solution was maintained at 37ºC over a heated stirrer. Four different flow rates (0.5, 0.8, 1 and 2 µl/min) were used in different experiments. The sampling time differed for each flow rate as the sample volume was kept consistent (20 µl). A 30 min sample was collected to clear the dead space and followed by a 30 min equilibration sample, both the samples were discarded. At 2 µl/min flow rate, the dialysate samples (C_{out}) was collected every 10 min for 60 min. At 1 µl/min flow rate, the dialysate samples (C_{out}) was collected every 20 min for 120 min. At 0.8 µl/min flow rate, the dialysate samples (C_{out}) was collected every 25 min for 150 min. At 0.5 µl/min flow rate, the dialysate samples (C_{out}) was collected every 40 min for 240 min. The recovery of each probe at each flow rate was then calculated as percentage by using the formula: Recovery (%) = (C_{out}/C_{medium}) * 100 and was plotted against respective flow rates.

2.2.3.4 Retrodialysis method

The monoprobes were connected to the perfusion system as described before. The experiment was performed in 2 parts; the probe tip was initially immersed in a beaker containing 100 nM dopamine or amphetamine and perfused with aCSF at flow rate of 1 µl/min. After dead space clearance and 30 min equilibration period, consecutive 30 min samples were collected for 120 min. Then the perfusate was changed to one containing 100 nM of dopamine or of amphetamine and the probe tips were immersed in distilled water. After 30 min dead space clearance and 30 min

equilibration period, consecutive 30 min samples were collected for further 120 min. The recovery of each probe was then calculated as percentage by using the formula: Recovery (%) by gain = (C_{out}/C_{medium}) * 100, for part1 and for part 2, the recovery was calculated by the formula: Recovery (%) by loss = ((C_{out}-C_{in})/ C_{in}) * 100.

2.2.3.5 Construction of dual probe NNF probes

Two monoprobes were constructed as described in section 2.2.1, then maintaining 1 mm between the two monoprobes tips; the monoprobes were glued at the body of the probes using epoxy resin.

2.2.3.5.1 Dual probe NNF method

In the dual monoprobes assembly, one of the two probes was perfused with aCSF and the other was perfused with 10 nM concentration of dopamine or of amphetamine. Both probes were perfused at 1 µl/min flow rate using Instech 2000 perfusion pump. A dynamic system was achieved by varying the beaker concentrations at 0, 5, 3 6 and 10 nM of dopamine or amphetamine respectively within an individual experiment. The beaker solutions were maintained at 37ºC over a heated stirrer. After dead space clearance and a 30 min equilibration sample, consecutive 30 min samples were collected for 120 min (see *figure 2.4* for experimental design). The C_{out}-C_{in} values of each probe were plotted against each C_{in} values respectively for each time point, and NNF point was obtained by linear regression.

Figure 2.8: Experimental timeline for determining dual probe NNF experiments in vitro.

2.2.4 Analytical method

2.2.4.1 Detection of dopamine, DOPAC, 5HIAA and HVA by high performance liquid chromatography

Standard solutions: Fresh standards of dopamine, DOPAC, 5HIAA and HVA in aCSF were prepared daily. The standards were made by serial dilutions to give concentrations of 1, 2.5, 5, 7.5 and 10 nM solutions which were used to calibrate the assay on a daily basis.

The HPLC system was composed of a Rheo 4000 pump (Pharmacia Biotech, Milton Keynes, Buckinghamshire, UK), an auto sampler (Triathlon, Spark, Holland) and a Luna C18, 5 μm, 100 x 1 mm (Phenomenex Ltd). Following chromatographic separation, sample constituents were detected electrochemically at a glassy carbon electrode set at 700 mV relative to Ag/AgCl electrode (ANTEC Intro, Netherlands) with ANTEC, VT-03 (low volume flow cell) at flow rate of 0.9 ml/min. The mobile phase comprised of a phosphate buffer (75 mM sodium dihydrogen phosphate, 1 mM EDTA, 1.6 mM of 1-octanesulfonic acid, 14% methanol). The solutions were prepared with purified water. The mobile phase was adjusted to pH 3.7 using concentrated hydrochloric acid and was vacuum filtered before use with 0.2 μm filter papers (Millipore Ltd., Watford, Hartfordshire, UK).

Dialysate samples (15 μl) were injected automatically onto the column. The concentrations of dopamine, DOPAC, 5HIAA and HVA were measured relative to standard solutions, using ChromPerfect data analysis software (Justice Laboratory Software).

2.2.4.2 Mass spectrometry detection of amphetamine

The concentration of amphetamine in the dialysate samples was measured by liquid chromatography with mass spectrometry (LC/MS). Brain dialysate samples were prepared for LC/MS by addition of 5 μl internal standard (IS: ranitidine, 2 ng/ml) followed by 195 μl

of MF5 buffer to 5 µl sample: 5 µl of this mixture was injected onto an API 4000 (Applied Biosystems/MDS SCIEX) mass spectrometer. The multiple reaction monitoring (MRM) transition 136 > 91 was monitored for amphetamine using a turbo ion spray (TIS) source in positive mode. The analyte was separated by the endogenous components in the sample using an Agilent 1200 Ultra High Performance Liquid Chromatography (UHPLC) system equipped with a Zorbax Eclipse XDB-C18 (4.6 x 50 mm, 1.8 µm) column. Gradient elution was performed by using MF5 buffer (10% methanol, 90% water v/v, 0.027 % Formic Acid, 2 mM Ammonium Acetate, pH 3.6) and MF4 (90% methanol, 10% water v/v, 0.027 % Formic Acid, 2 mM Ammonium Acetate, pH 3.6) buffer as mobile phases.

Data were acquired and processed using the Analyst software, version 1.4.2 (Applied Biosystems/MDS SCIEX) and amphetamine concentrations were expressed as free base.

2.2.4.3 Mass spectrometry detection of PD168077

The dialysate sample of PD168077 was measured by LC/MS/MS. Brain dialysate samples were prepared for LC/MS/MS analysis by addition of 5 µl IS (gabapentin) followed by 50 µl of mobile phase comprising MF4 and 2 mM ammonium acetate (pH 3.6) to 5 µl sample. 5µl dialysate samples were injected onto an API 4000 (Applied Biosystems/MDS SCIEX) mass spectrometer. The MRM transitions 335 > 200 and 172 > 137 were monitored for PD0168077 and the IS respectively, using a TIS source operating in positive mode. The analyte was separated by the endogenous components in the sample using an Agilent 1200 UHPLC system equipped with a Zorbax Eclipse XDB-C18 (3 x 50 mm, 1.8 micron) column. Gradient elution was performed by using 10 mM ammonium formate containing 0.1% formic acid as aqueous mobile phase and acetonitrile containing 1% formic acid as organic mobile phase.

Data were acquired and processed using the Analyst software, version 1.4.2 (Applied Biosystems/MDS SCIEX) and PD168077 concentrations were expressed as free base.

2.2.5 The principle chemicals and drugs used

Calcium chloride, d-amphetamine sulphate, DOPAC, dopamine hydrochloride, 5HIAA, HVA, *o*-phthaldialdehyde, mercaptoethanol, boric acid, sodium hydroxide, potassium chloride, sodium chloride, magnesium sulphate, potassium sulphate, sodium dihydrogen phosphate, EDTA (disodium salt), octanesulfonic acid, amphetamine, PD168077 were all supplied by Sigma, Poole, UK. Ranitidine were purchased from Sigma (Steinhem, Germany). Ammonium acetate was purchased from Aldrich (Steinhem, Germany) and ammonium formate was purchased from Fisher Chemicals (Fisher Scientific, UK). Methanol, MF5 and MF4 were purchased from Romil Ltd. (Cambridge, UK). All chemical used were analytical grade. The water used was deionised with a Milli-Q plus deioniser (Millipore, Bedford, MA, USA).

2.3 Results

2.3.1 Dopamine, DOPAC, 5HIAA and HVA assay detection

The detector response was obtained as change in the voltage (milliVolts) *(figure 2.5)* that occurs linearly in proportion to increasing concentration of injected dopamine over the range of 1, 2.5, 5, 7.5 and 10 nM *(figure 2.6).* Dialysate samples (15 µl) were injected automatically onto the column. The concentrations of dopamine, DOPAC, 5HIAA and HVA were measured relative to standard solutions.

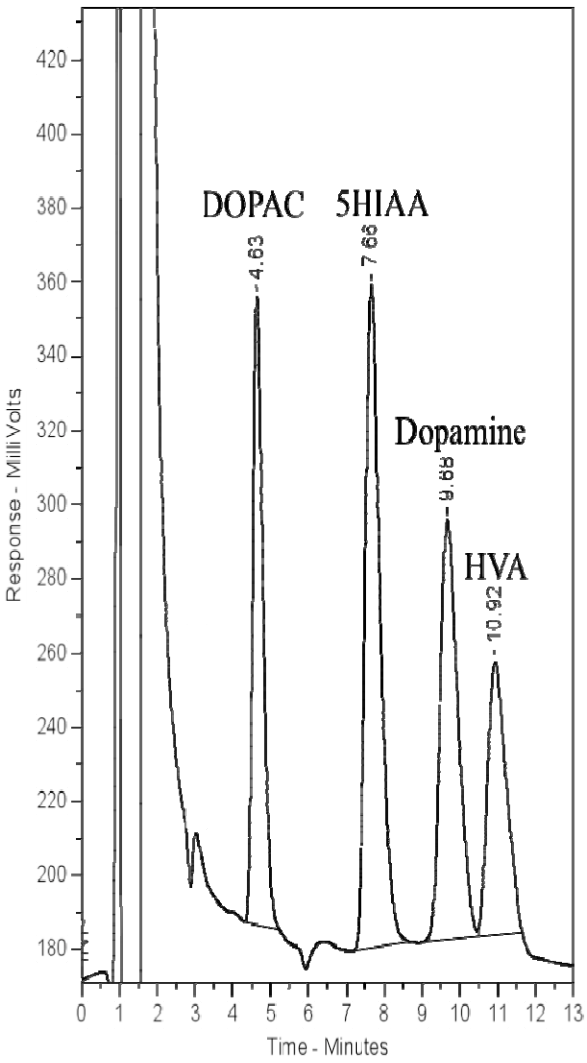

Figure 2.5: The original chromatogram showing the separation of DOPAC, 5HIAA, dopamine and HVA on a HPLC system.

The retention time was 4.6 ±0.2 min for DOPAC, 7.66 ±0.3 min for 5HIAA, 9.6 ±0.4 min for dopamine and 10.9 ±0.2 min for HVA.

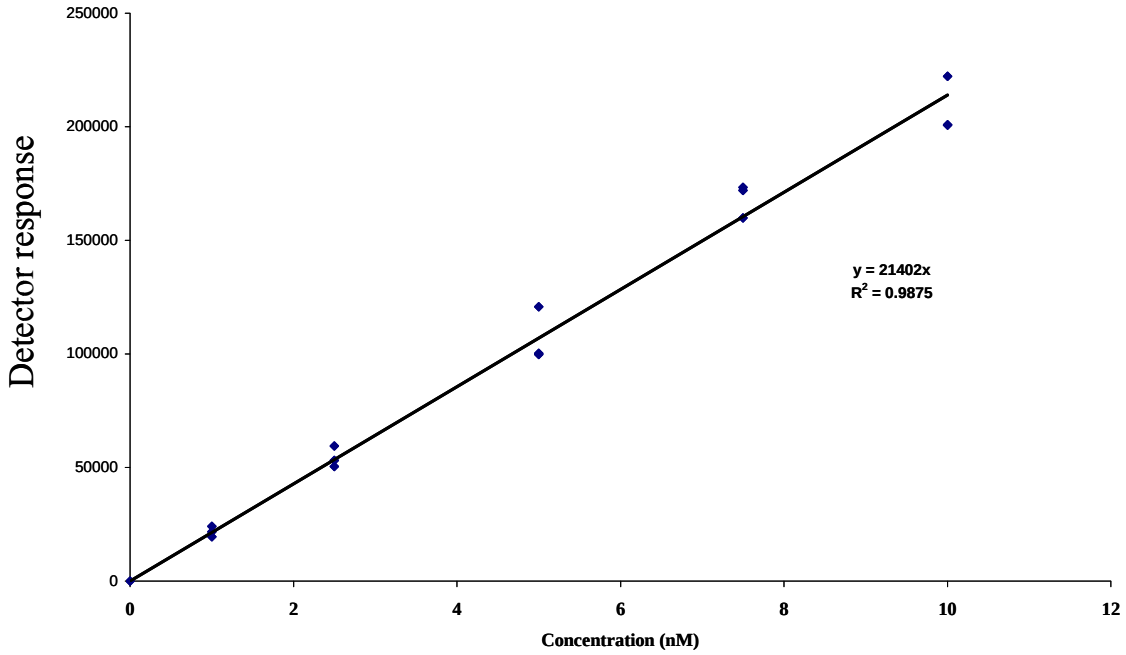

Figure 2.6: The plot of the linear relationship between the concentration of dopamine injected and the detector response (area under the curve obtained from figure 2.5).

Individual data points from three independent injection of each standard were plotted (n=3). The line of best fit is shown. The correlation coefficient for the concentration response curve was $r^2 = + 0.98$.

2.3.2 Recovery of dopamine and DOPAC using conventional monoprobe and MQ probes *in vitro*

The recovery of dopamine, and DOPAC, using conventional microdialysis monoprobes and MQ probes were carried out to assess the recoveries obtained by the two probe types. The recovery obtained by monoprobes gave the mean recovery (%) of dopamine of 19.0 ±2.5 and DOPAC of 13.0 ±2.1 (*figure 2.11 a*). MQ probes gave mean recovery (%) over the time period for dopamine of 96.9 ±2.3 and DOPAC of 95.4 ±3.4. The recovery (%) decreased with change of beaker to distilled water; dopamine was 5.2 ±1.8; DOPAC was 5.0 ±2.0 in S5 (at

150 min) and in S6 (at 180 min), the recovery (%) for both compounds was 0 *(Figure 2.11b).*The MQ probes gave near 100% recovery of dopamine and DOPAC compared to the conventional monoprobes *in vitro*.

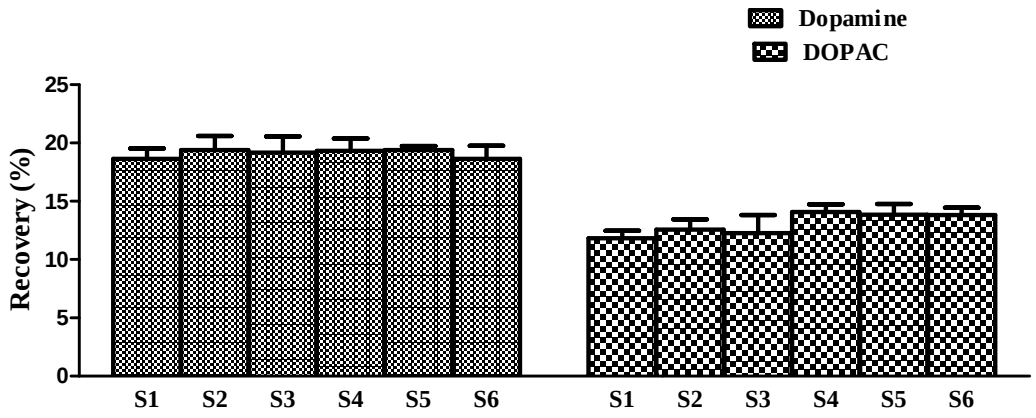

Figure 2.11 a): The recovery (%) of dopamine, and DOPAC, using conventional microdialysis monoprobe (n=5).

Consecutive 30 min dialysate samples were over the duration of the experiment. The conventional monoprobes were immersed in a beaker containing 100 nM concentration of dopamine or DOPAC. Then dialysate samples S1, S2, S3, S4, S5 and S6 were collected at 30, 60, 90, 120, 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes. For clarity samples following experiments carried out using dopamine or DOPAC, are shaded differently.

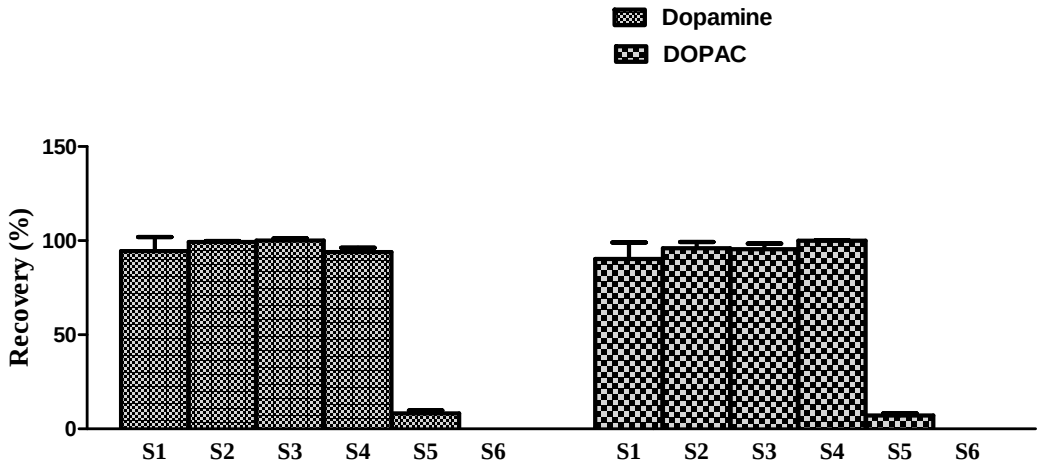

Figure 2.11 b): The recovery (%) of dopamine and DOPAC using MQ probes (n= 4).

Consecutive 30 min dialysate samples were over the duration of the experiment.
The MQ probes were immersed in a beaker containing 100 nM concentration of dopamine or DOPAC. Then dialysate samples S1, S2, S3 and S4 were collected at 30, 60, 90, and 120. Then the beaker solution was changed to distilled water and samples S5 and S6 were collected at 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes. For clarity samples following experiments carried out using dopamine or DOPAC, are shaded differently.

2.3.3 Recovery of amphetamine using conventional monoprobe and MQ probes *in vitro*

The recovery of amphetamine, using conventional microdialysis monoprobes and MQ probes were carried out to assess the recoveries obtained by the two probe types. Conventional monoprobes gave the mean recovery (%) of amphetamine as 15.2 ±3.2 *(figure 2.12 a)* and MQ probes gave mean recovery for amphetamine as 95.0 ±3.3. The recovery decreased with change of beaker to distilled water; amphetamine was 7.6 ±1.2; in S5 (at 150 min) and in S6 (at 180 min), the recovery (%) was 0 *(figure 2.12 b)*. The MQ probes gave near 100% recovery of amphetamine compared to the conventional monoprobes *in vitro*.

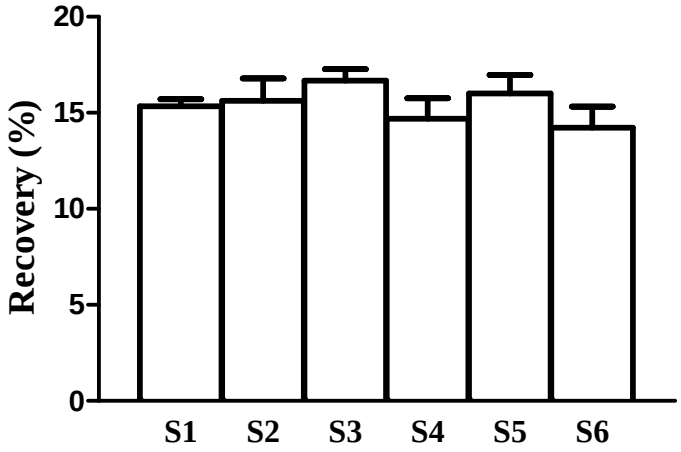

Figure 2.12 a): The recovery (%) of amphetamine using conventional microdialysis monoprobe (n=5).

Consecutive 30 min dialysate samples were over the duration of the experiment. The conventional monoprobes were immersed in a beaker containing 100 nM concentration of amphetamine. Then dialysate samples S1, S2, S3, S4, S5 and S6 were collected at 30, 60, 90, 120, 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes.

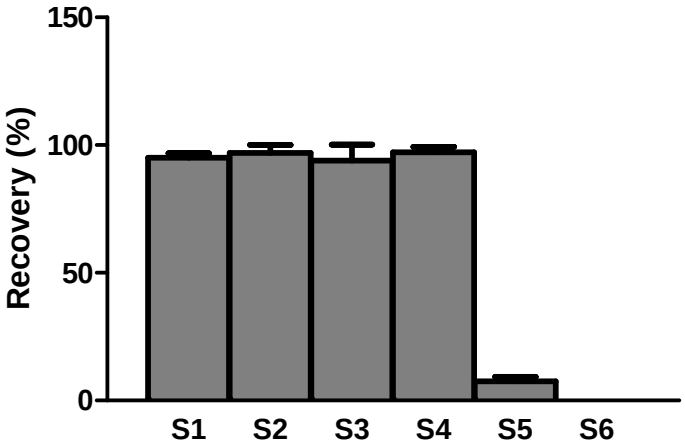

Figure 2.12 b): The recovery (%) of amphetamine using MQ probe (n=4).

Consecutive 30 min dialysate samples were over the duration of the experiment. The MQ probes were immersed in a beaker containing 100 nM concentration of amphetamine. Then dialysate samples S1, S2, S3 and S4 were collected at 30, 60, 90, and 120. Then the beaker solution was changed to distilled water and samples S5 and S6 were collected at 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes.

2.3.4 Recovery of PD168077 using conventional monoprobe and MQ probes *in vitro*

The recovery of PD168077 using conventional microdialysis monoprobes and MQ probes were carried out to assess the recoveries obtained by the two probe types. The recovery (%) of PD168077 obtained by conventional monoprobes was 11.0 *(figure 2.13 a).* For MQ probes, the mean recovery (%) of PD 168077 was 98.5 ±7. The recovery (%) decreased with change of beaker to distilled water; PD168077 was 7 ±1.8; in S5 (at 150 min) and in S6 (at 180 min), the recovery (%) was 0 *(figure 2.13 b).* The MQ probes gave near 100% recovery of PD168077 compared to conventional monoprobes *in vitro*.

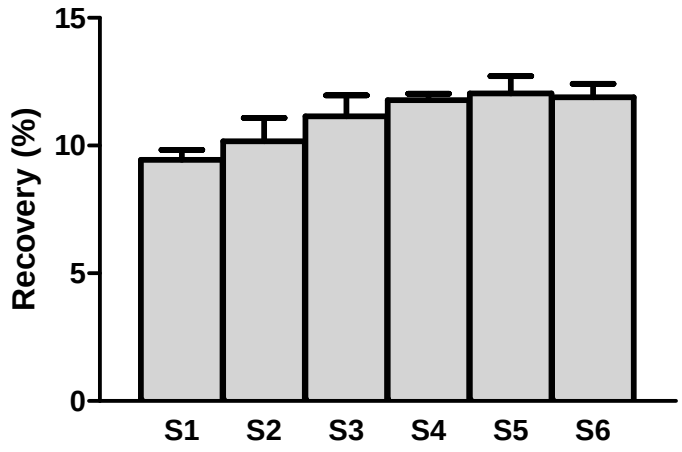

Figure 2.13 a): The recovery (%) of PD168077 using conventional microdialysis monoprobes (n=5).

Consecutive 30 min dialysate samples were over the duration of the experiment. The conventional monoprobes were immersed in a beaker containing 100 nM concentration of PD168077. Then dialysate samples S1, S2, S3, S4, S5 and S6 were collected at 30, 60, 90, 120, 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes.

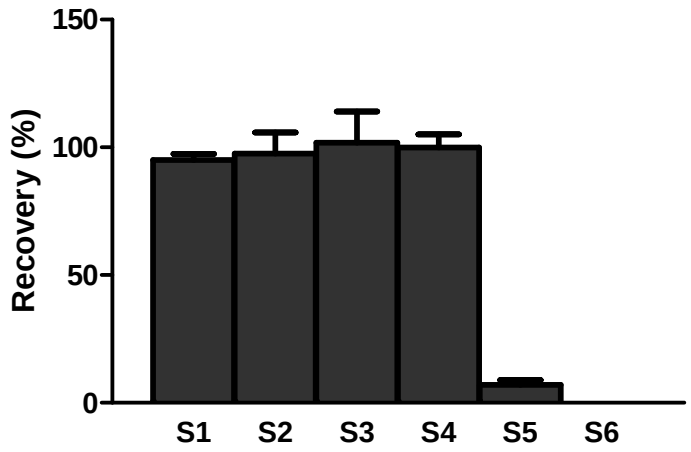

Figure 2.13 b): The recovery (%) of PD168077 using MQ probes (n=4).

Consecutive 30 min dialysate samples were over the duration of the experiment. The MQ probes were immersed in a beaker containing 100 nM concentration of PD168077. Then dialysate samples S1, S2, S3 and S4 were collected at 30, 60, 90, and 120. Then the beaker solution was changed to distilled water and samples S5 and S6 were collected at 150 and 180 min respectively. The dialysate concentrations were corrected for the dead time of the outlet tubes.

2.3.5 *In vitro* calibration of extrapolation to zero flow method

The recovery (%) of dopamine increased with decrease in the flow rate *(figure 2.14)*. The recovery (%) at 0.5 µl/min was 68.4 ±4.6; at 0.8 µl/min was 38.3 ±2.6; at 1 µl/min was 14.0 ±1.1 and at 2 µl/min was 8.0 ±0.2. The extrapolation of the curve *(figure 2.10)* to 100% recovery did not yield an accurate fit to 100% recovery for zero flow rate.

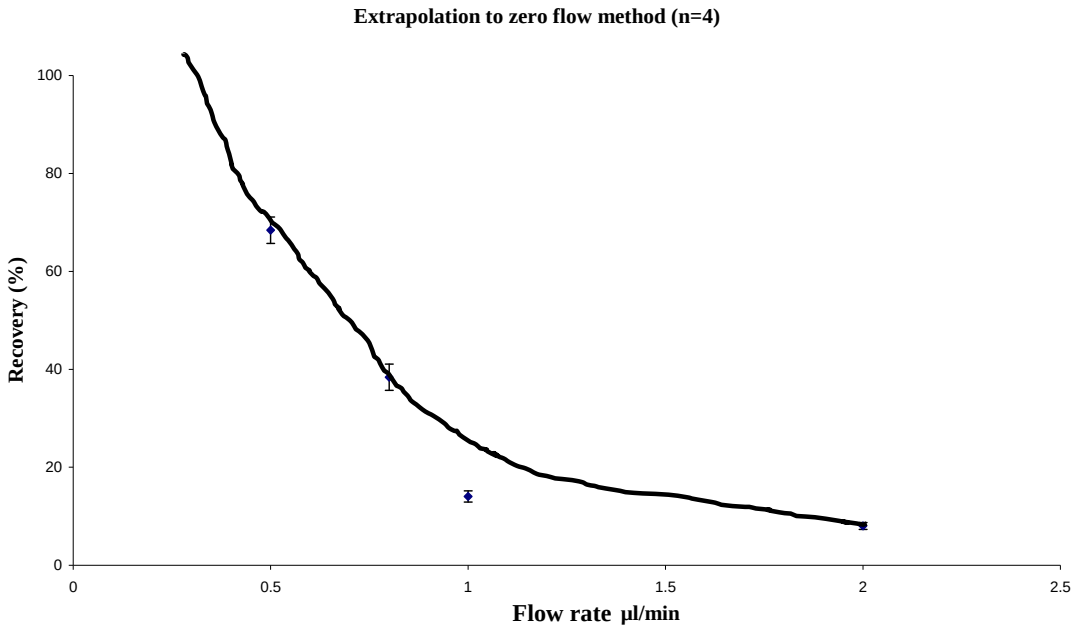

Figure 2.14: The recovery of dopamine using conventional microdialysis probes were run at different flow rates: 0.5, 0.8, 1 and 2 µl/min (n=4).

Conventional homemade monoprobes were run at four different flow rates (0.5, 0.8, 1 and 2 µl/min) in different experiments. The sampling time differed with each individual experiment and for each flow rate the sample volume was kept consistent (20 µl). The recovery of each probe at each flow rate (from respective experiments) was then calculated as percentage by using the formula: Recovery (%) = (C_{out}/C_{medium}) * 100 and was plotted against respective flow rates.

2.3.6 *In vitro* calibration of dopamine using retrodialysis method

Two-way ANOVA revealed no main effect of recovery of dopamine between part 1 and part 2 of the experiment ($F[1, 56] = 0.01$, $p < 0.9$) indicating that the influx of dopamine from the beaker to the probe was similar to the efflux of dopamine from the probe to the beaker in part 2 of the experiment; and moreover there was no main effect of sample ($F[3, 56] = 0.05$, $p < 0.58$; *figure 2.15*) indicating no changes in the recovery within the overall duration of the individual parts of the experiment. There was no interaction sample x recovery ($F[3, 56] =$

0.15, p < 0.38) indicating no different in the recovery-time profile between part 1 and part 2 of the experiment.

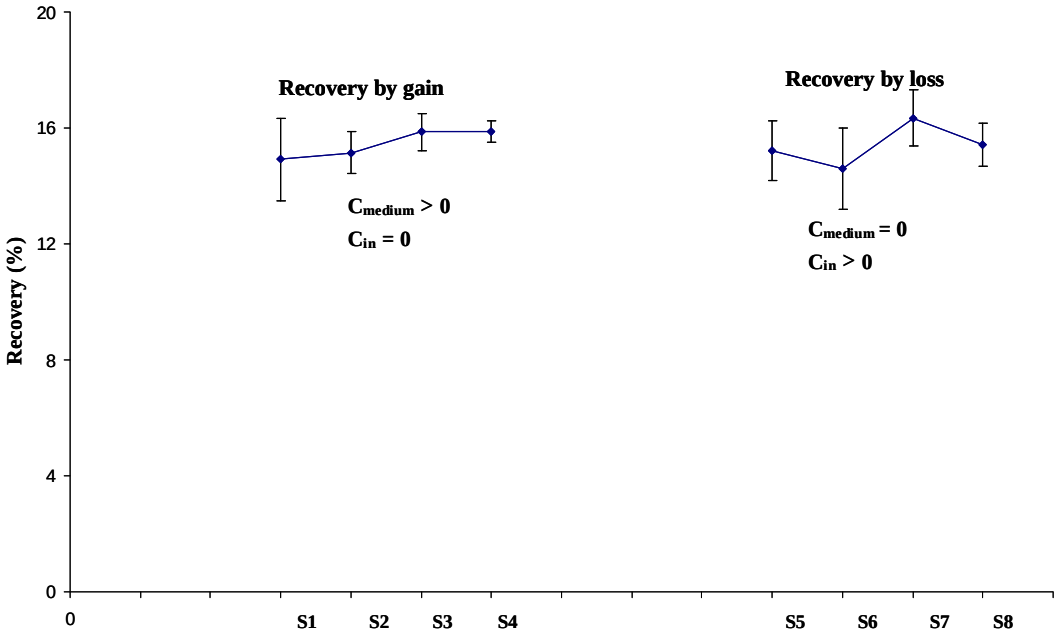

Figure 2.15: In vitro retrodialysis calibration method was performed with dopamine (n=8).

The samples S1, S2, S3 and S4 were collected for the recovery by gain (%) in part 1 of the experiment and the samples S5, S6, S7 and S8 were collected for the recovery by loss (%) in part 2 of the experiment. The recoveries from part 1 and part 2 were similar ($t(7) = 1.5$, $p = 0.9$; paired t-test).

2.3.7 *In vitro* calibration of amphetamine using retrodialysis method

For amphetamine experiments, there was significant main effect of recovery between part 1 and part 2 of the experiment ($F[1, 56] = 110.97$, $p < 0.001$) indicating that the influx of dopamine from the beaker to the probe was different to the efflux of dopamine from the probe to the beaker in part 2 of the experiment. However, no significant main effect of sample ($F[3, 56] = 0.10$, $p = 0.98$; *figure 2.16*) indicating no changes in the recovery within the overall duration of the individual parts of the experiment. However there was significant

interaction sample x recovery ($F[3, 56] = 11.01$, $p < 0.01$) indicating different in the recovery-time profile between part 1 and part 2 of the experiment.

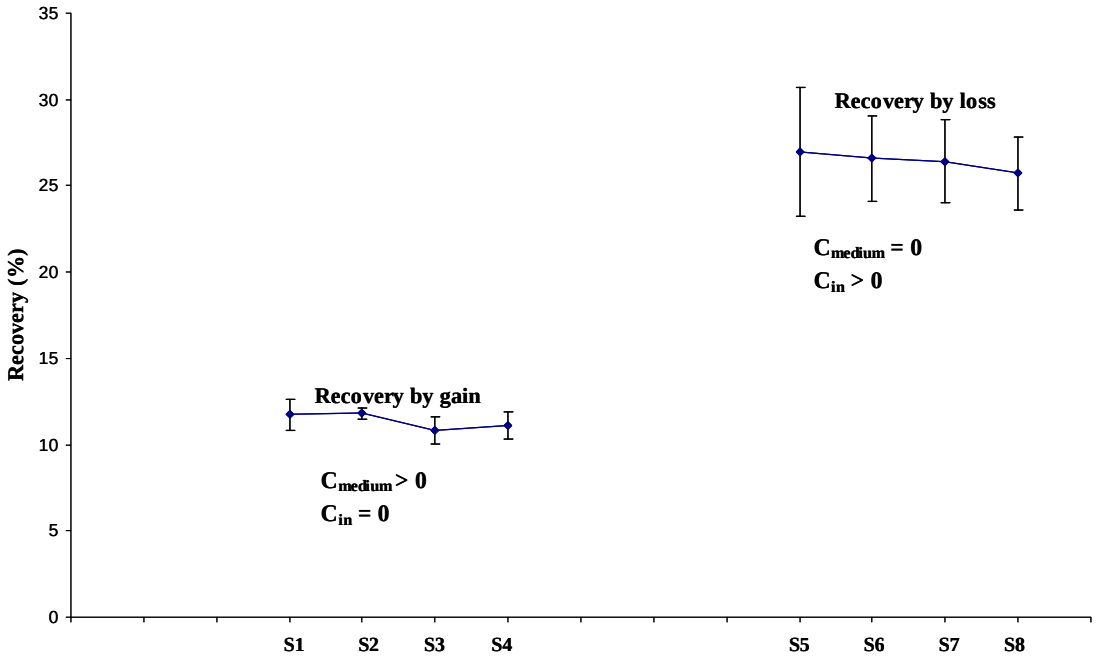

Figure 2.16: In vitro retrodialysis calibration method was performed with amphetamine (n=8).

The samples S1, S2, S3 and S4 were collected for the recovery by gain (%) in part 1 of the experiment and the samples S5, S6, S7 and S8 were collected for the recovery by loss (%) in part 2 of the experiment. The recoveries from part 1 and part 2 were significantly different ($t(7) = 7.1$, $p < 0.05$; paired t-test).

2.3.8 *In vitro* assessment of dual probe NNF using dopamine

The absolute concentration of dopamine measured was averaged from all experiments: 0.8 ±0; 4.2 ±1.2; 3.8 ±1.8; 7.2 ±2.5 and 12.5±1.1 nM measured for beaker concentrations of 0, 5, 3, 6 and 10 nM of dopamine respectively *(figure 2.17).* Each time points were averaged from all *in vitro* experiments using dual probe NNF method.

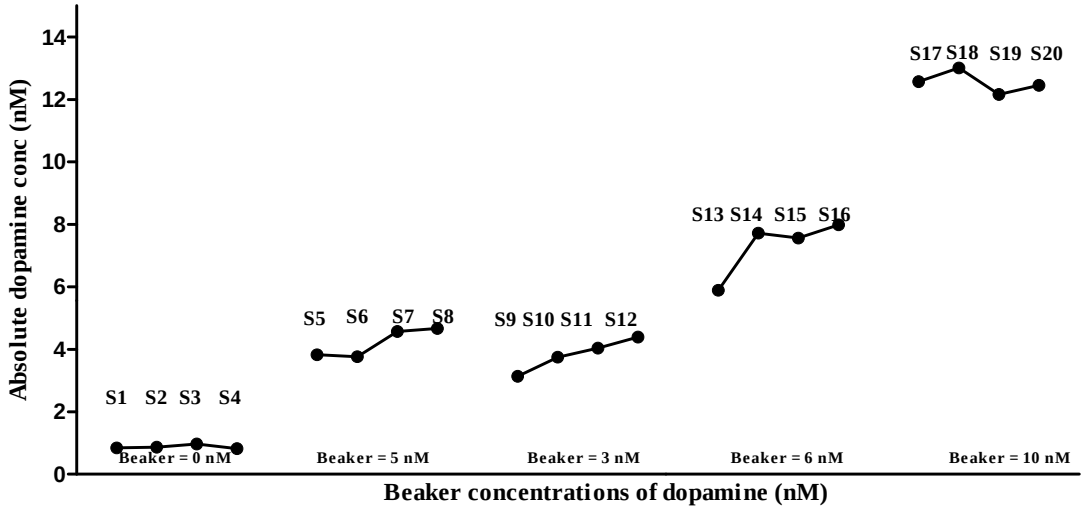

Figure 2.17: The absolute concentrations of dopamine for each beaker concentration using dual probe NNF (n=6).

Consecutive four 30 min samples were collected for each beaker concentration of dopamine. The sample S1, S2, S3 and S4 were collected for beaker solution containing concentration of 0 nM of dopamine, samples S5, S6, S7 and S8 were collected for beaker solution containing concentration of 5 nM of dopamine , samples S9, S10, S11 and S12 were collected for beaker solution containing concentration of 3 nM of dopamine, samples S13 S14, S15 and S16 were collected for beaker solution containing concentration of 6 nM of dopamine and samples S17 S18, S19 and S20 were collected for beaker solution containing concentration of 10 nM of dopamine within an individual experiment.

2.3.9 *In vitro* assessment of dual probe NNF using amphetamine

The absolute concentration of amphetamine measured was averaged from all experiments: 1.1 ±0.1; 5. 4 ±0.5; 3.3 ±0.1; 6.1 ±1.2; and 10.9 ±1.5 nM respectively measured for beaker concentrations of 0, 5, 3, 6 and 10 nM of amphetamine respectively *(figure 2.18).* Each time points were averaged from all *in vitro* experiments using dual probe NNF method.

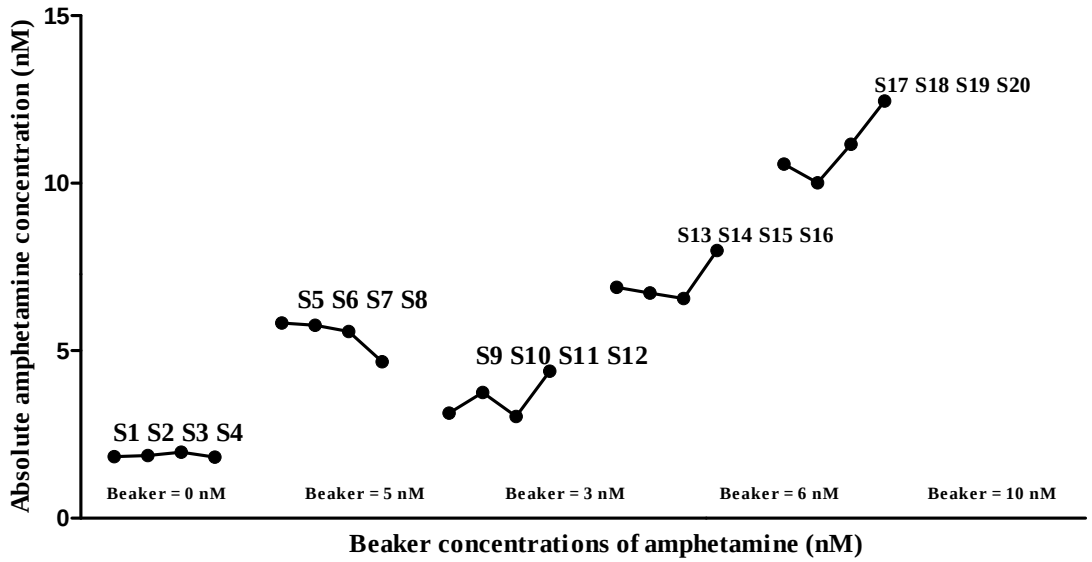

Figure 2.18: The absolute concentrations of amphetamine for each beaker concentration using dual probe NNF (n=8).

Consecutive four 30 min samples were collected for each beaker concentration of amphetamine. The sample S1, S2, S3 and S4 were collected for beaker solution containing concentration of 0 nM of amphetamine, samples S5, S6, S7 and S8 were collected for beaker solution containing concentration of 5 nM of amphetamine , samples S9, S10, S11 and S12 were collected for beaker solution containing concentration of 3 nM of amphetamine, samples S13 S14, S15 and S16 were collected for beaker solution containing concentration of 6 nM of amphetamine and samples S17 S18, S19 and S20 were collected for beaker solution containing concentration of 10 nM of amphetamine within an individual experiment.

2.4 Discussion

The initial experiments were performed *in vitro* to gain an insight into "recovery" of compounds of interest. Under *in vitro* conditions, the concentration of the medium surrounding the probe is known, and the system can be easily validated in obtaining the absolute/true concentrations surrounding the probes. The conventional homemade probes gave recovery approximately 15%-20% for dopamine, DOPAC, amphetamine, and PD168077 in comparison MQ probes gave a near 100% recovery for the compounds. The main finding of these sets of *in vitro* experiments were that MQ probes yielded a near 100%

recovery for the compounds tested *in vitro*. Furthermore, the recovery of compounds reflected the change in the external medium, when beaker concentration was changed to one containing no compound of interest. Hence these experiments provided a validation of the use of MQ probes in measuring absolute/true concentration in external medium in a dynamic system.

In addition, three other quantitative microdialysis methods were tested to measure absolute recovery of compounds *in vitro*: extrapolation to zero flow method, retrodialysis and dual probe NNF. The results of extrapolation to zero flow method showed an inverse relationship between recovery and the flow rate at which the probes (Jacobson *et al.,* 1985). As expected the recovery of dopamine increased with decrease in the flow rate. However, the main drawback in the use of the method was that when the probes were run at the flow rate below 0.5 µl/min the samples collection and analyses became difficult. The data for experiments where the probes were run at 0.3 µl/min flow rate were not obtained, as not enough sample volume was collected to analyse. Hence, the recovery values obtained using four flow rates 0.5, 0.8, 1 and 2 µl/min did not give an accurate extrapolation to 100% recovery. These experiments, therefore, highlighted the main drawback of sample collection at very slow flow rates and only gave an estimation of recovery value. Whilst running a very slow flow rate (0.15 µl/min) through the dialysis tip was not problematic with MQ probes, therefore gave an accurate measurement of external concentration around the probe tip.

The retrodialysis calibration method relies on the principle that mass transfer that occurs by diffusion of compounds across the membrane is same in each direction. Thus when a probe is immersed in a medium containing a given concentration of compound the diffusion occurs into the probe and when the probe is infused with a given concentration of compound of interest, the compound diffuses out of the probe. The main factor that influence the diffusion

is the concentration gradient and thereby this method, relies on the assumption that recovery by loss is equal to recovery by gain. The results showed that the diffusion of dopamine by gain was same as recovery by loss. However, in the case of amphetamine, the recovery by gain was not equal to recovery by loss. The recovery by gain of amphetamine was however similar to recovery by gain of dopamine but the recovery by loss of amphetamine was higher than recovery of gain of amphetamine. This implies that the diffusion of amphetamine is not uniform in both directions. This can be the major limiting factor in using retrodialysis for studying the recovery of amphetamine. This may be because the diffusion of some compounds also depends on the difference in the volume across the probe and not solely on the concentration gradient across the probe. Thus, the constantly stirred beaker with 10 ml of solution devoid of compound of interest may induce higher diffusion for some compounds from the probe that contain a small volume (around 2 µl) of concentrated fluid, into the beaker as in case of amphetamine in this experiment (Morrison 1991 a and b).

In addition to above described traditional quantitative methods we also tested novel method: dual probe NNF method, a method that was modified from traditional NNF. The main feature of our modified method was that it enabled the measurement of absolute concentration surrounding the probes in a dynamic system. This was achieved by perfusing two different concentrations through the two probes while both immersed in the same varying external (beaker) concentration. Thus, NNF points were obtained from the same system at varying external concentration. This was not possible with traditional NNF method as each probe (perfused with one concentration) were used during inter experimental days and every experiment required a steady state (same external concentration). The results from our experiments, dual probe NNF points did estimate near beaker concentrations. The main outcome was that the NNF points (C_{ecf}) reflected the changes in the beaker (external) concentrations. This showed that dual probe NNF did measure the changes in the external concentrations in a dynamic system. Similarly, MQ probes gave near 100% recovery, in a

dynamic system. Thus, dual probe NNF and MQ dialysis provide a quantitative measure of external concentrations moreover measure dynamic changes in the external concentrations.

The experiments described in this chapter were important in terms of gaining insight into recovery of various compounds of interest and at the same time to assess quantitative methods that can be used successfully in *in vivo* experiments. The compounds: amphetamine and PD168077 were chosen for *in vivo* studies. The quantitative methods: dual probe NNF method and MQ probes were selected for the experiments in chapter 3, to measure absolute/true concentrations of amphetamine and dopamine. In studies included in chapter 4 and 6, MQ probes were employed in freely moving animals to measure absolute basal concentration of dopamine, and PD1680777 (a dopamine D_4 receptor agonist) simultaneously; to monitor PD168077 induced changes in true concentration of dopamine in a dynamic system. Moreover the data obtained determined the PK/PD parameter of PD168077. The true/absolute concentration of compounds is an essential requirement in studying the PK/PD parameter of any given drug, which was achieved using MQ probes.

Chapter 3: Application of intracerebral microdialysis in PK/PD studies: Measurement of absolute brain concentration of amphetamine and dopamine in a dynamic system in anaesthetised rats

3.1 Introduction

Intracerebral microdialysis is an analytical tool that can be used for continuous sampling of the endogenous substances in the ecf space of the brain. Many studies have used microdialysis to measure the levels of endogenous neurotransmitters and their response to pharmacological and behavioural challenge (see reviews by Ungerstedt 1991; Young 1993; Westerink 1995), while other studies have measured free unbound drug levels in the brain following systemic drug administration (Morrison *et al.,* 1991; Ungerstedt 1997). However, one of the drawbacks of microdialysis has been the inability to measure absolute/true ecf concentrations of compounds. The dialysis tip comprises a semi-permeable membrane across which solutes move down their concentration gradients. Under equilibrium conditions, where there is no flow through the dialysis tip, the dialysate concentration is equal to the ecf concentration. However, when there is a flow through the dialysis tip the diffusion does not reach equilibrium and the concentration of the solutes found in the dialysate samples represents only a fraction of their concentration in the ecf. The ratio of the two concentrations is termed the relative recovery; this recovery is easily obtained *in vitro* as the concentration surrounding the probe *in vitro* is known. However, assessing recovery *in vivo* is problematic as the actual brain concentration of the substance surrounding the probe is unknown. This problem is generally circumvented in microdialysis studies, where changes in endogenous substances (e.g., neurotransmitters) are expressed as dialysate concentrations rather than actual brain concentrations. This approach overcomes the necessity of knowing the *in vivo*

recovery for the substance and is appropriate for many studies where the *changes* in concentration to drug challenge (as opposed to absolute brain concentrations) are important. However, it is severely limited for studies, such as PK studies, where knowledge of the absolute brain concentrations of drugs and neurotransmitters becomes important (see reviews by Elmquist and Sawchuk 2000; Sawchuk and Elmquist 2000).

Several approaches have been used to attempt to address this problem, and make reliable estimate of the ecf concentrations of solutes. One method is simply to assume that recovery *in vitro* provides a reliable estimate of recovery *in vivo*. This method is severely limited as physiological factors *in vivo* interact with the dialysis membrane performance (e.g., extracellular tissue tortuosity, active processes in the tissue) and are not accounted for *in vitro* recovery. The other approaches are (see section in chapter 2 for details): extrapolate to zero flow (Jacobson *et al.,* 1985); NNF method (Lonnroth *et al.,* 1987; Justice 1993; Olson and Justice 1993); retrodialysis method (Larrson 1991). All these methods have severe limitations in application to studies of dynamic process *in vivo*, such as changes in drug and neurotransmitter concentration after drug application. Both extrapolation to zero flow and NNF require multiple measurements over a relatively long period of time, and therefore require the solute of interest to be in steady state for a period of hours, while retrodialysis calibration requires the addition of the calibration compound prior to the experiment which may interfere with its distribution and its effect for the actual study.

Given that when there is no flow through the tip, equilibrium is achieved, and the recovery is 100%, it follows that at very low flow rates, the recovery is close to 100%. However, at such low flow rates the sample handling and analysis of the amount of dialysate becomes problematic. To circumvent this problem, a novel MQ method has been developed (Cremers and Ebert 2007). This incorporates ultra-slow flow through the dialysis tip to achieve near 100% recovery and a second 'carrier' flow, which dilutes the outflow from the dialysis tip

within the body of the dialysis probe to give manageable flow in the output line, and sufficient sample volumes. This allows accurate quantitative microdialysis with convenient sample volumes for fluid handling and short lag times inside the outlet tubing (Cremers *et al.,* 2009; Sood *et al.,* 2009).

In my lab, I developed an alternative approach which relies on the NNF principle but using dual probes for measuring absolute ecf levels (Sood *et al.,* 2007). The main advantage of dual probes NNF is that it can be employed in dynamic system (see section in chapter 2 for details). *In vitro* results (Chapter 2) validated the use of dual probe NNF method and MQ probes in measuring the absolute concentrations, in this chapter these methods were assessed in anaesthetised rats (*in vivo*). Amphetamine was chosen for these studies, since its central actions are well characterised (e.g. Sulzer *et al.,* 1995; Rothman and Baumann 2003). Amphetamine induced dopamine dynamics have been well documented using microdialysis. At the cellular level, amphetamine interferes with neurotransmitter systems by increasing extracellular dopamine levels (Ellison *et al.,*1978) by interactions with dopaminergic cells through several different mechanisms. The putative mechanisms involved in amphetamine induced dopamine release from dopaminergic neurons *(figure 3.1)* are:

1) Amphetamine is a substrate for the dopamine transporter (DAT) (Sitte *et al.,* 1998). At low concentrations, amphetamine is transported by the DAT to the cytosol (into the cells) and resulting in the exchange of extracellular amphetamine by intracellular dopamine, and leading to an increase in extracellular dopamine (Jones *et al.,* 1999).
2) At higher concentrations, increased extracellular concentrations, leading to additional passive diffusion into the cell. This passive diffusion is attributed to amphetamine's liphophilic characteristics (Sulzer *et al.,* 1995; Kahlig *et al.,* 2005).

3) When inside the cells, intracellular amphetamine also interferes with the vesicular monoamine transporter (VMAT) function, rapidly redistributing VMAT to a nonvesicular location (Riddle *et al.,* 2002), impairing the active transport of dopamine into synaptic vesicles, where it would be normally stored. Hence increasing intracellular cytosolic dopamine levels, leads to release into the synaptic cleft through active/passive diffusion.

4) In addition, amphetamine may enter in the vesicles by diffusion, due to its weak base properties. A low pH inside the vesicles is essential to maintain the proton gradient used by VMAT for active transport of monoamines into the vesicles. Since amphetamine is a weak base, at acidic pH it accepts protons leading to alkalinisation inside the vesicles. Therefore, amphetamine induces the release of vesicular dopamine to the cytosol and impairs the storage of dopamine in the vesicles. Cytosolic dopamine is then released to the extracellular space via reverse transport by the DAT (Sulzer *et al.,* 2005).

5) Amphetamine also interferes with dopamine synthesis. At low concentrations, amphetamine promotes dopamine synthesis (Kuczenski 1975), whereas at higher concentrations or upon prolonged exposure, reduced TH activity which may be mediated by TH inhibition (Ellison *et al.,* 1978) or decreased TH protein levels (Bowyer *et al.,* 1998).

6) Amphetamine also impairs dopamine metabolism by inhibiting MAO at the synaptic cleft (Ramsay and Hunter 2002). Therefore leading to decrease in DOPAC levels, at the synaptic cleft.

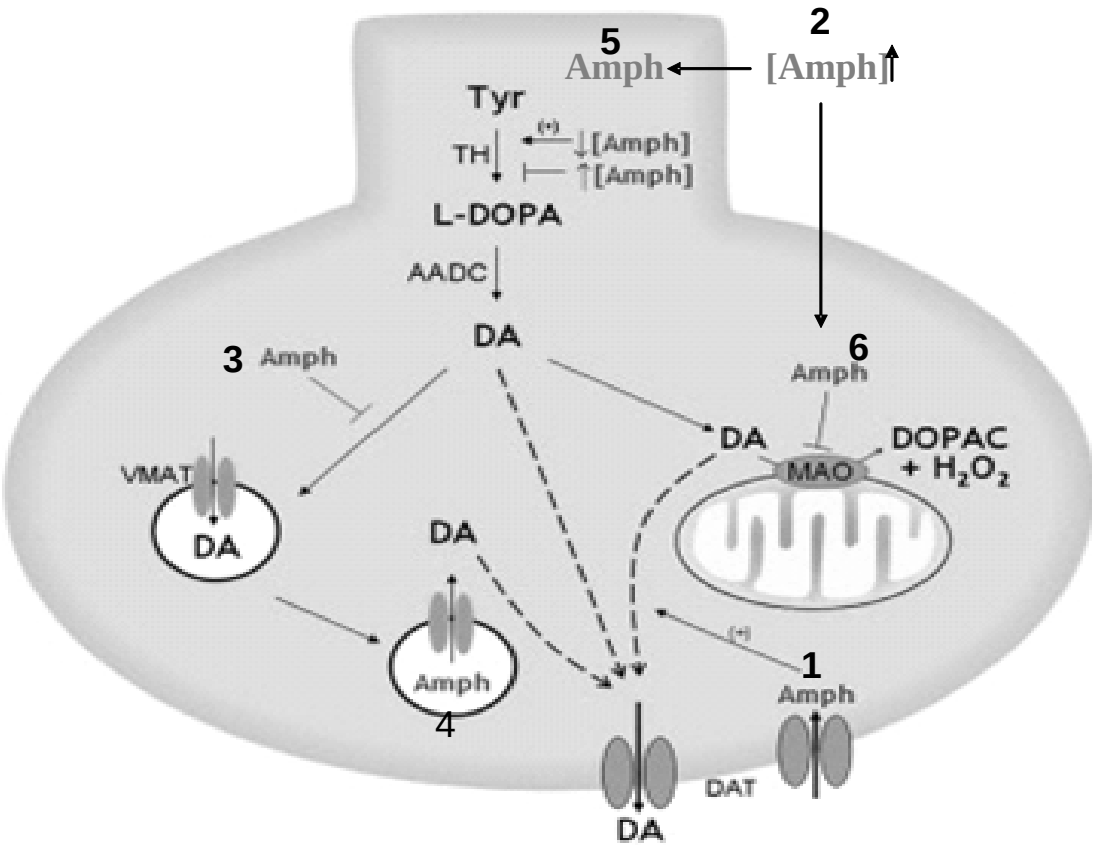

Figure 3.1: Putative mechanism involved in amphetamine induced dopamine release from dopaminergic neurones (figure from Stahl 1997).

Ever since the synthesis of the first of amphetamines in 1887, there has been extensive interest in characterising their behavioural and pharmacological effects (see review by Seiden *et al.,* 1993). They have been valuable tools in exploring the biochemical basis of psychotic and mood disorders (Ellison 1993). Due their abuse potential and capability of exacerbating or inducing mood and psychotic disorders in humans, investigations into their behavioural effects are commonly studied with the laboratory rat (Seiden *et al.,* 1993). In rodents, studies have shown that amphetamine (0.1-15 mg/kg, i.p.) produced significant dose- and time-

dependent changes in responses such as locomotion and stereotypy responses (rearing and sniffing). The present study aims to quantify the neurochemical effect of amphetamine, at the doses of 0.5 mg/kg and 1 mg/kg in anaesthetised rats.

3.1.1 Aims

Experiment 1: To assess the use of two novel microdialysis methods in measuring absolute ecf concentrations of amphetamine: (1) dual probe NNF method and (2) MQ ultra-slow flow method in a dynamic system.

Experiment 2: To compare the *in vivo* measurement of amphetamine and dopamine by conventional microdialysis monoprobes and MQ probes, simultaneously in a dynamic system. Moreover in the same animals, the blood concentrations of amphetamine were measured to study the distribution profile of amphetamine from the blood to brain obtained by: conventional monoprobes and MQ probes.

3.2 Materials and methods

3.2.1 *In vitro* recovery of dopamine and amphetamine using conventional monoprobes and MQ probes

The *in vitro* microdialysis perfusion was carried out for both conventional and MQ dialysis probes as described in chapter 2. The dialysis tips were inserted in a continuously stirred beaker containing 10 ml of mixture solution: 1 µM mixture of dopamine and amphetamine. For conventional probes, perfusion was at 1 µl/min: for MQ probes the tip was perfused at 0.15 µl/min and the carrier flow was 0.8 µl/min. The dialysate samples were collected and the content of dopamine and amphetamine were analyses as described in chapter 2.

3.2.*2 In vivo* microdialysis

3.2.2.1 Animals, housing and surgery

Male Sprague Dawley rats (275±30 g; Charles River) were housed in groups of two in a temperature and humidity controlled environment and under regular lighting conditions (12-hour light/dark cycle) with food and water available *ad libitum*. Microdialysis experiments were performed 7-10 days after arrival.

3.2.2.2 Microdialysis surgery and probe implantation

Rats were anaesthetised with urethane (ethyl carbamate: 1.75 g/kg, i.p.). Once pedal and eye blink reflexes were lost, the rats were prepared for surgery by shaving the hair on the head with a hand held electric shaver. The rats were then mounted in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, USA), with the incisor bar set at 3.3 mm below the intraural line. During surgical procedures, in order to ensure appropriate depth of anaesthesia, a low level of isofluorane (1% isofluorane in 1l/min oxygen) was also applied via a face mask. The scalp was cut and retracted to expose the surface of the skull. A 1 mm diameter hole was drilled overlaying: a) for experiment 1: in the right caudate nucleus (H: +3.2, Tr: -0.5) and two 1mm hole in left caudate nucleus for dual probe NNF (H: -3.2, Tr: -0.4; -0.5) relative to bregma and b) for experiment 2: A 1 mm diameter hole was drilled above: right caudate nucleus (H: +3.2, Tr: -0.5) and in left caudate nucleus (H: -3.2, Tr: -0.5) relative to bregma; according to Paxinos and Watson 1998, leaving the dura mater intact. A further two smaller holes were drilled to mount the stainless steel anchor screws. The skull was cleaned and dried. The microdialysis probes were placed in the probe holder (Biotech Instruments Ltd; UK) attached to the stereotaxic frame. Appropriate co-ordinates were relocated from the bregma and after dura mater membrane was punctured with a needle, the probes were

lowered vertically over c. 2 min into: For experiment 1: A MQ probe was implanted in the right caudate nucleus (tip: V,7.0) and dual dialysis monoprobes were implanted in the left caudate nucleus, with a tip separation of 1mm (tip: V,7.0). For experiment 2: A MQ probe was implanted into the right caudate nucleus (tip V,7.0), and conventional dialysis monoprobes were implanted into the left caudate nucleus (tip: V,7.0): stereotaxic positions are quoted relative to dura (Paxinos and Watson 1998). The probes were secured to the skull with the two anchor screws and dental acrylic cement. Particular care was taken to ensure that the cement mound and the seal to the wound were sound. The animals were then administered a post operative 5 ml of 0.9% saline with 1% glucose. Following the surgical procedure, the animals were taken off isoflurane and maintained on urethane (0.5 mg/kg, i.p.) for the rest of the experiment. The pedal reflex and eye blink were monitored every 15 min.

3.2.2.3 *In vivo* microdialysis procedure

The animals were connected to microdialysis perfusion system (the perfusion system as described in chapter 2). Following a 60 min of initial stabilisation period, perfusion was started immediately. For experiment 1 the MQ probes were perfused with aCSF at 0.95µl/min (tip flow, 0.15 µl/min; carrier flow 0.8 µl/min) and for the dual probe assembly; one probe was perfused with aCSF and the other with 10 µM amphetamine, both probes perfused at flow rate of 1 µl/min. For experiment 2; both MQ (tip flow: 0.15 µl/min; carrier flow: 0.8 µl/min) and conventional homemade probes (1 µl/min) were perfused with aCSF. The dead time between the outlet of the probe and to the sample vials was 30 min, which was taken into account when analysing the data.

A 30 min sample was collected to clear dead space, and then a 30 min equilibration period sample was collected. Then two consecutive 30 min basal dialysate samples were collected. Amphetamine (0.5 or 1 mg/kg in a volume of 1 ml/kg saline, i.p.) was then given and

dialysate sampling continued for a further 150 min (see *figure 3.2* for experimental design and timeline of sample collection). The samples were collected into 3 µl of 1M H_3PO_4 on dry ice. Each sample was split in two equal volumes, to allow separate measurement of amphetamine and amines (see chapter 2). The vials used for collecting MQ probe samples were weighed before and after the experiments to monitor the dialysate volume obtained.

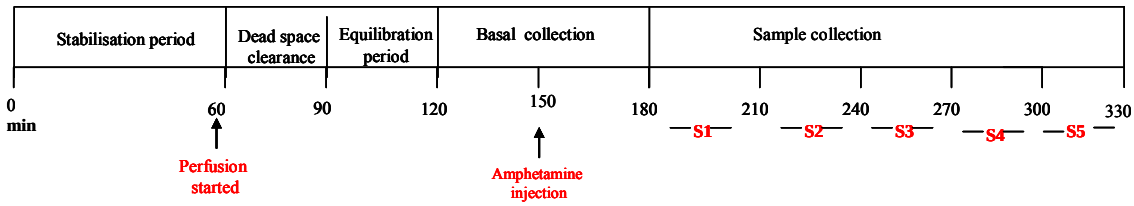

Figure 3.2: Experimental design for sample collection for experiment 1 and 2.

3.2.3 Femoral vein cannulation for blood sampling

In addition for experiment 2; the femoral vein was cannulated to allow repeated blood sampling: a sterile blood withdraw catheter (O.D 0.67 mm, L 19 mm; surflo i.v. catheter), with heparinised saline (20 units/ml; sterile), was inserted into the femoral vein and secured in place with a suture.

3.2.3.1 Blood sampling procedure

Blood samples (0.15 ml) were collected into heparinised vials before amphetamine injection and at 15, 30, 45, 60 and 90 min after injection. These samples were centrifuged at 3000 rpm for 10 min and the supernatant was collected for measurement of amphetamine by mass spectrometry.

At the end of the experiment the animals were killed by pentobarbital overdose, the brains were removed immediately and stored in 10% formal saline for 12-15 days. The brains were

frozen and the probe locations were verified in 20 µm cryostat cut transverse brain sections with reference to the atlas of Paxinos and Watson 1998 *(figure 3.3).*

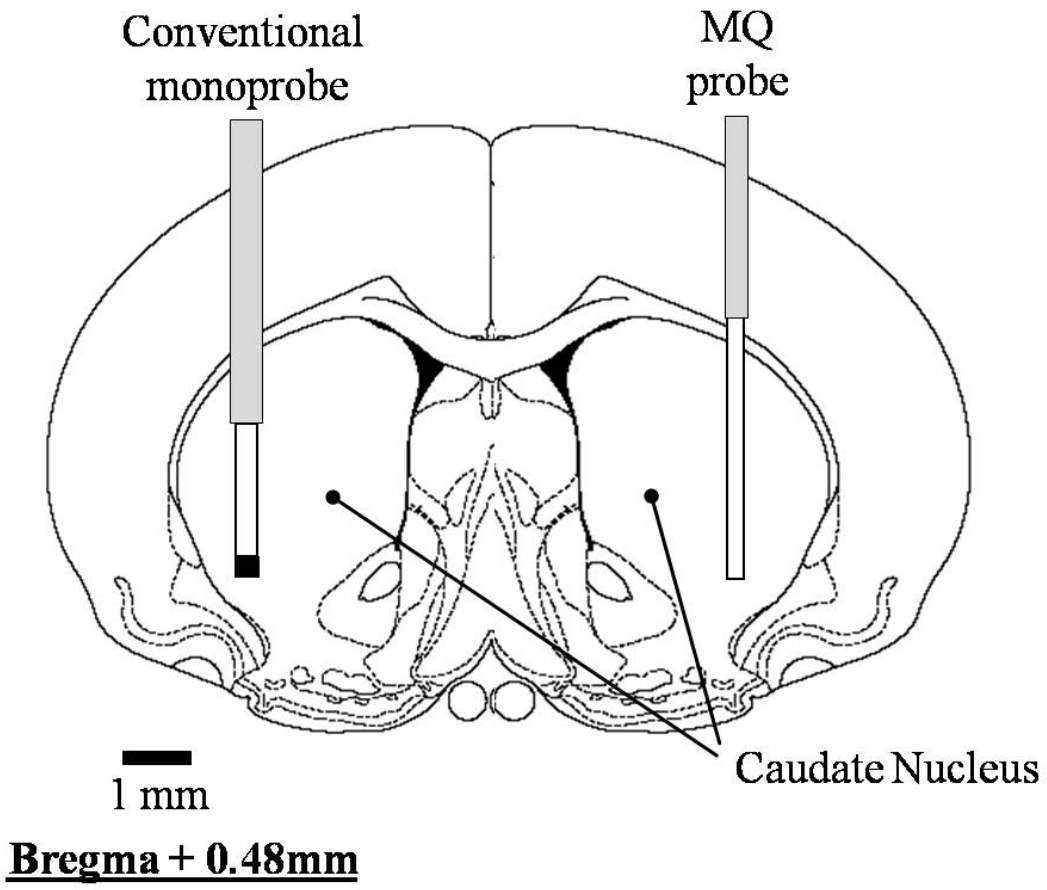

Figure 3.3: Drawing of coronal section of rat brain, 0.45mm anterior to bregma (Paxinos and Watson, 1998), showing positions and relative sizes of a MQ probe (right) and a conventional monoprobe (left). The open portion of each probe represents the dialysis tip: lightly shaded portion represents the shaft, and the darkly shaded portion (monoprobe only) represents the epoxy plug sealing the end of the membrane.

All procedures were carried out in accordance with University of Leicester ethical review procedures, with appropriate personal and project licence authority under the Animals (Scientific Procedures) Act, 1986, UK.

3.2.4 Analyses of dopamine, DOPAC and amphetamine

3.2.4.1 Dialysis sample analyses

The contents of dialysate for dopamine and DOPAC were analysed by HPLC and amphetamine was analysed by mass spectrometry as described in chapter 2**.**

3.2.4.2 Blood samples extraction for amphetamine analysis

The concentration of amphetamine in the blood was measured by LC/MS. For measuring blood concentrations, amphetamine was extracted from blood samples using the micro elution OASIS MCX (Waters) solid phase extraction in 96-well format. Blood samples (50 µl) were mixed with 400 µl of 2% formic acid after addition of 10 µl IS. The 96 well SPE plate was activated with 200 µl methanol followed by 200 µl water and the buffered samples were loaded onto the plate. Following sample application, each of the SPE 96 well containing samples were washed with 200 µl of 2% formic acid followed by 200 µl methanol. Amphetamine and IS were eluted into a 2 ml deep 96-well collection plate with 50 µl of 5% ammonium hydroxide in methanol. The extracts were evaporated to dryness under a nitrogen stream (37°C) and resuspended in 400 µl of MF5 buffer. 40 µl extracted blood samples were injected onto an API 4000 (Applied Biosystems/MDS SCIEX) mass spectrometer (further details in chapter 2).

Data were acquired and processed using the Analyst software, version 1.4.2 (Applied Biosystems/MDS SCIEX) and amphetamine concentrations were expressed as free base.

3.2.4.3 Statistical analyses

Dual probe NNF estimation: The mean change in concentration between the dialysates and perfusate (C_{out}-C_{in}) of each amphetamine concentration (y-axis) was plotted against the perfusate concentration alone (C_{in}) (x-axis) for each individual time point. Using linear regression analyses, the amphetamine concentration in the ecf (i.e., point of NNF) was determined (x-intercept; when y=0) and expressed as absolute ecf amphetamine levels.

Two-way ANOVA was used to evaluate the main effect of method or probe types and time in the absolute concentration of amphetamine and dopamine measured. The results showing significant overall changes were subjected to Dunnett's post hoc test in order to identify the samples which differed significantly from the basal levels. All statistical tests were carried out by using Graphpad Prism v5. Data are presented as mean ± SEM.

3.3 Results

3.3.1 Experiment 1: assessment of dual probe NNF and MQ probes in determining absolute ecf amphetamine concentrations

Following amphetamine administration, the absolute ecf concentrations of amphetamine showed significant increase in the samples S1, S2, S3 and S4 measured by both methods; compared to basal samples (B: pre drug samples) (*figure 3.4*). Two- way ANOVA revealed no main effect of method ($F[1, 98] = 0.0$, $p = 0.9$) indicating both dual probe NNF and MQ probes gave similar absolute ecf concentrations of amphetamine. There was a main effect of sample ($F[6, 98] = 33.2$, $p < 0.001$) indicating significant increases in the drug levels over the duration of the experiment. Moreover showed no interaction (method x drug; $F[6, 98] = 1.2$, $p = 0.7$) indicating similar time profile for distribution of amphetamine in the caudate

nucleus, by both methods across the duration of the experiment. Post hoc analysis using Dunnett's test showed significant increases the absolute ecf concentrations of amphetamine showed significant increase in the samples S1, S2, S3 and S4 measured by both methods; compared to basal samples (B: pre drug samples; *figure 3.4*). The maximum absolute ecf concentrations of amphetamine measured in dual probe NNF and MQ probes were 0.4 ±0.02 μM and 0.5 ±0.09 μM, which were not statistically different from each other ($t(12) = 0.5$, $p = 0.5$; paired t-test).

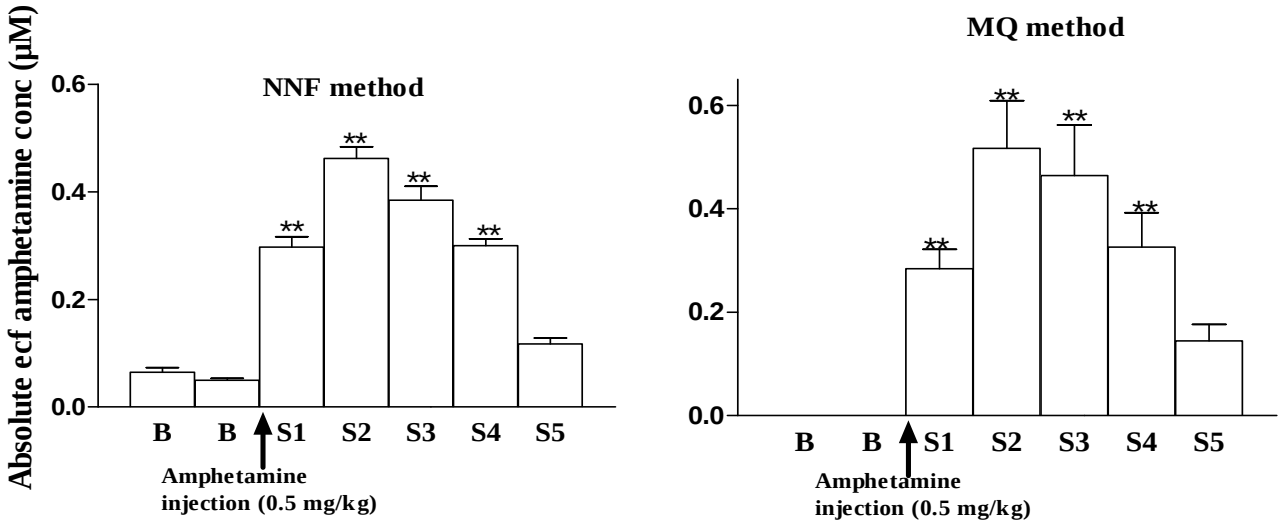

Figure 3.4: The absolute ecf concentrations of amphetamine obtained using dual probe NNF method and MQ probe in contralateral caudate nucleus in the same animals (n=8).

Consecutive 30 min dialysate samples were collected from contralateral sides of caudate nucleus (right side and the left side of caudate nucleus was implanted with MQ probe and dual probe NNF probes respectively) over the duration of the experiment. After 60 min basal collection (labelled as sample B in *figure 3.4*), amphetamine (0.5 mg/kg; i.p. injection) was administered as indicated by the arrows. The samples S1, S2, S3, S4 and S5 were collected for 0.5 mg/kg i.p. dose of amphetamine. * $p < 0.05$, ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

3.3.2 Experiment 1: assessment of dual probe NNF and MQ probes in determining absolute ecf dopamine concentrations

In the same experiment, the absolute ecf levels of dopamine were also measured from MQ probes and dual probe NNF method. The absolute basal levels of dopamine measured in the MQ probe was 8.6 ±0.4 μM (samples B). Following amphetamine injection there was significant increase in absolute ecf dopamine concentrations in S1, S2, S3 and S4 from basal samples *(figure 3.5).* The maximum dopamine level of 32.0 ±1.01 nM was attained in sample S2 *(figure 3.5).* ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

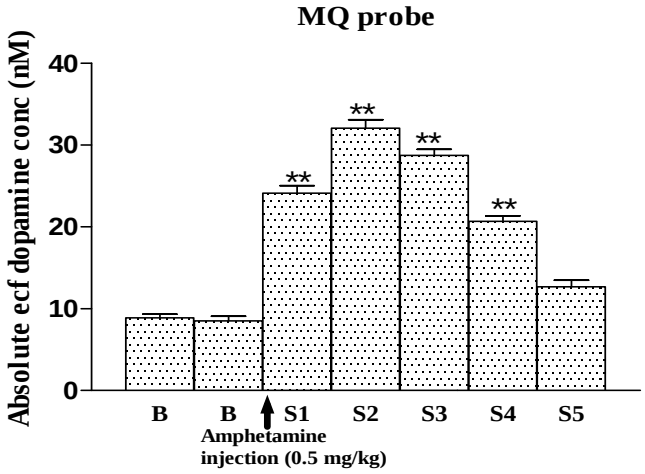

Figure 3.5: The absolute ecf concentrations of dopamine measured using MQ probes (n= 8).

Consecutive 30 min dialysate samples were collected from right side of the caudate nucleus over the duration of the experiment. After 60 min basal collection (samples B), amphetamine (0.5 mg/kg; i.p. injection) was administered as indicated by the arrow in the *figure 3.5.* The samples S1, S2, S3, S4 and S5 were collected for the ecf concentration of dopamine after 0.5 mg/kg i.p. dose of amphetamine. ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

The ecf dopamine concentrations estimated from dual probes NNF points were expressed as dialysate dopamine concentrations *(figure 3.6).* The basal dopamine levels in the dialysate (pre drug samples: B) measured in probe 1 and probe 2 was 12.8 ±1.1 nM and 34.9 ±3.5nM respectively from the dual probe NNF method. The elevated basal dopamine levels were due to amphetamine induced dopamine efflux caused by the local perfusion of amphetamine through probe 2, which was perfused with 10 μM of amphetamine. Following amphetamine injection, in probe 1 there was significant increase in dialysate dopamine levels in S1, S2, S3, S4 and S5 from basal samples (B) *(figure 3.6).* ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test). However in probe 2 the dialysate levels of dopamine did not significantly differ from the basal levels after the injection of amphetamine. The dopamine levels from probe 2 remained elevated before and after amphetamine injection due to the local perfusion of 10 μM of amphetamine through probe 2 *(figure 3.6).*

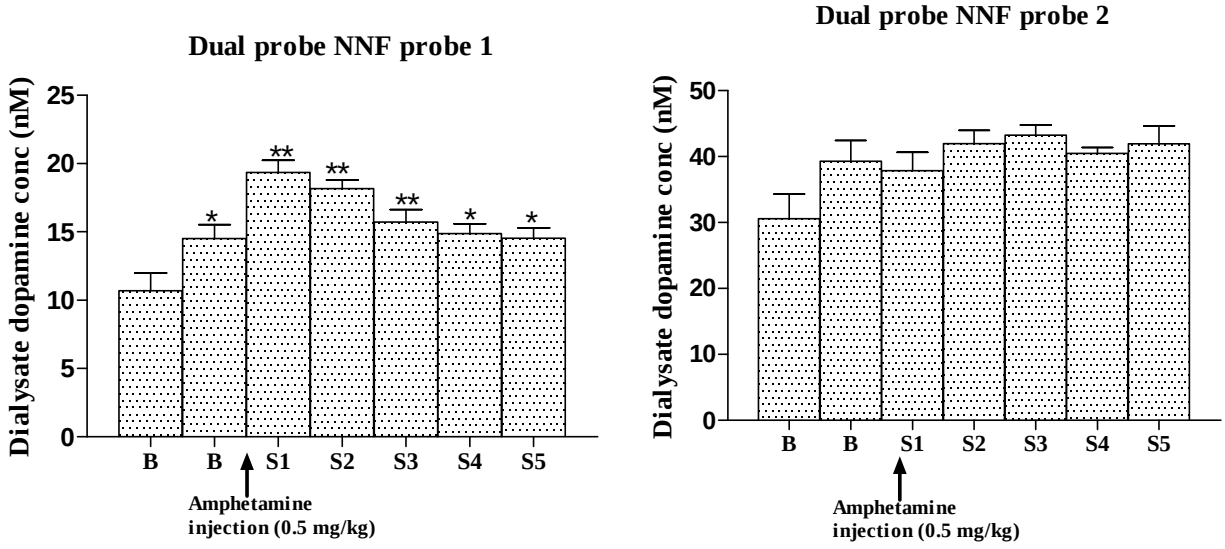

Figure 3.6: The dialysate concentrations of dopamine obtained from probe 1 and probe 2, using dual probe NNF method (n=8).

Consecutive 30 min dialysate samples were collected from the dual NNF probe set (probe 1 and probe 2) from left side of the caudate nucleus over the duration of the experiment. After 60 min basal collection (samples B), amphetamine (0.5 mg/kg; i.p. injection) was administered as indicated by the arrow in the figure 3.6. The samples S1, S2, S3, S4 and S5 were collected for the ecf concentration of dopamine after 0.5 mg/kg i.p. dose of amphetamine.

3.3.3 Experiment 2: comparison of conventional monoprobes and MQ probes in measuring ecf concentrations *in vitro*

In vitro probe recovery (%) for MQ probes (n=5) for dopamine was 96.9 ±2.3 and for amphetamine was around 95.0 ±5.0. *In vitro* recovery for conventional monoprobes (n=5) for dopamine was around 19.0 ±0.9 and for amphetamine was around 15.2 ±0.7.

3.3.4 Experiment 2: Comparison of conventional monoprobes and MQ probes in measuring ecf concentrations of amphetamine

Two-way ANOVA revealed a main effect of probe types indicating a significant difference between the amphetamine levels measured in the two probe types ($F[1, 48] = 32.2$, $p < 0.001$) with the MQ probes showing around 4-fold higher concentrations than conventional probes. Moreover, there was main effect of sample for both probes; ($F[5, 84] = 6.5$, $p < 0.001$) indicating significant increases in the drug levels over the duration of the experiment. However there was no significant interaction, probe type x drug ($F[5, 48] = 2.3$, $p=0.07$); indicating similar time profile for both probe types (*figure 3.7*). Post hoc analysis using Dunnett's test showed that following amphetamine injection (1.0 mg/kg; i.p.) the absolute ecf amphetamine concentrations obtained from MQ probes showed significant increases in S1, S2 and S3 compared to basal concentration (sample B). For conventional monoprobes the increase in dialysate amphetamine level was seen in S1 and S2 post injection compared to basal concentration (B). The maximum concentrations of amphetamine obtained were 743.0 ±156.8 nM and 226.0 ±73.4 nM (in sample S2) from MQ probe and conventional probe respectively *(figure 3.7)* which were statistically different from each other ($t (8) = 2.9$, $p < 0.001$; paired t-test).

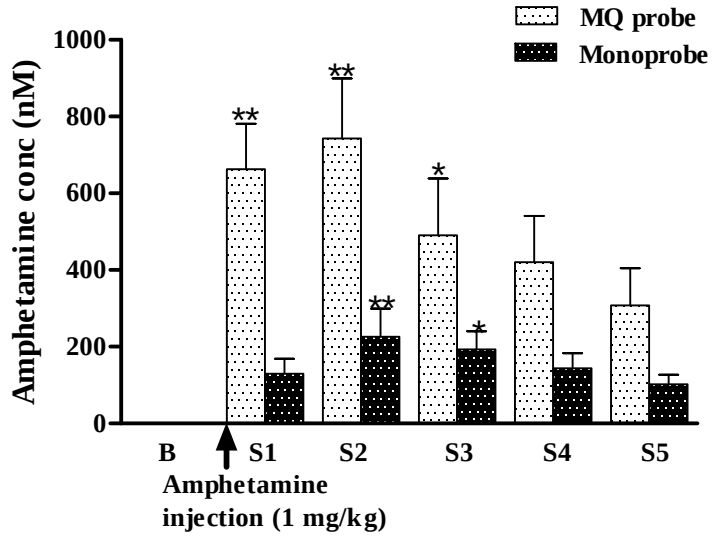

Figure 3.7: Comparison of ecf amphetamine concentrations obtained by MQ and conventional monoprobes in caudate nucleus (n=5).

Consecutive 30 min dialysate samples were collected from contralateral sides of caudate nucleus (right side and the left side of caudate nucleus was implanted with MQ probe and conventional monoprobe respectively) over the duration of the experiment. After 60 min basal collection (average of two 30 min basal samples labelled as B in *figure 3.7*), amphetamine (1 mg/kg; i.p. injection) was administered as indicated by the arrows. The samples S1, S2, S3, S4 and S5 were collected for 1 mg/kg i.p. dose of amphetamine. * $p< 0.05$, ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

3.3.5 Comparison of conventional homemade probes and MQ probes in measuring ecf concentrations of dopamine

The ecf concentrations of dopamine were also measured in the same animals. The basal levels of dopamine measured in the MQ probe and the conventional monoprobe were 9.4 ±0.4 nM and 6.3 ±0.3 nM, respectively, a difference which was statistically significant ($t(8) = 5.5$, $p < 0.001$; paired t-test). Two-way ANOVA revealed a main effect of probe type: ($F[1, 48] = 796.4$, $p <0.001$) reflecting the difference in overall levels of dopamine in the two probe types and also main effect of sample ($F[5, 48] =70.0$, $p<0.001$) indicating a change in

dopamine over the course of the experiment. But no interaction (F[5, 48] = 3.02, p=0.09) indicating that the increases in dopamine measured in the two different probe types (*figure 3.8*) showed a similar profile. Following amphetamine injection, the absolute ecf dopamine concentrations obtained from MQ probes and dialysate dopamine levels in conventional homemade probes showed significant increase in S1, S2, S3, S4 and S5 compared to basal level (B) for both probes. Post hoc analysis (Dunnett's test) showed significant changes in the samples S1, S2, S3, S4 and S5 after the injection of amphetamine (1.0 mg/kg, i.p.). The maximum dopamine concentration was observed in S2 which was 30.8 ±1.0 nM and 15.7 ±1.1 nM for MQ probe and conventional homemade probe respectively *(figure 3.8)* which were statistically different from each other (t(8) = 11.4, p < 0.001; paired t-test).

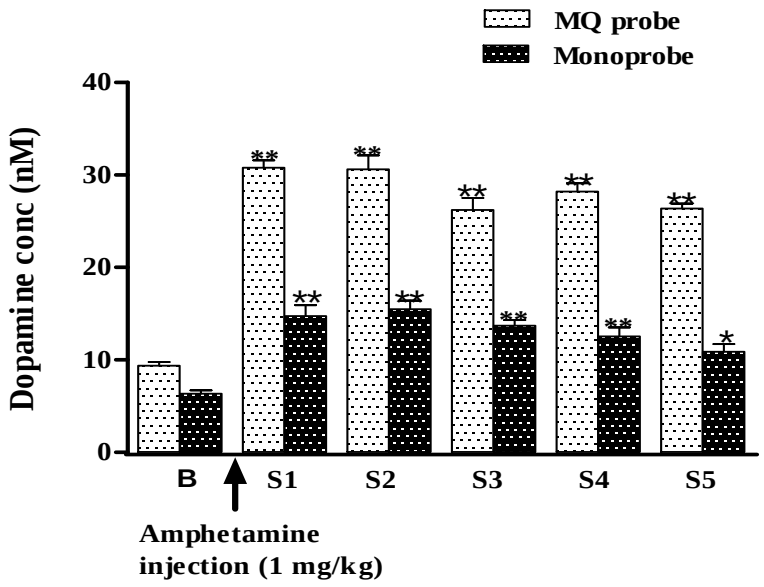

Figure 3.8: The caudate nucleus concentrations of dopamine using MQ probes and conventional monoprobes (n=5).

Consecutive 30 min dialysate samples were collected from contralateral sides (right side for MQ probe and left side for conventional probe) of the caudate nucleus over the duration of the experiment. After 60 min basal collection (samples B), amphetamine (1 mg/kg; i.p. injection) was administered as indicated by the arrow in the *figure 3.8*, The samples S1, S2, S3, S4 and S5 were collected for the ecf concentration of dopamine after 1 mg/kg i.p. dose of amphetamine. * p < 0.05, ** p < 0.01 indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

3.3.6 Comparison of conventional monoprobes and MQ probes in measuring ecf concentrations of DOPAC

The ecf concentrations of DOPAC were also measured in the same animals. The basal levels of DOPAC measured in the MQ probe and the conventional probe were 807.1 ±23.7 nM and 454.5 ±19.9 nM, respectively, a difference which was statistically significant ($t(8) = 2.5$, $p < 0.001$; paired t-test). Two-way ANOVA revealed a main effect of probe type ($F[1, 48] = 473.2$, $p < 0.001$) reflecting the difference in overall levels of DOPAC in the two probe types and also main effect of sample ($F[5, 48] = 70.0$, $p < 0.001$) indicating a change in DOPAC over the course of the experiment. But no interaction, probe type x sample ($F[5, 48] = 4.3$, $p = 0.07$) indicating that the increases in DOPAC measured in the two different probe types *(figure 3.9)* showed a similar profile. Amphetamine caused a decrease in DOPAC levels in caudate nucleus. Following amphetamine injection, the absolute ecf DOPAC concentrations obtained from MQ probes and dialysate DOPAC levels in conventional probes showed significant decrease in the samples S1, S2, S3, S4 and S5 compared to basal level (B) for both probes. Post hoc analysis (Dunnett's test) showed significant changes in the samples S1, S2, S3, S4 and S5 after the injection of amphetamine (1.0 mg/kg, i.p.; *figure 3.9)*.

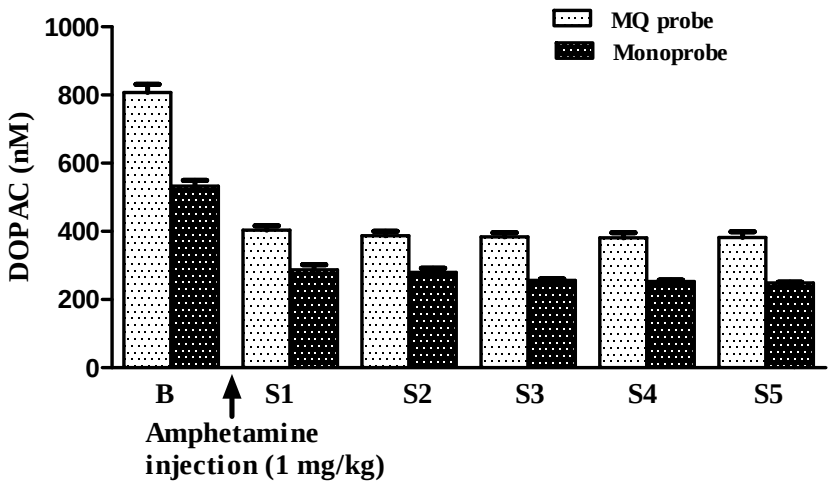

Figure 3.9: The caudate nucleus concentrations of DOPAC obtained for two probe types (n =5).

Consecutive 30 min dialysate samples were collected from contralateral sides (right side for MQ probe and left side for conventional probe) of the caudate nucleus over the duration of the experiment. After 60 min basal collection (B: average of two 30 min basal samples), amphetamine (1 mg/kg; i.p. injection) was administered as indicated by the arrow in the *figure 3.9*. The samples S1, S2, S3, S4 and S5 were collected for the ecf concentration of DOPAC after 1 mg/kg i.p. dose of amphetamine. ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

3.3.7 Comparison of ecf amphetamine concentrations obtained from conventional monoprobes and MQ probes to plasma amphetamine concentrations

The distribution of amphetamine in the caudate nucleus obtained from MQ and monoprobe was compared with amphetamine levels in the plasma followed by 1mg/kg i.p. dose of amphetamine *(figure 3.10)*. The maximum concentration of amphetamine obtained from plasma was 838.0 ±171.7 nM at 30 min post amphetamine injection (1 mg/kg, i.p.). The absolute ecf amphetamine concentration obtained from the MQ closely followed plasma levels following amphetamine injection. Two-way ANOVA revealed no main effect of method indicating no difference in the concentration of amphetamine obtained from plasma

and MQ probe (F[1, 48] = 0.2, p = 0.6) and no main effect of interaction, method x sample (F[5, 48] = 0.1, p = 0.9) indicating similar time profile of amphetamine in the plasma and MQ probe. There was significant main effect of sample (F[5, 48] = 9.8, p < 0.001) indicating that the changes in amphetamine measured over the course of the experiment *(figure 3.10).* Post hoc analysis (Dunnett's test) showed significant amphetamine levels in the plasma were significant at 15, 30 and 45 min post injection (* p<0.05; ** p<0.01).

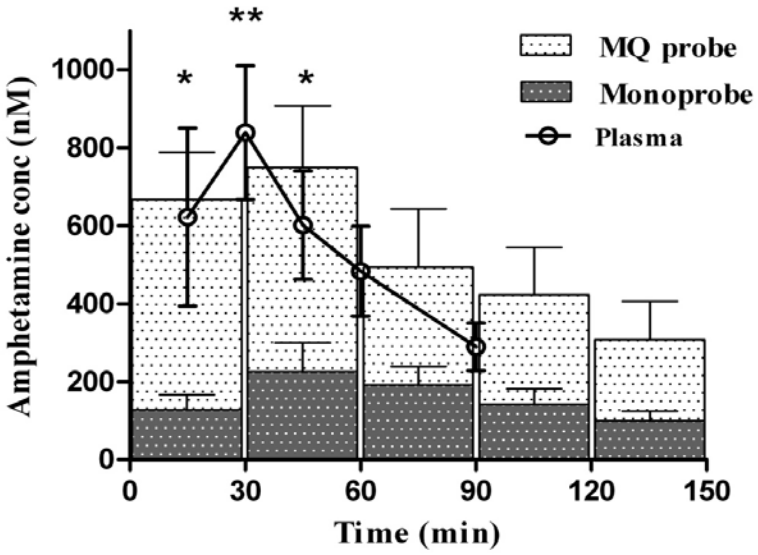

Figure 3.10: The distribution of amphetamine in the plasma and in the MQ and conventional monoprobe in caudate nucleus followed by 1mg/kg i.p. dose of amphetamine (n=5).

The plasma samples were collected at 15, 30, 45, 60 and 90 min post amphetamine injection (1 mg/kg, i.p.), in addition to the ecf concentrations of amphetamine obtained from MQ probe and conventional probe (samples S1, S2, S3, S4 and S5) post amphetamine injection (*figure 3.10*). The concentrations of amphetamine in the plasma were significant at 15, 30 and 45 min compared to pre drug levels. * p < 0.05, ** p < 0.01 indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

3.4 Discussion

The main aim of experiment 1 was to assess the effectiveness of two novel quantitative microdialysis methods for measuring absolute/true C_{ecf} in a dynamic *in vivo* system. Quantitative microdialysis data are extremely useful in many investigations to determine brain distribution of CNS active drugs. Accurate and continuous *in vivo* measurements of brain extracellular concentrations are a prerequisite for many neurobiological studies. This is because the free ecf concentration contributes to its neurochemical effects. The purpose of the current study was to improve the use of novel quantitative microdialysis methods, and thereby apply these methods in further neurochemical research. To achieve these goals, the first stages of this research project were focussed on developing and validating method for measuring absolute ecf concentration of compounds. To this end we modified the existing traditional NNF method. Traditional NNF method has been widely used to estimate free ecf concentration of compounds of interest. Although the NNF method has been widely recognized as the "golden standard" among quantitative microdialysis methods, it has several major disadvantages (see introduction), which makes its application to dynamic systems, problematic (Chaurasia *et al.,* 2007).

After a single i.p. injection of 0.5 mg/kg amphetamine, absolute caudate nucleus concentration of free unbound amphetamine was measured using dual probe NNF method and MQ probes in the same animal. Both the methods gave similar ecf concentrations of amphetamine, and moreover showed similar time profile of amphetamine distribution in the caudate nucleus (Sood *et al.,* 2009). Amphetamine readily entered caudate nucleus within 30 min of drug administration. The highest amphetamine concentration in caudate nucleus was achieved at 60 min post injection; measured by dual probe NNF method and MQ probes respectively. The time course of amphetamine changes in the caudate nucleus followed a

decline in the concentration and after 150 min post injection it was nearly cleared from caudate nucleus. The half life of amphetamine in the caudate nucleus was observed to be around 120 min. Both sets of data obtained from dual probe NNF method and MQ probes indicates that amphetamine is readily taken into caudate nucleus (30 min-120 min). Moreover, these data showed dose-related brain levels in a dynamic system. The close correspondence in the levels measured by the two different methods suggests that both methods provide the absolute measurement of CNS drugs. Moreover, both methods provide a within-animal means of measuring dynamic changes in brain concentrations of drugs.

The dual probe NNF method has many advantages over the traditional NNF and circumvents the major disadvantages of NNF. The first disadvantage of the NNF method is in its experimental design as for determination of ecf concentration of compound or a dose a large number of experimental groups (and hence often a large number of animals) are needed. This negates, at least in part, the inherent advantage of using microdialysis for minimizing the use of animals. The method uses the principle of quantifying the extracellular levels by addition of the compound being studied to the perfusate in different concentrations. This implies that, a minimum of two or more experimental groups are required for each concentration of compound added to the perfusate for each dose administrated. Data collecting over wide range of animal groups incorporates a huge inter group variability. The other main disadvantage of NNF is the requirement of a steady-state of the dose at the tissue level. This means that the dynamic changes that occur to a particular dose can not be determined. The dual probe NNF method circumvents these downfalls of traditional NNF method. Dual probe NNF method requires fewer animals, reduces inter-subject variability and moreover enables a within animal data collection for a dynamic condition (Sood *et al*., 2008).

In parallel with ongoing development of dual probe NNF method, Cremers *et al*., 2007 showed an alternative method for measuring absolute ecf concentrations using MQ probes. I incorporated the use of MQ to validate the use of MQ probes in measuring absolute ecf concentration of compounds. Hence, the second main outcome of this study was to validate the use of the newly developed MQ probes to measure absolute concentration of amphetamine in the caudate nucleus. The results showed that MQ probes gave similar ecf concentrations to dual probe NNF method. As with dual probe NNF, MQ probes can be used successfully to study absolute brain distribution of drugs of interest in a dynamic system. However, the dual probe NNF method has disadvantage in terms of its use to measure the ecf concentration of drugs and their effect on neurotransmitters, simultaneously. As the very nature of this method, includes adding the compound of interest to the ecf, through the probes. The dopamine concentration obtained from dual probe NNF method were much higher, this was due to the local perfusion of amphetamine that cause huge increase in dopamine release. The effect of amphetamine injection on dopamine increase can not be clearly predicted using dual probe NNF as the basal levels were elevated, compared to MQ dopamine measurements, to start with. This was due to perfusion of amphetamine from probe 2 that caused sustained increase in dopamine from pre dose levels (Sood *et al*., 2008). The aspect of the perfusing drug through the probe interferes with the changes in the neurotransmitters due effect of the drugs, to be studied. On the other hand, MQ probes gave absolute measurement of basal and amphetamine induced dopamine release. The increase in dopamine release in MQ probes were around 250% compared to basal levels. Hence, MQ probes can be successfully used within animal to study the brain concentration of drugs and its neurochemical effect simultaneously (Sood *et al*., 2009). MQ slow flow method is practically a relatively simple method. This study was designed to assess and validate a quantitative, highly practical and direct method for measurement of absolute ecf concentrations of CNS targeted drugs.

In experiment 2, I determined absolute brain concentrations of dopamine in the caudate nucleus under both non-stimulated conditions and after amphetamine administration using MQ dialysis and compared this to concentrations measured by conventional microdialysis in the contralateral caudate nucleus. This study for the first time compares the use of MQ microdialysis probes for measuring absolute brain concentrations of transmitter and drug, to conventional microdialysis probes within an animal. These experiments were designed to assess the difference in recoveries between a conventional dialysis probe and MQ probe that gives near 100% recovery *in vivo*. The measurements of both amphetamine and dopamine using the two probe types showed consistent time courses of responses. The dilution factor in MQ, as a result of the carrier flow mixing with the tip flow is a constant (constant flow rates), and so can be accounted for in calculating the concentration in the dialysis tip, given the near 100% recovery achieved at the tip, this provides a good estimate of the ecf concentration surrounding the tip. However, in conventional microdialysis, no such calculation can be made, since the recovery *in vivo* is unknown. Since it has been well documented that in vitro recovery does not reflect *in vivo* recovery (e.g. Bungay *et al*., 1990), it is not appropriate to calculate the ecf concentration using the *in vitro* recovery factor. Thus the concentration quoted for the conventional probes represents the dialysate concentration, without having corrected for recovery and the difference between this concentration and the ecf concentration derived from the MQ dialysis, allows us to calculate the *in vivo* recovery of the conventional probes: this was approximately 20% for amphetamine and around 50% for dopamine. These data therefore provide empirical evidence that the recovery *in vivo* is very different from that in vitro, measured as 12% for amphetamine and 15% for dopamine.

It has long been realised that recovery *in vivo* is very different from that *in vitro*, and that *in vitro* recovery cannot be used as a dilution factor to convert dialysis concentration to ecf concentration, principally due to the diffusion resistance of the tissue and the active processes

going on in the living tissue (Bungay *et al*., 1990; Morrison *et al*., 1991). Indeed, *in vivo* recovery has proved difficult to estimate, which has been a major barrier to using microdialysis in PK and PD studies, where the absolute ecf concentrations of drugs and neurotransmitters are required. Studies using NNF have estimated true extracellular concentrations of a number of compounds, including dopamine (for example, see Justice 1993), but these studies rely on the compound of interest being in steady state, and require the compound of interest to be infused through the probe over a time period of several hours, thus creating potential confounds. In addition, however to estimating the true extracellular concentrations, NNF also enables calculation of the *in vivo* recovery. This has been variously estimated at between 60 and 67% for dopamine, several-fold higher than the *in vitro* recoveries (Parsons and Justice 1992; Parsons and Justice 1994). The present data, using the MQ approach, support this view, and show that *in vivo* recovery is indeed appreciably higher than *in vitro* recovery.

Amphetamine induced a substantial increase in dopamine efflux, which attained its maximum response 30-60 min after drug injection; this neurochemical profile was seen in both the probes. Thus, although the levels measured by the two dialysis methods were significantly different, as expected the time-course of the response was the same. Indeed, if the concentrations are expressed as percentages of basal values rather than as dialysate concentrations, the changes evoked by dopamine look very similar in the two probes. Moreover, the concentrations of amphetamine obtained from conventional and MQ probes were related to plasma concentration of amphetamine. This study was essential in providing the necessary parameters for studying PK: distribution of amphetamine from the blood to the brain area *in vivo*. In addition to measuring dopamine levels, we also measured brain and plasma levels of amphetamine. As with dopamine measurements, the dialysate concentrations of amphetamine measured by the two dialysis methods was significantly different, but the time course was the same. The measurements of plasma concentrations also

showed a similar profile, with peak levels achieved 30 min after injection. Thus we were able to follow the time course of the drug's entry into the blood compartment, and show its rapid transfer from the blood to the brain similar to that previously described after intravenous amphetamine injection (Fuh *et al*., 2002). However, the current study extends this previous work by showing for the first time, the relationship between blood concentrations of amphetamine, the absolute brain concentration of the drug, and the neurochemical response in terms of dopamine increases, within the same animals over the time course of the drugs pharmacological effect.

I used MQ microdialysis to measure the time course of changes in dopamine levels following amphetamine administration and to measure the brain levels of amphetamine itself. It has not been possible in the past to measure dynamic changes of absolute amphetamine concentrations in a single animal. The NNF (Lonnroth *et al*., 1987, Justice 1993) was able to estimate absolute extracellular concentrations of compounds, but required multiple measurements to be made at different perfusion concentrations over a period of many hours and so required the solute of interest to be at steady state. A variation in this method, in which the different perfusion concentrations were applied across separate animals (rather than across time) was capable of measuring dynamic changes (Olson and Justice 1993). However, because it relied on between-animal comparisons the number of animals used was substantially increased, and data showed a great deal more variability. The advantage of MQ is that it provides a reliable measure of dynamic changes in absolute extracellular concentrations. This method, therefore provides a within subject means of measuring changes in drugs levels and transmitter responses in a dynamic system, which can be used for assessment of the PK/PD profile of novel psychoactive drugs.

Chapter 4: Measurement of neurochemical effect of systemic administration and local perfusion of PD168077 in freely moving rats: A quantitative microdialysis study

4.1 Introduction

Dopamine exerts its neuro-physiological effects by binding to its relevant dopamine receptors. The action of dopamine is mediated by two different classes of G-protein coupled dopaminergic receptors: D_1-like (D_1 and D_5) receptors, which on activation increases intracellular adenylate cyclase activity through coupling to stimulatory G-protein and D_2-like (D_2, D_3, D_4) which inhibit adenylate cyclase through coupling to inhibitory G protein ($G_{i\,/\,o}$) (Missale *et al*., 1998; Oak *et al*., 2000). The evidence of dopamine involvement in schizophrenia has been reported in tandem with the discovery of this neurotransmitter in the 1950s. Early studies by Carlsson speculated, nearly 50 years ago that an imbalance in dopamine release might underlie the cause of psychosis (Seeman and Lee 1975; Lieberman *et al*., 2005). Around the same time, the drug reserpine was discovered to block the accumulation of dopamine and other monoamines in secretory vesicles thus decreasing presynaptic terminals of dopamine levels, leading to the approval for antipsychotic use in the 1954. Converging studies show that dopaminimetics amphetamine can induce schizophrenic symptoms and can be psychotogenic. Amphetamine releases massive amounts of dopamine from presynaptic terminals, and the repeated amphetamine or methamphetamine use by individuals without schizophrenia can lead to a state similar to schizophrenic psychosis. It may also lead to a propensity for psychotic reaction to subsequent psychostimulant exposure even decades after the initial use. Thus, this led to approaches in elucidating mechanisms that control the level of presynaptic dopamine stores, and contributing to fundamental

understanding of the basic patho-physiologic mechanism in schizophrenia. The studies suggest that schizophrenic patients, including those never exposed to antipsychotic drugs, maintain high presynaptic dopamine accumulation in the striatum. Furthermore, a role for dopamine release in the hallucinations and other positive symptoms associated with schizophrenia has long been inferred from the antipsychotic response to D_2 dopamine receptor antagonist.

More recent evidence from studies measuring dopamine D_2 receptor occupancy in patients suggests that there are increased levels of dopamine in the striatum of schizophrenic brains Karoum and Egan 1992; Kapur and Remington 2001). The dopamine D_2 receptors are located in nearly every class of neurone in the striatum, including D_2 autoreceptors located on striatal dopaminergic axonal terminals, mediate negative feedback by inhibiting dopamine synthesis and release. Also they are located on the GABA mediated (GABAergic) medium spiny neurons and are highly expressed on neurons within the "indirect" striatopallidal pathway particularly on dendritic synapses formed by dopamine terminals. Moreover a presence of dopamine D_2 receptors on the axons of some of the glutamatergic afferents from the cortex is supported by anatomical and electrophysiological studies. Furthermore, dopamine D_1 and D_2 receptors are also found on GABAergic interneurones of the PFC and striatum (Oak *et al.,* 2000). The studies using imaging found that altered dopamine D_2 receptor binding in striatum could reflect changes in any or all of these striatal dopamine D_2 receptor populations. A meta-analysis comparing D_2 receptor parameters in schizophrenic patients and healthy controls revealed a small (12%) but significant elevation of striatal dopamine receptors in untreated patients with schizophrenia (Khan *et al.,* 1998).

Therefore, the role of dopamine D_2 receptors in schizophrenia is well documented. In this current study I was interested in testing the role of activation of dopamine D_4 receptor (one of

the group of D_2 like receptor) in synaptic levels of dopamine (this chapter) and its role in NOR (chapter 5 and 6). In general, the pharmacological profile of the dopamine D_4 receptor is very similar to that of the dopamine D_2 and D_3 receptors (Oak *et al.,* 2000). Dopamine D_4 receptor activation may trigger multiple intracellular pathways such as inhibition of cAMP synthesis (Seeman 1995; Seeman and Van Tol 1995) and modulation of G-protein regulated channels (Werner *et al*., 1996; Lavine *et al*., 2002). Numerous studies have analysed the functional and pharmacological properties of several human polymorphic dopamine D_4 receptor variants (e.g., Huff 1996). The ability of the various human dopamine D_4 receptor variants to bind dopamine, and to induce dopamine-mediated inhibition of adenylyl-cyclase activity or stimulation of $GTP_{\gamma S}$ binding, was evaluated *in vitro* in transfected cell lines (Asghari *et al*., 1995; Jovanovic *et al*., 1999; Czermak *et al*., 2006). The insight into the function of the dopamine D_4 receptor has been limited until very recently by the lack of selective antagonists and agonists. However, experiments with cultured cells expressing this receptor have provided insight into its signalling properties and the classification of the compounds acting at this receptor as an agonist or as an antagonist (as listed in table 4.1).

I was interested in determining *in vivo* effects of D_4 receptor activation in terms of its modulation of dopamine levels at the synaptic terminals. The importance of dopamine D_4 receptors in movement, mood and cognition is well established (Emilien *et al*., 1999 a and b; Browman *et al.,* 2005). The dopamine D_4 receptors mediate changes in neuronal excitability (Van Tol *et al*., 1991; Neve *et al*., 2004) and synaptic plasticity in the brain (Rubinstein *et al*., 2001; Calabresi *et al*., 2007). This receptor is predominantly expressed in PFC (Ariano *et al*., 1997; Mrzljak *et al*., 1996), where it is thought to play a major role in the control of integrative functions underlying the organization of complex behaviours (Goldman-Rakic 1996 and 1998; Rubinstein *et al*., 1997 and 1998). It is also localised predominately to the hippocampus and hypothalamus and with moderate density in caudate nucleus and NAc

(Ariano *et al*., 1997 a and b). The data from radioligand binding studies using a selective dopamine D_4 receptor agonist correlate the immuno-histochemical localization of the receptor (Moreland *et al*., 2004 a and b). The main focus of this study is in line with the search for D_4 receptor selective compounds based on the speculation that antagonism of this receptor may underlie the activity of atypical antipsychotics (such as clozapine). It has been proposed that the ability of clozapine to treat the positive, negative symptoms and cognitive deficits is associated with schizophrenia as well as having a lower propensity to induce extrapyramidal side effects may be partly related to the fact that it has a higher affinity for the dopamine D_4 receptor than for dopamine D_2 or D_3 receptors (Wilson *et al*., 1998). Moreover, in the recent years, the potential novel dopamine selective compounds (Bristow *et al.,* 1997 a and b; Gazi *et al.,* 1998; 1999 a and b; 2000), mainly developed as an antipsychotic target are dopamine D_4 receptor-selective ligands (as listed in the Table 4.1). In the table 4.1, PD168077 and CP226269 are characterised as agonists while all the other compounds are characterised as antagonists; classified as an antagonist or agonist based on *in vitro* functional assays. However, these studies as mentioned above only looked into the actions of these drugs *in vitro* and the action of drug or its mechanism was not assessed *in vivo*.

Code Name	Activity	References
PNU101958	antagonist	(Kula *et al*., 1999
PNU101958	ND	(Arlt *et al*., 1998)
S18126	antagonist	(Millan *et al*., 1998)
NGD94-1	antagonist	(Tallman *et al*., 1997)
PD108635	ND	(Belliotti *et al.*, 1998)
L745870	antagonist	(Patel *et al*., 1997)
SCH66712	antagonist	(Kim *et al*., 1999)
SCH66712	antagonist	(Kesten *et al*., 1999)
CP293019	antagonist	(Schlachter *et al*., 1997)
CP293019	ND	(Perrone *et al*., 1999)
PD168077	agonist	(Glase *et al*., 1997)
CP226269	agonist	(Faraci *et al*., 1998)

Table 4.1: D4 receptor selective compounds that have been developed to date (as potential antipsychotics), classified as an antagonist or agonist based on in vitro functional assays using cloned receptors which measured coupling to adenylyl cyclase and/or mitogesis as measured by [^{3}H]thymidine incorporation and/or dopamine mediated GTPγ^{35}S binding (ND means not determined).

My interest was to elucidate the neurochemical effects of PD168077 in freely moving rats. PD168077 is a potent D_4 dopamine receptor agonist with greater than 400-fold selectivity over D_2 and greater than 300-fold selectivity against D_3 subtypes respectively (Glase *et al*., 1997). Previous microdialysis studies by Melis *et al* in 2005, determined the effect of PD168077 found that injection of PD168077 into the paraventricular nucleus of the hypothalamus caused penile erection episodes, which occurred concomitantly with an increase in ecf dopamine and DOPAC concentration in the shell of the NAc. In this case the increase in dopamine in NAc shell was a downstream effect, as drug was injected into the paraventricular nucleus and therefore was not present in the NAc. For my studies the MQ probes were implanted in the mPFC (a high density dopamine D_4 receptor area) and in the NAc (a low density dopamine D_4 receptor area). The PFC dopamine function has been well studied in terms of cognitive dysfunction in schizophrenia; the pathway involved is the dopamine containing neurons originating in the VTA (Lindvall *et al*., 1974) that project to the PFC. Moreover, the dopamine system in the PFC is interlinked with other neurotransmitter systems such as glutamatergic and GABAergic system. The PFC contains pyramidal neurons of glutamatergic and also GABAergic interneurones, which are connected reciprocally and also, have self-innervations. All populations of PFC neurones are under dopaminergic modulation. The transient dopaminergic input to the pyramidal neurons triggers the dynamics of the circuit. In one view, the excitatory activity of cortical pyramidal neurons is thought to be reduced in schizophrenia due to NMDA receptor hypofunction (as discussed in chapter 5 and 6), and/or decreased dopamine neurotransmission through D_1 receptors. Similar alterations may contribute to GABAergic dysregulation in the PFC interneurones, a segment of pyramidal neurones thus leading to increased dopamine activity in the striatum and decreased dopamine activity in the cortex, with compensatory but functionally insufficient upregulation of dopamine D_1 receptors. This current thesis focuses on the role of dopamine D_4 receptor in the PFC circuit. As shown in *figure 4.1*, the dopamine D_4 receptors co exist

with D_1 receptors, and NMDA in the PFC pyramidal neurones modulating dopamine-glutamate system in this circuit, postulated by various studies as discussed below.

First, several studies have highlighted the need of dopamine/glutamate co-activation for a number of PFC functions (Gurden *et al*., 1999; Baldwin *et al*., 2002; Jay 2003). The interactions between dopamine and glutamate receptors may be essential for a proper PFC function, and a thorough understanding of the cellular underpinnings of those interactions needs to be gathered. The dopamine mediated alteration of glutamate function has different effects depending on the receptors involved (Cepeda *et al*., 1993; Nicola *et al*., 2000) and the neurone type in which this interaction occurs (Nicola *et al*., 2000). The dopamine D_4 receptors present on the pyramidal neurones in the PFC co exists with NMDA receptors. Furthermore, a mechanistic link has been established between D_4 receptor and NMDA receptors in the prefrontal pyramidal neurones (Wang *et al*., 2002 and 2003) based on the observation that blocking D_4 receptor activation modulated NMDA receptor function in cultured cells.

Second, other dopamine receptors such as D_1 and D_2 also co-localises with D_4 receptors and mediate the cellular mechanisms involved in the modulation of responses caused by D_1 and D_2 receptors in PFC pyramidal neurones. Third, D_4 receptors are also present on GABA producing interneurones in the PFC. This has modulatory effects on GABAergic signalling and transient inputs can alter NMDA receptor function and/or dopamine neurotransmission through GABAergic system. Thus it can be seen that modulating D_4 receptor function can result in enhancement or inhibition of the PFC circuit (dopamine-glutamate-GABA systems; *figure 4.1)*.

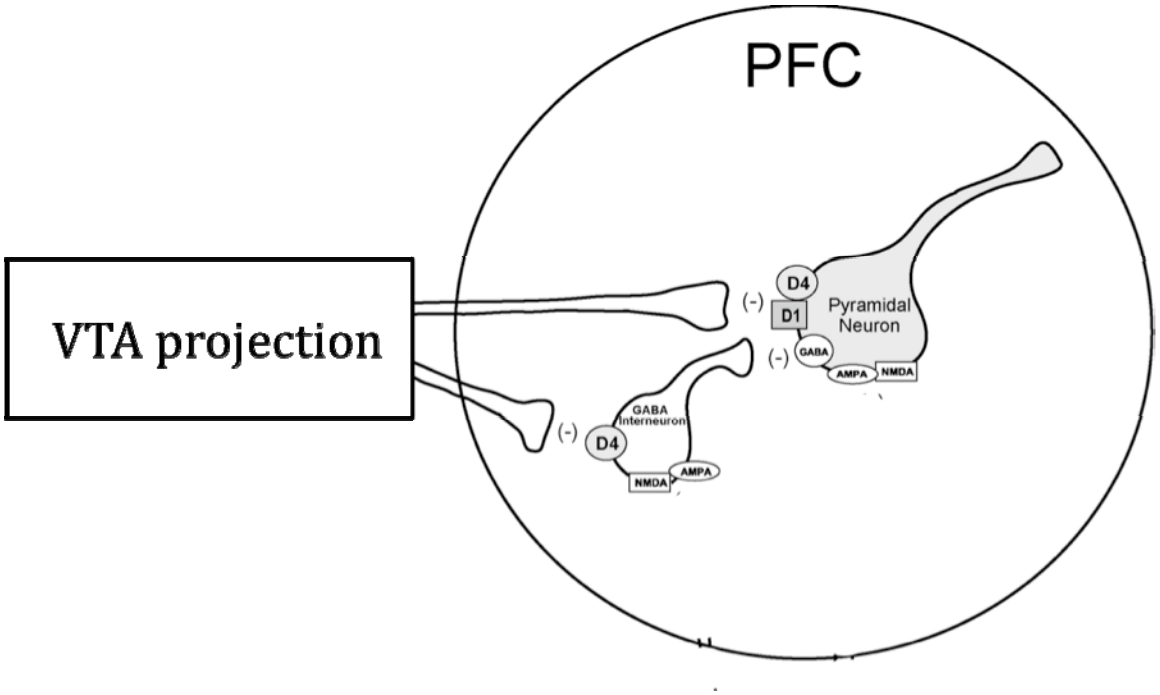

Figure 4.1: A dopamine D_4 receptor mediated model of dopamine-glutamate-GABA systems in the PFC (adapted from Papoutsi et al., 2009).

The figure demonstrates some of the known functional connections between the interneurones, and the pyramidal neurone in the PFC. The VTA provides dopamine input to the PFC via the mesocorticolimbic pathway. These dopamine inputs provide emotional and/or motivational salience information to target neurons within the PFC. However it is important to note that PFC neuronal populations send functionally important descending projections to the VTA which may in turn regulate recurrent inputs from these regions back up to the PFC. The D_4 receptors are found on both interneurones and pyramidal neurone populations. There is a functional localization and interaction of the D_4 receptor on intrinsic, inhibitory interneurones and pyramidal neurones within the PFC. Thus, activation of dopamine D_4 receptors in the PFC circuit, can influence the integrative networks within pyramidal neurones.

The experiments described in this chapter targets the D_4 receptors and its connections to NMDA receptors, and to do so a dopamine D_4 receptors agonist PD168077 was employed. The experiments were designed to quantify the distribution of PD168077 in two brain areas: mPFC and NAc. Moreover, dopamine dynamics in these two areas were measured using MQ probes that provide near 100% recovery of compounds *in vivo*. MQ probes recovery becomes crucial here due to the fact that the dopamine levels in the mPFC area are very low, therefore

accurate absolute (true) measurement of the dopamine dynamics in this area can only be achieved with a technique that yields high recovery. Two sets of experiments were carried out.

4.1.1 Aims

Experiment 1: Systemic administration of 0.1 and 0.5 mg/kg doses of PD168077. Nayak and Cassaday in 2003 used PD168077 doses over the range 0.064-1.0 mg/kg, to identify doses of drug with contrasting behavioural effects. The dose that increased locomotor activity was 0.064 mg/kg and dose of 0.5 mg/kg that did not significantly increase in activity. These doses were then tested using aversive and appetitive procedures. Both doses of PD168077 were without effect on conditioning, whether appetitive or aversive. For this current neurochemical study, I chose 0.1 mg/kg dose that induced locomotor activity and 0.5 mg/kg dose that did not affect the locomotor activity.

Experiment 2: Intracerebral perfusion of 200 nM and 400 nM concentrations of PD168077 in the mPFC and NAc. These experiments were carried out to study the regional effect of D_4 receptor activation on dopamine dynamics.

4.2 Materials and methods

4.2.1 Animals, housing and surgery

Male Sprague Dawley rats (275 ±30 g; Harlan) were housed in groups of two in a temperature and humidity controlled environment and under regular lighting conditions (12 hour light/dark cycle) with food and water available *ad libitum*. Surgery was performed 7-10 days after arrival. Rats were anaesthetised with 2% isoflurane in O_2: 1l/min in an induction chamber for 10-15 min. The rats were prepared for surgery by shaving the hair on the head

(around the area of interest) with a hand held shaver. The rats were then mounted in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, USA), with the incisor bar set at 3.3 mm below the intraural line as described by Paxinos and Watson (1998). Anaesthesia was maintained during surgery under 1-2% isoflurane (in O_2: 1-2l/min) to the rats via a face mask (Stoelting Instruments). Once the pedal reflex was lost, the scalp was cut and retracted to expose the surface of the skull. A 1 mm diameter hole was drilled in the skull above the right mPFC (H: +3.2, Tr: -0.5) and above the left NAc (H: +1.20, Tr: +1.4) relative to bregma, according to Paxinos and Watson 1998, leaving the dura mater intact.

A further two holes were drilled in the left and right parietal bones to mount the stainless steel anchor screws. The skull was cleaned and dried. The cannulae (with stylettes) were placed in the probe holder (Biotech Instruments Ltd) attached to the stereotaxic frame. Appropriate co-ordinates were relocated from the bregma and after the dura mater membrane was penetrated with a needle, the guide cannulae were lowered vertically over c. 2 minutes into mPFC (V: -1.4) in the right side of the brain and at NAc (V: -3.5) in the left side of the brain (relative to the skull). The cannulae were secured to the skull with the two anchor screws and dental acrylic cement. Particular care was taken to ensure that the cement mound and the seal to the wound were sound. Animals were then administered a post operative analgesic (Vetergesic; 0.03 mg/kg s.c), 5 ml of 0.9% saline with 1% glucose following the surgery and allowed to recover for 7-8 days prior to microdialysis. After surgery, animals were housed in single cages with food and water available *ad libitum* during recovery.

4.2.2 Microdialysis procedure in freely moving rats

Microdialysis was performed in freely moving animals 7-8 days after cannulae implantation. The stylettes were removed and MQ probes were inserted into the cannulae under brief isoflurane (typically 1% at 1 l/min oxygen) anaesthesia. MQ probe tips were at mPFC (V: -

5.4) in the right side of the brain and at NAc (V: -7.5) in the left side of the brain. Once the animals regained consciousness, they were placed in a skinner box. The animals were connected to a perfusion system (as described in chapter 3). After 60 min of initial stabilisation period, perfusion was started and the probes were perfused with aCSF for experiment 1 and experiment 2 *(see figure 4.2 and 4.3)*. The MQ probes were run at a flow rate of 0.95 µl/min (tip flow, 0.15 µl/min; carrier flow, 0.8 µl/min) delivered using a microinfusion pump. The outlet of the probe was connected to the sample vials by PEEK tubing of a length such that the dead space was 25 min; which was taken into account when analysing the data.

4.2.2.1 Microdialysis sample collection

For experiment 1 a 30 min sample was collected to clear the dead space, and then a 30 min equilibration period sample was collected. After equilibration, two consecutive 30 min basal samples were collected, then a vehicle (saline) 0.1 ml/kg., s.c. injection was given and consecutive 30 min samples were collected for 60 min. After this injections of PD168077 (0.1 mg/kg, and 0.5 mg/kg, s.c. in the volume of 1 ml/kg saline) were given at 120 min intervals, and consecutive 30 min samples were collected for 240 min (see *figure 4.2* for experimental design and timeline of sample collection).

For experiment 2, after clearing the dead space and equilibration (as above), three consecutive 30 min basal samples were collected. Then the perfusion medium was changed to one containing 200 nM of PD168077 and consecutive 30 min samples were collected for 120 min, and then the perfusion medium was changed again to one containing 400 nM of PD168077 and samples were collected for further 120 min (see *figure 4.3* for experimental design and timeline of sample collection).

Samples were collected into 3 µl of 1M H_3PO_4 on dry ice. Each sample was split in two, to allow separate measurement of PD168077, and dopamine and DOPAC. The vials used for MQ probe samples were weighed before and after the experiments to monitor the dialysate volume obtained.

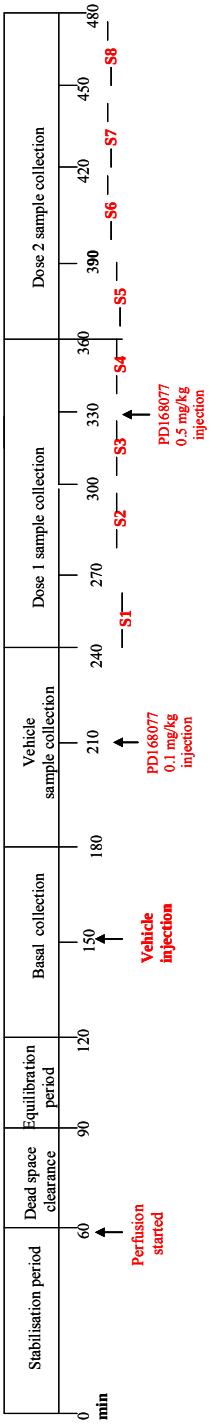

Figure 4.2: Experimental timeline for experiment 1.

Figure 4.3: Experimental timeline for experiment 2.

At the end of the experiment the animals were killed by pentobarbital overdose, the brains were removed immediately and stored in 10% formal saline. Then the probe locations were verified in 100 µm cut of transverse brain sections *(figure 4.4)*.

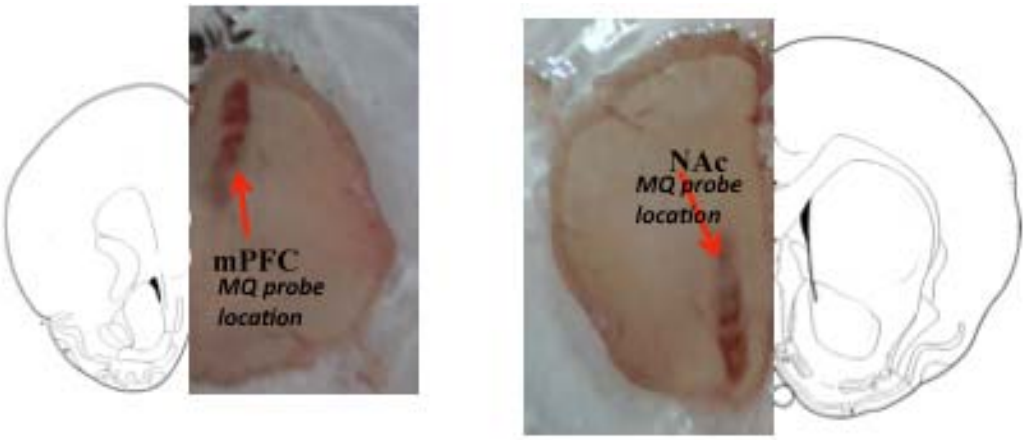

Figure 4.4: MQ probe tip location in mPFC and NAc; *with reference to the atlas of Paxinos and Watson 1998.*

All procedures were carried out in accordance with University of Leicester ethical review procedures, with appropriate personal and project licence authority under the Animals (Scientific Procedures) Act, 1986, U.K.

4.2.3 Statistical analyses

Two-way ANOVA was used to evaluate differences between concentrations of PD168077 and dopamine measured in the two brain regions. The results showing significant overall changes were subjected to Dunnett's post hoc test in order to identify the samples which differed significantly from the basal levels. All statistical tests were carried out by using Graphpad Prism v5. Data are presented as mean ± SEM.

4.3 Results

4.3.1 Experiment 1: Systemic administration of 0.1 mg/kg and 0.5 mg/kg doses of PD168077

Sub cutaneous administration of PD168077 (0.1 and 0.5 mg/kg) caused an increase in the ecf concentration of the drug in both mPFC and NAc, which reached a maximum in the sample immediately following the drug injection in each case (in the samples labelled as S1 and S5 in the *figure 4.5*). Although two-way ANOVA revealed a significant main effect of sample ($F[9,126] = 16.1$, $p < 0.001$), indicating significant increases in the drug levels over the duration of the experiment, there was no significant main effect of brain region ($F[1, 126] = 0.6$, $p = 0.4$) nor any brain region x sample interaction ($F[9,126] = 0.3$, $p = 0.9$) indicating that the concentrations of the drug was similar in both brain regions across the duration of the experiment. Post hoc analysis using Dunnett's test showed significant increases in drug concentrations only in mPFC in the samples (S1) immediately following injection of the lower dose of PD168077 (0.1 mg/kg) and a significant increase in both regions in the two samples (S5 and S6) following the higher dose of PD168077 (0.5 mg/kg, *figure 4.5*).

The maximum absolute ecf concentrations of PD168077 measured in mPFC and NAc were 19.1 ±3.2 nM and 13.9 ±2.7 nM respectively at lower dose of 0.1 mg/kg *(table 4.3.1),* which were not statistically different from each other ($t(7) = 1.0$, $p = 0.3$; paired t-test). Following a higher dose of 0.5 mg/kg PD168077, the maximum ecf concentrations obtained were 27.6 ±9.1 nM and 29.5 ±7.5 nM in the mPFC and NAc respectively *(table 4.3.2),* which were also not statistically different from each other ($t(7) = 0.1$, $p = 0.8$; paired t-test). The 5-fold higher dose of PD168077 evoked only approximately 2-fold increases in ecf concentration of the drug in the S5 compared to S1 in the both brain regions. Statistical analysis using paired t-test comparing S1 and S5 also revealed no significant difference in the increase in the ecf

concentration of PD168077 in the mPFC (t(7) = 0.8, p = 0.4; paired t-test) and in the NAc (t(7) = 2.0, p = 0.08; paired t-test).

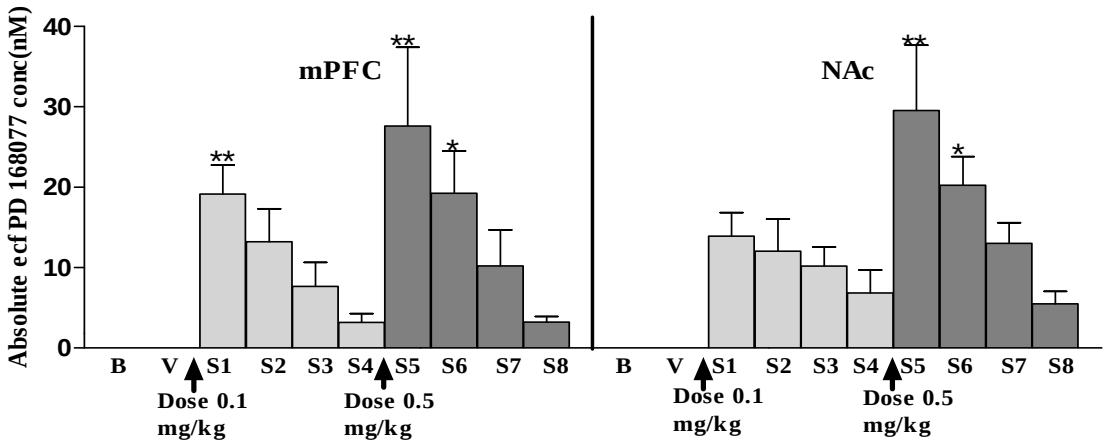

Figure 4.5: The absolute ecf concentration (nM) of PD168077 in the mPFC and NAc after s.c doses of 0.1 and 0.5 mg/kg PD168077 (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), a vehicle injection (1 mg/kg; saline s.c.) was given and sample collection proceeded for a further 60 min (V: mean of two samples). PD168077 (0.1 and 0.5 mg/kg; s.c. injection) was then administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes). The samples S1, S2, S3 and S4 were collected for 0.1 mg/kg and S5, S6, S7 and S8 were collected for 0.5 mg/kg s.c. dose of PD168077. For clarity samples following different injections are shaded differently. * $p< 0.05$, ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test).

In the same experiment, the absolute ecf levels of dopamine were also measured. The absolute basal levels of dopamine measured in the mPFC and NAc were 5.8 ±0.2 nM and 21.1 ±0.6 nM, respectively, a difference which was statistically significant ($t(7) = 19.4$, $p < 0.001$; paired t-test). PD168077 evoked an increase in ecf levels of dopamine in both brain regions. Two-way ANOVA revealed a significant main effect of sample ($F[9, 126] = 2.2$, $p <0.001$; *figure 4.6*) indicating a change in dopamine over the course of the experiment and a significant main effect of brain region ($F[1, 126] = 831.8$, $p <0.001$), reflecting the difference

in overall levels of dopamine in the two brain regions. Also there was a significant sample x brain region interaction indicating that the response profiles in the two brain regions were different (F[9,126] = 45.3, p = 0.03; *figure 4.6).* Post hoc analysis (Dunnett's test) showed no significant changes in individual samples after the lower dose in either brain region, but a significant elevation of dopamine (to 9.5 ±0.4 nM) in the mPFC in sample S6 after the higher dose *(figure 4.6)*.

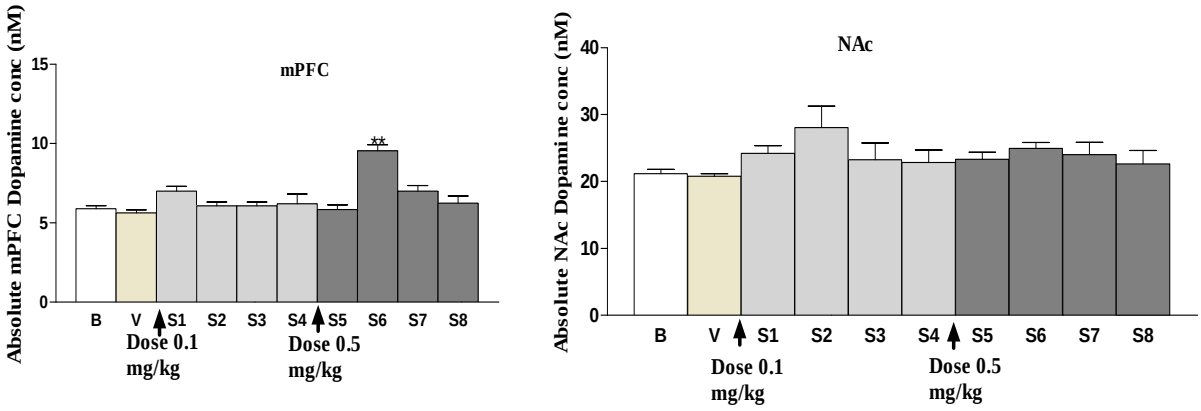

Figure 4.6: The absolute ecf concentrations (nM) of dopamine in mPFC and NAc (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), a vehicle injection (1 mg/kg; saline s.c.) was given and sample collection proceeded for a further 60 min (V: mean of two samples). PD168077 (0.1 and 0.5 mg/kg; s.c. injection) was then administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes). The samples S1, S2, S3 and S4 were collected for 0.1 mg/kg and S5, S6, S7 and S8 were collected for 0.5 mg/kg s.c. dose of PD168077. For clarity samples following different injections are shaded differently. * $p< 0.05$, ** $p < 0.01$ indicate samples which are significantly different from the basal levels (Dunnett's post hoc test). Note that the y- axis scales are different in the two graphs, reflecting the difference in the basal levels of dopamine in the two brain regions.

In the same experiment, the absolute ecf levels of DOPAC were also measured. DOPAC levels showed a similar pattern to the dopamine levels in response to the PD168077 challenge. The absolute basal levels of DOPAC measured in the mPFC and NAc were 162.8 ±8.6 nM and 911.6 ±26.3 nM, respectively, a difference which was statistically significant ($t(7) = 31$, $p < 0.001$; paired t-test). Two-way ANOVA revealed a significant main effect of

sample (F[9, 126] = 3.9, p <0.001; *figure 4.7*) indicating a change in DOPAC over the course of the experiment and a significant main effect of brain region (F[1,126] = 9520.6, p <0.001), reflecting the difference in overall levels of DOPAC in the two brain regions. Also there was a significant sample x brain region interaction indicating that the response profiles in the two brain regions were different (F[9,126] = 89.0, p = 0.04; *figure 4.7)*. Post hoc analysis (Dunnett's test) showed no significant changes in individual samples after the lower dose in either brain region, but a significant elevation of DOPAC (to 193.6 ±7.9 nM) in the mPFC in sample S5 after the higher dose *(figure 4.7)*.

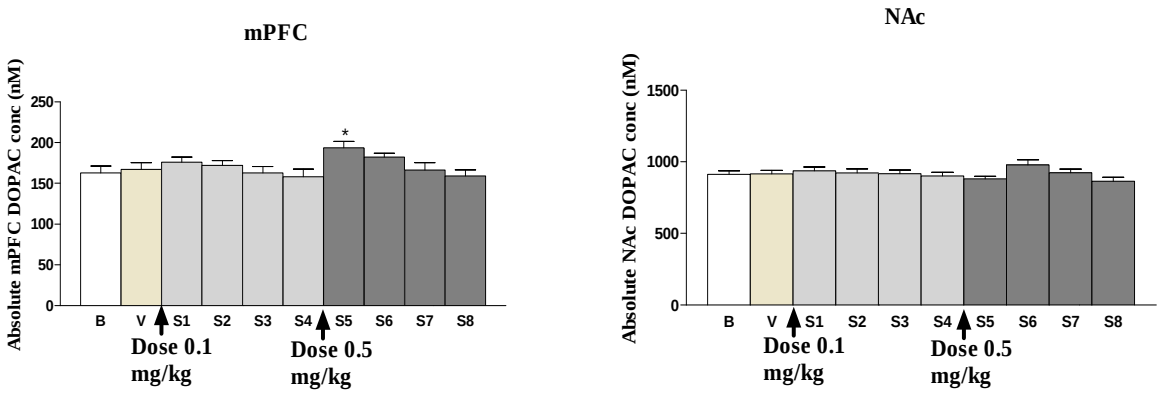

Figure 4.7: The absolute ecf DOPAC concentrations (nM) in mPFC and NAc (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), a vehicle injection (1 mg/kg; saline s.c.) was given and sample collection proceeded for a further 60 min (V: mean of two samples). PD168077 (0.1 and 0.5 mg/kg; s.c. injection) was then administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes). The samples S1, S2, S3 and S4 were collected for 0.1 mg/kg and S5, S6, S7 and S8 were collected for 0.5 mg/kg s.c. dose of PD168077. For clarity samples following different injections are shaded differently. * p< 0.05 indicate samples which are significantly different from the basal levels (Dunnett's post hoc test). Note that the y- axis scales are different in the two graphs, reflecting the difference in the basal levels of DOPAC in the two brain regions.

4.3.2 Experiment 2: Brain perfusion of PD168077

In experiment 2, the absolute basal ecf levels of dopamine were measured before the intracranial perfusion of PD168077. The absolute basal levels of dopamine measured in the mPFC and NAc were 5.8 ±0.4 nM and 20.3 ±0.6 nM, respectively, which were statistically different from each other ($t(14) = 18.4$, $p < 0.001$; paired t-test). Perfusion with dialysis perfusate containing 200 nM and 400 nM of PD168077 (120 min each concentration) caused an increase in ecf dopamine levels in both brain regions, which was shown by two-way ANOVA to be statistically significant (main effect of sample; ($F[8,108] = 41.9$, $p <0.001$; *figure 4.8*). There was also a significant main effect of brain region ($F[1, 108] = 879.9$, $p <0.001$), reflecting the different overall levels seen in the two brain regions, and a significant sample x brain region interaction ($F[8, 108] = 675.0$, $p < 0.05$; *figure 4.8*). Post hoc analysis with Dunnett's test showed significant increases in dopamine concentrations after perfusion of 200 nM (from samples S1 to S4) and 400 nM (from samples S5 to S8) concentrations of PD168077 in both brain regions *(figure 4.8)*.

The maximum concentrations reached in the two brain regions during perfusion with 200 nM of PD168077 were 14.4 ±1.0 nM (mPFC) and 33.7 ±0.7 nM (NAc) *(Table 4.3.1 and 4.3.2)*, representing 244.9% (mPFC) and 166.2% (NAc) of basal respectively, whereas during perfusion with 400 nM of the drug, the maximum levels were 22.6 ±1.8 nM (mPFC) and 51.2 ±1.9 nM (NAc) or 383.8% (mPFC) and 251.8% (NAc) of basal respectively *(Table 4.3.1 and 4.3.2)*. Further statistical analysis (paired t-test) showed that the maximum levels after 400 nM of PD168077 were significantly greater than after 200 nM of the drug in both brain regions (mPFC, $t(14) = 3.1$, $p = 0.03$ and NAc, $t(14) = 6.4$, $p < 0.001$), although the 2-fold increase in drug concentration did not produce a 2-fold increase in dopamine release.

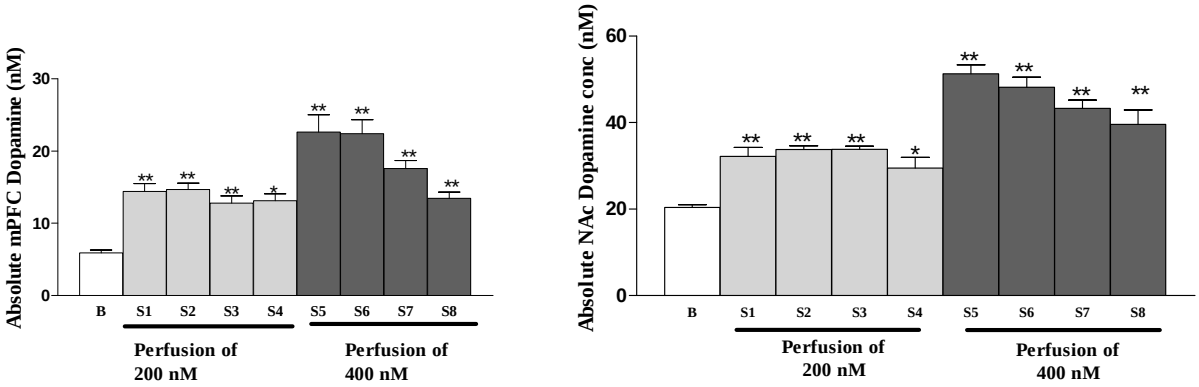

Figure 4.8: The absolute ecf concentration of dopamine following intracerebral perfusion of 200 nM or 400 nM PD168077 via the dialysis probe (n=9).

*Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), the dialysis perfusion was switched to aCSF containing 200 nM PD168077 for 120 min (pale bar), and then to aCSF containing 400 nM PD168077 (dark bar: the timings shown take into account the dead time of the outlet tubings). For clarity, samples during the different infusions are shaded differently. * p<0.05; ** p<0.01: indicate samples which are significantly different from basal levels (Dunnett's post hoc test). Note that the y-axis scales are different in the two graphs, reflecting the difference in basal levels between the two brain regions.*

In this same experiment, the absolute basal ecf levels of DOPAC were measured before the intracranial perfusion of PD168077. The absolute basal levels of DOPAC measured in the mPFC and NAc were 175.5 ±5.1 nM and 918.7 ±4.2 nM, respectively, which was statistically different from each other ($t(14) = 39.0$, $p < 0.001$; paired t-test). Perfusion with dialysis perfusate containing 200 nM and 400 nM of PD168077 (120 min each concentration) caused an increase in ecf DOPAC levels in both brain regions, which was shown by two-way ANOVA to be statistically significant (main effect of sample; ($F[8, 108] = 6.9$, $p < 0.001$; *figure 4.9*). There was also a significant main effect of brain region ($F[1, 108] = 1828.0$, $p < 0.001$), reflecting the different overall levels seen in the two brain regions, and a significant sample x brain region interaction ($F[8, 108] = 4.5$, $p < 0.05$; *figure 4.9*). Post hoc analysis with Dunnett's test showed significant increases in DOPAC concentrations after perfusion of

200 nM (from samples S1 to S4) and 400 nM (from samples S5 to S8) concentrations of PD168077 in both brain regions *(figure 4.9)*.

Further analysis showed that maximum concentrations reached in the two brain regions during perfusion with 200 nM of PD168077 were 250.6 ±6.7 nM (mPFC) and 1269.7 ±5.1 nM (NAc), whereas during perfusion with 400 nM of the drug, the maximum levels were 276.1 ±8.1 nM (mPFC) and 1517.9 ±9.0 nM. Further statistical analysis (paired t-test) showed that the maximum levels after 400 nM of PD168077 were significantly greater than after 200 nM of the drug in both brain regions (mPFC, $t(14) = 4.2$, $p < 0.001$ and NAc, $t(14) = 5.1$, $p < 0.001$), although the 2-fold increase in drug concentration did not produce a 2-fold increase in DOPAC release.

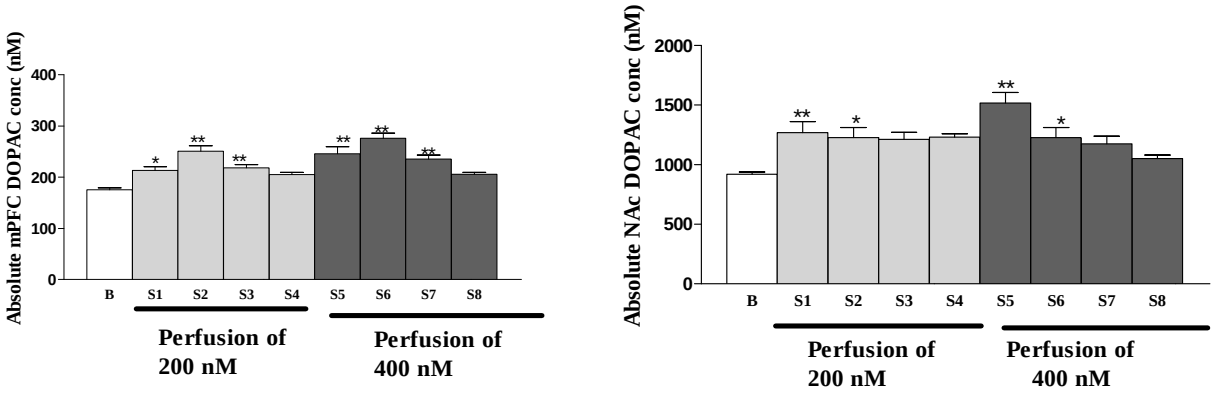

Figure 4.9: The absolute ecf concentration of DOPAC following intracerebral perfusion of 200 nM or 400 nM PD168077via the dialysis probe (n=9).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), the dialysis perfusion was switched to aCSF containing 200 nM PD168077 for 120 min (pale bar), and then to aCSF containing 400 nM PD168077 (dark bar: the timings shown take into account the dead time of the outlet tubings). For clarity, samples during the different infusions are shaded differently. * $p<0.05$; ** $p<0.01$ indicate samples which are significantly different from basal levels (Dunnett's post hoc test). Note that the y-axis scales are different in the two graphs, reflecting the difference in basal levels between the two brain regions.

4.3.3 Summary of results from systemic administration of PD168077 (experiment 1) and local perfusion of PD168077 (experiment 2)

4.3.3.1 Summary of results for mPFC

Dose (mg/kg)	Samples	mPFC PD168077 concentration (nM) (average)	Dopamine (nM) (average)	Dopamine release (normalised % of basal)
0.1	B	0	5.8	100
0.1	V	0	5.6	95.7
0.1	S1	19.2	6.9	118.8
0.1	S2	13.2	6.0	103.1
0.1	S3	7.6	6.0	103.1
0.1	S4	3.1	6.1	105.3
0.5	S5	27.6	5.8	99.3
0.5	S6	19.2	9.5	162.4
0.5	S7	10.2	7.0	119.2
0.5	S8	3.2	6.2	106.1
Brain perfusion (nM)	**Samples**	**mPFC PD168077 concentration (nM) (average)**	**Dopamine (nM) (average)**	**Dopamine release (normalised % of basal)**
0	B	0	5.8	100
200	S1	200	14.4	244.9
200	S2	200	14.6	248.6
200	S3	200	12.8	217.2
200	S4	200	13.1	223.0
400	S5	400	22.6	383.8
400	S6	400	22.4	380.4
400	S7	400	17.5	298.5
400	S8	400	13.4	228.5

Table 4.2: Summary of results obtained from the brain region: mPFC for systemic administration of s.c. doses of 0.1 mg/kg and 0.5 mg/kg of PD168077 and local perfusion of mPFC of 200 nM and 400 nM of PD168077. There was an increase in dopamine release in response to PD168077 challenge shown as % of the basal levels.

4.3.3.2 Summary of the results for NAc

Dose (mg/kg)	Samples	NAc PD168077 concentration (nM) (average)	Dopamine (nM) (average)	Dopamine release (normalised % of basal)
0.1	B	0	21.1	100
0.1	V	0	20.7	98.2
0.1	S1	13.9	24.2	114.4
0.1	S2	12.0	28.0	132.4
0.1	S3	10.1	23.2	109.8
0.1	S4	6.8	22.8	107.9
0.5	S5	29.5	23.3	110.2
0.5	S6	20.2	24.9	117.8
0.5	S7	13.0	24.0	113.5
0.5	S8	5.4	22.6	106.9
Brain perfusion (Nm)	**Samples**	**NAc PD168077 concentration (nM) (average)**	**Dopamine (nM) (average)**	**Dopamine release (normalised % of basal)**
0	B	0	20.3	100
200	S1	200	32.1	158.0
200	S2	200	33.7	166.9
200	S3	200	33.8	165.2
200	S4	200	29.4	144.8
400	S5	400	51.2	251.8
400	S6	400	48.1	236.6
400	S7	400	43.3	212.9
400	S8	400	39.5	194.5

Table 4.3: Summary of results obtained from the brain region: NAc for systemic administration of s.c. doses of 0.1 mg/kg and 0.5 mg/kg of PD168077 and local perfusion of NAc of 200nM and 400nM of PD168077. There was an increase in dopamine release in response to PD168077 challenge shown as % of the basal levels.

4.4 Discussion

One of main finding of this study is the regional distribution of PD168077 after s.c doses 0.1 mg/kg and 0.5 mg/kg in the mPFC and NAc. The results show that at a dose of 0.1 mg/kg the true ecf concentration did not show any regional difference in distribution of the drug between the two brain regions. However, at 0.1 mg/kg dose PD168077 attains a significant ecf concentration immediately in mPFC and not in NAc when compared to pre-injection levels. Also PD168077 attained the highest ecf concentration in mPFC compared to NAc after the lower dose of 0.1 mg/kg. At a higher dose of 0.5 mg/kg, PD168077 attained significant ecf concentration both in mPFC and NAc immediately, compared to pre-injection levels. The increase in the ecf concentrations of PD168077 after 0.5 mg/kg dose was 30% and 57% for mPFC and NAc respectively compared to the levels after 0.1 mg/kg dose. Therefore, a 5-fold increase in the dose did not result in a 5-fold PD168077 concentration in the ecf in both the brain regions. The ecf concentrations in mPFC and NAc did not show any significant difference in regional distribution of PD168077. Moreover, the concentration-time profile was similar for both brain regions for both the doses.

Another main finding of this study is the true/absolute dopamine dynamics in mPFC and NAc measured by MQ probes. To best of our knowledge, true basal levels of dopamine in the mPFC have not been measured by quantitative microdialysis so far. At a dose of 0.1 mg/kg PD168077 failed to induce any changes in the dopamine levels. Even at the highest ecf concentration of PD168077 in the mPFC (19.1 nM; *table 4.2*) immediately after the drug administration there was no significant change in the dopamine levels, suggesting that 19.1 nM is not a pharmacologically relevant concentration in terms of inducing dopamine changes. However, following a dose of 0.5 mg/kg, a significant increase in the true dopamine concentrations from the basal concentrations was observed in the mPFC. The dialysate concentration obtained from the area of interest reflects the events that occur at

synapses levels. Hence implicating that PD168077 increases dopamine levels at the synapses of mPFC region. Moreover, it is interesting to note that the ecf concentration of PD168077 in mPFC (27.6 nM; *table 4.2*) after 0.5 mg/kg dose was only pharmacologically relevant in the mPFC, as there was no significant change in the dopamine levels in the NAc. This suggests that PD168077 induced dopamine release at a target site with a higher D_4 receptor density. The data suggests PD168077 *in vivo* is pharmacologically active at ecf concentration of 27.6 nM which is higher than in vitro K_i value of 8.4 nM (Glase *et al*., 2000). PD168077 also increased DOPAC levels in mPFC but only at the higher dose (0.5 mg/kg). There was no significant change in the levels of DOPAC in NAc for either dose. In normal physiological events, dopamine in the synapses is broken down into its metabolite DOPAC by the enzyme MAO present at the synapses. Hence the data suggests that in mPFC, the increase in DOPAC levels were concomitant with the increase in dopamine concentration in challenge to the higher dose.

In experiment 2, the changes in the regional dopamine dynamics due to activation of local D_4 receptors with PD168077 was studied by perfusing both the brain regions with concentrations that are 10 times higher than the ecf concentrations attained in experiment 1. Local perfusion of PD168077 induced a substantial increase in dopamine release in both areas at concentrations of both 200 nM and 400 nM. The increased levels remained significantly elevated for 120 min for each concentration. The increase in dopamine levels from the baseline levels in the mPFC was 244.9% and 383.8% *(table 4.2)* and for NAc it was 166.9% and 251.8% *(table 4.3)*; at 200 nM and 400 nM perfusion respectively. This again suggests that PD168077 is more potent in its pharmacological action in mPFC a higher D_4 receptor density area. Moreover, this increase in dopamine levels resulted in concomitant increase in DOPAC levels in both the brain regions. The results obtained from the current study shows similarity with the results reported by Millan *et al.,* in 1998. This group employed S18126 a D_4 receptor antagonists to study dopamine release in mPFC, NAc and striatum using

microdialysis in freely moving rodents, and the results showed that at higher concentrations of S18126 an increased dopamine levels were recorded in mPFC without affecting dopamine levels in NAc and striatum. The increase in dopamine levels in the mPFC via D_4 receptor agonist (PD168077) and antagonist (S18126) is difficult to explain.

On the basis of my proposed model as shown in *figure 4.1*; I have two possible explanations- first: the dopamine efflux effect of PD168077 observed in this current study can be a downstream effect of VTA projections to the mPFC. The dopaminergic afferents arising from the VTA and the two major targets of these afferents are the mPFC and the NAc. In fact, VTA mediated dopamine release in mPFC and NAc can be differentially modulated and the firing patterns of dopaminergic neurones projecting to these brain regions are distinct (Di Chiara *et al*., 1999). The increase in dopamine levels in the mPFC, but not the NAc, suggest that the dopamine release mediated by PD168077 in specific brain regions can be independently targeting a subpopulation of VTA dopamine neurones. Moreover selective control of VTA dopamine neurones projecting to the mPFC has important implications for understanding addiction, attention disorders, and schizophrenia, all of which are associated with dopamine dysfunction in the mPFC (Margolis *et al.,* 2006).

Second, the local perfusion of PD168077 in the contralateral sides, suggests involvement of D_4 receptors localised on both interneurone and pyramidal neurone populations. The activation of dopamine D_4 receptors in the PFC circuit *(figure 4.1)* via PD168077 can influence the integrative networks within pyramidal neurones causing an increase in the dopamine release. Here I postulate that the increase in dopamine levels is mediated by PD168077 blockade on intrinsic, inhibitory GABAergic interneurones and pyramidal neurones within the PFC. Thus, activation of dopamine D_4 receptors in the PFC circuit, can indirectly influence the integrative networks within pyramidal neurones *(figure 4.1).* The increased ecf dopamine and DOPAC levels was predominant in the mPFC due to higher D_4

receptor density in mPFC compared to NAc. Moreover this increase in dopamine levels caused by PD168077 may implicate behavioural outcomes. Hence, it will be interesting to assess the effect of PD168077 in a behaviour that is concomitant with increase in dopamine levels in mPFC.

CHAPTER 5: PD168077 reverses sub-chronic PCP induced cognitive deficits in the NOR in rats

5.1 Introduction

Dopamine receptors are the principal target for many drugs used to treat schizophrenic patients; consistent with the view that aberrant dopamine neurotransmission is involved in the pathogenesis of schizophrenia (Grace 1991; Deutch 1992). However, many of these drugs are only effective in treating positive symptoms of the condition, with no effect on, or even exacerbation of negative and cognitive symptoms (e.g., Lewis and Lieberman 2000). The atypical antipsychotic drug, clozapine, however, is effective in treating most of the symptom types. Clozapine has a very diverse pharmacology, but among other things, it has been found to be a reasonably potent antagonist at dopamine D_4 receptors (Seeman 1994 a and b). The therapeutic and pharmacological profile of clozapine has led to speculation that the dopamine D_4 receptor subtype may play a role in the efficacy of clozapine and other similar atypical antipsychotic drugs (Wong and Van Tol 2003a). D_4 receptors may be critically involved in PFC functioning and neuropsychiatric disorders (Oak *et al.,* 2000). Since, elevated D_4 receptors are found in the PFC part of the brain of patients with schizophrenia (Seeman 1994 b) and to this end studies show that atypical antipsychotic drugs have high affinities for D_4 receptors (Van Tol *et al.,* 1991; Kapur and Remington 2001). In agreement with this D_4 receptor antagonists ameliorate cognition deficits caused by the psychotomimetic drug PCP (Jentsch *et al.,* 1997; 1998 a and b; Jentsch and Roth 1999).

In parallel with the above hypothesis, glutamate is also implicated to be involved in schizophrenia, demonstrated by the psychotomimetic properties of several non-competitive NMDA receptor antagonists such as PCP, MK-801 and ketamine (Javitt and Zukin 1991). The observation that PCP, induces symptoms in man which resemble the positive, negative

symptoms and cognitive dysfunction of schizophrenia (Javitt and Zukin 1991) has reinforced the hypothesis that glutamate hypofunction occurs in schizophrenia (Kim *et al.,* 1980). The NMDA receptor is an excitatory ionotropic receptor (ligand-gated ion channel) and this receptor complex has been extensively characterised physiologically and pharmacologically. It is widely distributed in mammalian brain, with high receptor densities found in hippocampus and cerebral cortex, and appears to have a pivotal role in long term depression, long term potentiation and developmental plasticity (Tsien *et al.,* 1996; Sprengel *et al.,* 1998). The NMDA receptor is a complex molecular entity with a number of distinct recognition sites for endogenous and exogenous ligands, each with discrete binding domains. There are at least six pharmacologically distinct sites through which compounds can alter the activity of the NMDA receptor (*figure 5.1*) (1) a transmitter binding site which binds L-glutamate and related agonists and promotes the opening of a high conductance Na^{+} and Ca^{2+} channel, (2) this action of L-glutamate is virtually ineffective unless a second site which binds glycine is also occupied, (3) a site within the channel which binds PCP (PCP site) and related non competitive antagonists (MK-801, ketamine). These agents act most effectively when the receptor is activated (i.e., open channel block). (4) a voltage dependent magnesium (Mg^{2+}) binding site, (5) an inhibitory divalent cation site near the mouth of the channel that binds zinc (Zn^{2+}) to produce a voltage dependent block, (6) a polyamine regulatory site whose activation facilitates NMDA receptor mediated transmission. For the normal physiological function of the NMDA receptor, fluxes of positive ions through the channel part of the activated receptor are required. PCP exerts its effect by entering the ion channel from the outside of the neurone and binding, reversibly, to a site in the channel pore (PCP site), thus blocking the flux of positive ions into the cell and interferes with cognitive and other functions of the nervous system. Moreover, in the view of the known interaction in the brain between glutamate and dopamine, it is interesting that PCP pretreated animals show significant reductions in dopamine utilization (the ratio of dopamine metabolite DOPAC to

dopamine) in regions of the PFC (e.g., Kargieman *et al.,* 2007), suggesting reduced dopaminergic transmission in frontal regions. Therefore, it has also been suggested that PCP may provide a relevant tool for examining the antipsychotic potential of novel agents in preclinical animal studies.

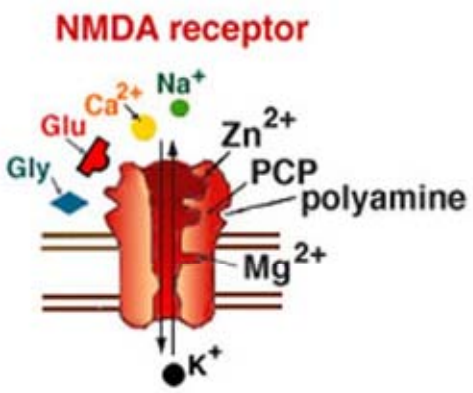

Figure 5.1: NMDA receptor, structurally complex with separate binding sites for glutamate, glycine, Mg2+, Zn2+ and polyamines (adapted from Kandel et al., 2000).

In humans, exposure to either acute or repeated PCP produces a variety of behaviours resembling schizophrenia, such as cognitive deficits, loss of short-term memory and occurrence of negative symptoms (Snyder 1980; Tamminga 1998). Furthermore, in schizophrenics, administration of PCP dramatically exacerbates their cognitive deficit symptoms (Itil *et al.,* 1967; Lahti *et al.,* 1995). These observations in human studies are supported by animal studies where PCP has been found to produce cognitive deficits (Jentsch and Roth 1999; Adams and Moghaddam 1998) disruption in sensorimotor gating (Mansbach and Geyer 1989) and impaired social interaction (Sams-Dodd 1995). Moreover animals receiving repeated treatment with PCP show long-lasting effects, which persist well beyond the period of drug treatment, on cognition (Sturgeon *et al.,* 1982; Stefani and Moghaddam

2002) suggestive of profound neuropathological changes (Jentsch *et al*., 1997; Jentsch *et al*., 1998 a and 1999) which may mimic the cognitive impairment seen in schizophrenia (Jentsch *et al*., 1997 and 1998 b; Jentsch and Roth 1999). Interestingly it has also been observed that PCP treatment causes altered levels of both dopamine and glutamate in the brain (Deutch 1992; Moghaddam *et al*., 1997), indicative of long term changes in these transmitters, which may provide a link to neurochemical theories of schizophrenia. PCP also impairs performance in memory tasks thought to be mediated by prefrontal cortical function (for review see Goldman-Rakic and Selemon 1997), which is particularly pertinent given that schizophrenia sufferers often exhibit deficits in cognitive tasks which are sensitive to the functional integrity of prefrontal PFC (Gold *et al.,* 1994, Weinberger and Lipska 1995).

Recent studies have shown that a sub-chronic treatment regimen of PCP (2 mg/kg) twice daily for seven days produces a cognitive deficit in NOR in rats, which endures beyond the end of drug treatment (Grayson *et al.,* 2007). This task relies on the rat's natural propensity to explore novel objects and utilises memory processes, perhaps episodic memory, which are disrupted in schizophrenia (Saykin *et al.,* 1991; see also introduction section of this thesis). Rats readily approach objects and investigate them physically by touching and sniffing the objects, rearing upon and trying to manipulate them with their forepaws. Moreover they show an innate preference for novel over familiar objects even when the objects have no natural significance to the animal, and spend more time exploring the novel objects than familiar ones. This discrimination between novel and familiar objects can easily be quantified and can be utilized to study simple recognition memory as well as more complex spatial-, temporal- and episodic-like memory, and forms the basis of the NOR task. Episodic memory involves binding items and spatiotemporal context to form an episode that then can be more or less permanently stored (Saykin *et al.,* 1991). Episodic memory storage is critical in acquisition of new information and its function in schizophrenia was one of the first cognitive

abilities studied by Hull in 1917. Schizophrenic patients generally remember stories, verbal paired associates and visual designs more poorly than do normal subjects (Duncan *et al.,* 1999) and the difference between normal controls and patients may be large (Saykin *et al.,* 1991; Gold *et al.,* 1994). Importantly the NOR task is ethologically relevant and non rewarding, and assesses visual learning and memory aspects of behaviour, criteria which have led NOR testing to be listed under the TURNS initiative as assessing visual learning and memory deficits in schizophrenia. Thus deficits in NOR brought about by chronic PCP pre-treatment may provide a behaviourally relevant model for studying cognitive deficits in schizophrenia.

5.1.1 Aims

The aims of the study were twofold: first to assess the effect of PD168077 on basic NOR; and second to ascertain whether the drug would reverse the disruption of NOR brought about by sub-chronic pre-treatment with PCP.

5.2 Materials and methods

5.2.1 Animals and housing conditions

Subjects were 99 female hooded-Lister rats (Harlan, UK; 200g). On arrival, they were housed in groups of 5 and adapted to laboratory conditions (12 h light/dark cycle with lights on at 07.00 h; 21 ± 2^{o}C; 40-50% humidity) with free access to food and water for at least 14 days. All experimental procedures were carried out in accordance with the Animals Scientific Procedures Act, UK, 1986 and approved by the University of Bradford ethical review process.

5.2.2 Apparatus and objects

The apparatus consisted of an open arena made of Plexiglas (52 cm L; 52 cm W; 31 cm H) which was positioned 27 cm above the floor. The walls of the box were black and the floor was white and divided into nine identical sectors by black lines. Animal behaviour was recorded by a video camera connected to a video recorder and monitor. Two different objects, each in triplicate, were used: clear Plexiglas pyramids and white bottles. In each experiment one object type was used as the familiar object, and the other as the novel object, with the identity counterbalanced across animals. The dimensions of the objects were similar (base 6 x 6 cm; height 10 cm; all ± 2 cm), and their weight was such that the animals could not displace them: to achieve these bottles were filled with NaCl. These objects, as far as could be ascertained, had no natural significance for rats and had never been associated with reinforcement. When objects were placed in the test arena, they were located 6 cm away from the walls of the box, in opposite corners: the animal was then introduced into one of the other corners, with its head facing the corner. After testing of each animal, the arena and the objects were cleaned with 10% alcohol to remove any lingering olfactory cues on the objects and in the box.

5.2.2 NOR procedure

The protocol used for NOR testing was carried out as described in Grayson and Neill 2004. During the week prior to behavioural testing, animals were handled daily, and habituated to the experimental test environment, by placing them in the behavioural arena for 30 min on the 3 days immediately prior to testing.

5.2.2.1 Behaviour testing

On the day of testing, animals first received a further 3 min habituation session, after which they received drug or vehicle injection. The NOR task consisted of a 3 min acquisition trial, followed, after an appropriate ITI, by a 3 min test (retention) trial. For the acquisition trial, two similar objects were placed in opposite corners of the arena, 6 cm in from the walls, and the rat was introduced to the testing arena by placing it is a third corner, facing the corner (see *figure 5.2*). The rat was allowed to explore the arena freely for 3 min, after which it was returned to its home cage for a delay period of either 1 min (experiment 1) or 6 hours (experiment 2). While the animal was out of the arena, the objects were replaced with one object similar to that used in the acquisition trial (familiar object): objects were placed in the same positions as in the acquisition trial, and the location of the novel object was counterbalanced across experiments. The rat was then returned to the arena for 3 minute for the retention (test) trial, and again allowed to explore freely. In order to prevent carry over of olfactory cues between acquisition and test, the familiar object used at test was an identical copy of those used at acquisition, rather than the actual ones. In this task depending on the ITI imposed between the acquisition and retention trail, untreated control animals spend more time exploring the novel object, suggesting that the familiar object was recognised (Dix and Aggleton 1999; Grayson *et al*., 2007; *figure 5.2*).

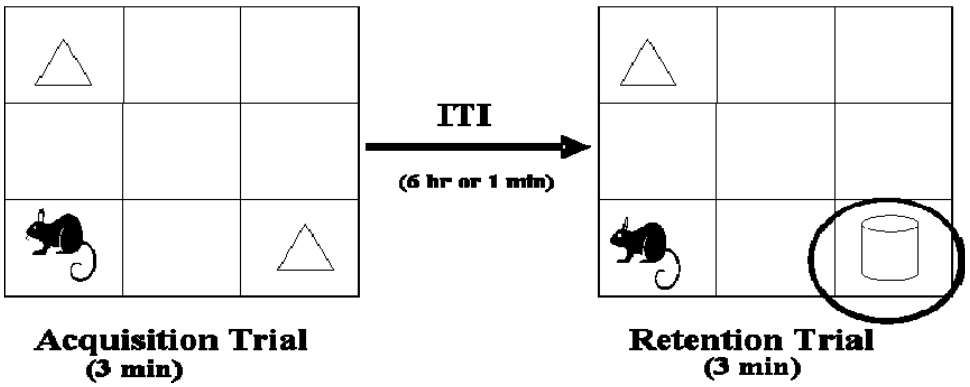

Figure 5.2: Novel object recognition task procedure description.

The animal is introduced into the arena in one of the other corners, facing the corner. (a) For acquisition two objects are placed in opposite corners of the arena, 6 cm form the walls. (b) For retention testing, one object similar to those used in acquisition, and one novel object are placed in the same positions, and the animal is introduced into the arena as for acquisition.

5.2.2.1 Drug treatment and preparation

Experiment 1: Following the habituation period, the rats (n=10 per group) were randomly assigned to receive vehicle (saline; 1 ml/kg; s.c.), or PD168077 (doses 0.3, 1.0, 3.0 and 10.0 mg/kg; in a volume of 1 ml/kg; s.c.), and 45 min later they started the acquisition trail. Following acquisition the animal was returned to its home cage for 6 hour delay (ITI) before the retention trial.

Experiment 2: Following the settling in period in the animal unit, and before habituation, the rats were randomly assigned to receive 0.9% saline (n=9), or PCP (2.0 mg/kg; n=40) in a volume of 1.0 ml/kg i.p. twice daily for seven days. Following PCP and saline treatment, the animals were given a one-week drug free period prior to NOR testing. The PCP dosing regimen was based on previous work by Grayson *et al*., 2007; Jentsch *et al*., 1997. On the NOR test day, sub-chronic PCP treated rats were treated with PD168077 (doses 0.3, 1.0, 3.0, 10.0 mg/kg, in a volume of 1 ml/kg; s.c.; n=8 per group) or vehicle (saline; 1.0 ml/kg; s.c.; n=8): saline pre-treated animals received vehicle (saline; 1.0 ml/kg; s.c.; n=9) and 45 min

later, the acquisition trial was carried out. Following the acquisition trial, a 1 min delay (ITI) was implemented before the retention trial.

5.2.2.1 Data Collection

The acquisition and retention trials were video recorded using a camera mounted above the test arena and the exploration time on the objects and locomotor activity were scored from the video recording, with the scorer blind to the treatment conditions. The exploration times (s) on each object in the two trials were recorded using two stopwatches and the discrimination index (DI), was calculated as: {(Time spent exploring novel object – Time spent exploring familiar object) / Total time spent exploring both the objects}. Animals were deemed to be exploring the object when the head of animal was facing it within 2 cm of it or touching it with any part of its body except the tail: turning around or sitting on the object was not considered as exploratory behaviour. Locomotor activity was recorded by counting the total number of line crossings (all four feet crossing a line) by the rats during the acquisition and retention trails.

5.2.2.1 Statistical Analyses

The data are expressed as mean ± SEM. Student's paired t-test was performed to compare the effect of treatment on the time spent exploring the familiar versus the novel object. Locomotor activity data are expressed as mean ± SEM of the total number of lines crossed. Statistical analysis of locomotor activity, total exploration time and DI data was performed using a one-way ANOVA followed by post hoc Dunnett's test, using GraphPad Prism v5 for Windows.

5.3 Results

5.3.1 Experiment 1: Effect of PD168077 on NOR testing in the control rats

Treatment with PD168077 (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.), 45 min prior to the acquisition trial had no significant effect on exploratory preference of the two familiar objects ($F[1,90] = 0.1$, $p = 0.6$; *Figure 5.3*).

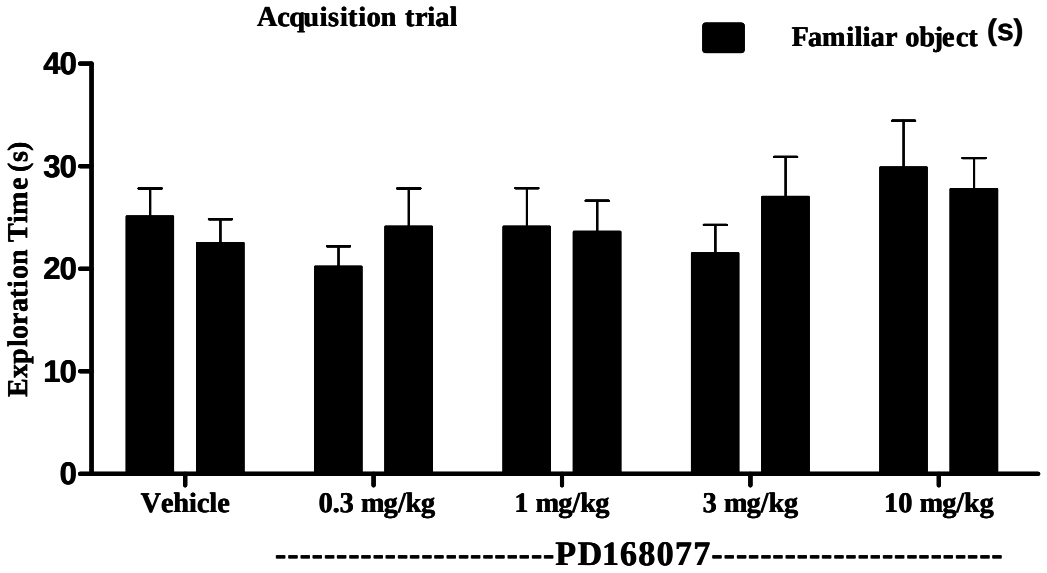

Figure 5.3: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) on exploration time of two identical objects in the 3 min acquisition trial of the NOR task. Data are expressed as mean ± SEM (n = 10 per group).

During the retention trial, paired t-test for individual groups showed that the vehicle treated rats show no significant difference in time spent exploring the familiar and novel object, showing that after 6 hours ITI delay control animals did not differentiate between the novel and familiar objects. In PD168077 treated animals, however, there was a significant difference at 3 mg/kg ($p < 0.05$) and at 10.0 mg/kg ($p < 0.01$) of

PD168077, showing a propensity for animals to explore the novel object more than the familiar object (*Figure 5.4*). The data were examined further using one-way ANOVA to look at the effects of the drugs on the exploration times on each object individually. There was no effect of drug treatment on exploration of either the novel object ($F[4,36] = 0.1$, $p = 0.9$) , or the familiar object ($F[4,36] = 1.5$, $p = 0.2$). However there was a significant linear trend of increasing drug dose on exploration of the familiar object ($R^2 = 0.07$; $p = 0.03$), but not the novel object ($R^2 = 0.004$; $p = 0.5$), showing that the effect on NOR is through recognition of the familiar object.

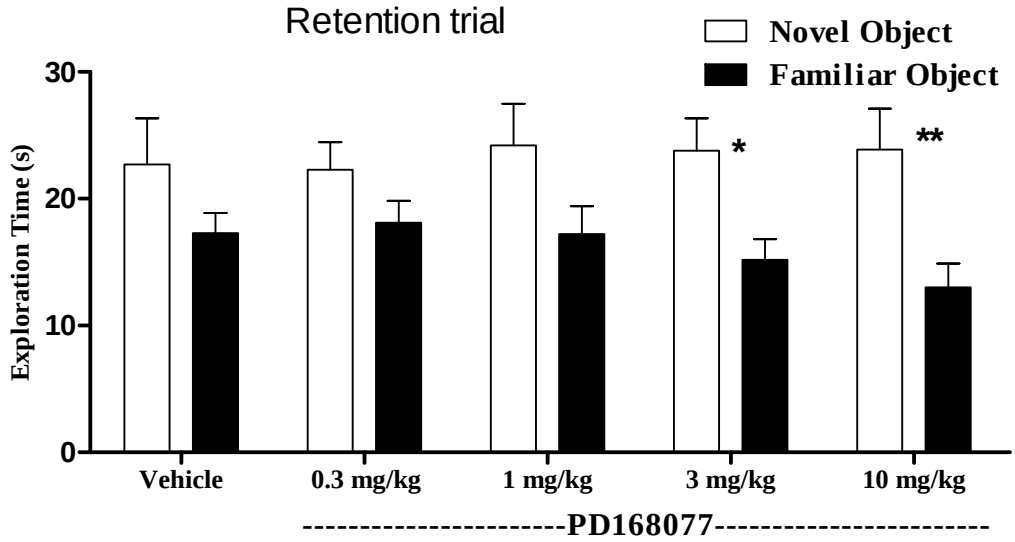

*Figure 5.4: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) on exploration time of a familiar and a novel object in the 3 min retention trial. Data are expressed as mean ± SEM. (n = 10 per group). * $p < 0.05$, ** $p < 0.01$, significant difference in time spent exploring novel and familiar objects during the retention trial analysed by paired t-test.*

Although the PD168077 treated groups showed an apparently dose dependent increase in the DI compared to the vehicle group, one-way ANOVA revealed no significant difference ($F[4,45] = 1.2$, $p = 0.3$) in DI between any of the groups (*figure 5.5*). Further analysis by linear trend did show a significant linear trend ($R^2 = 0.09$; $p = 0.01$) for DI to increase with dose (*figure 5.5*) suggesting that there was a dose dependent increase in the animals' ability to discriminate between the novel and familiar object (*figure 5. 5*).

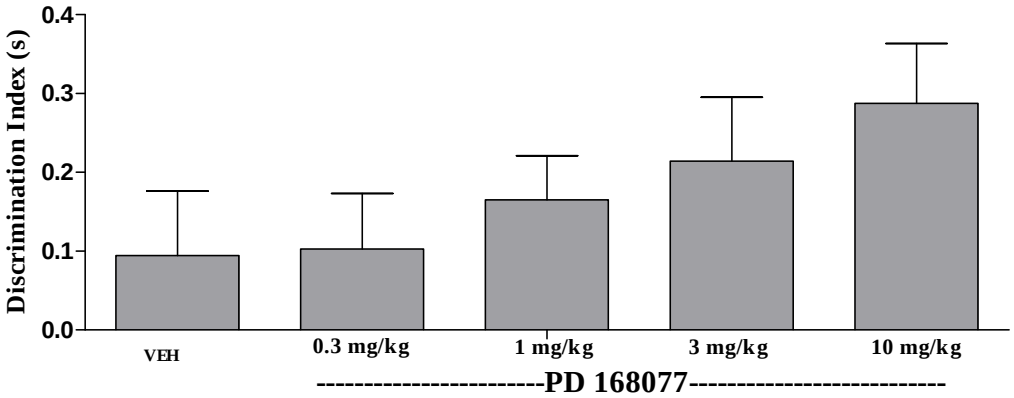

Figure 5.5: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) on DI during the retention trial following a 6 hour ITI. Data are expressed as mean ± SEM (n=10).

One-way ANOVA revealed was no significant effect on the total object exploration time (acquisition + retention trial) between the groups ($F[4,49] = 0.2$, $p = 0.9$; *figure 5.6*), nor on locomotor activity ($F[4,49] = 1.8$, $p = 0.1$; *figure 5.7*).

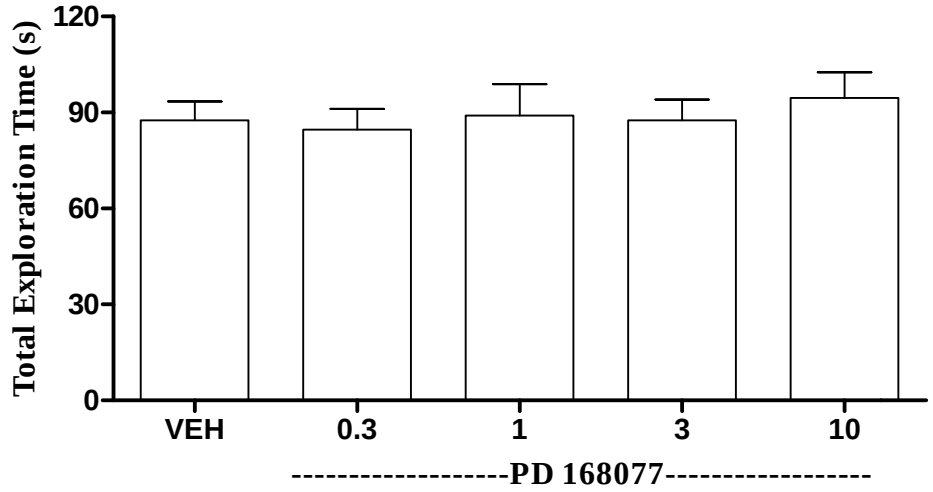

Figure 5.6: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) on total time spent exploring objects. Data are expressed as mean ± SEM (n=10).

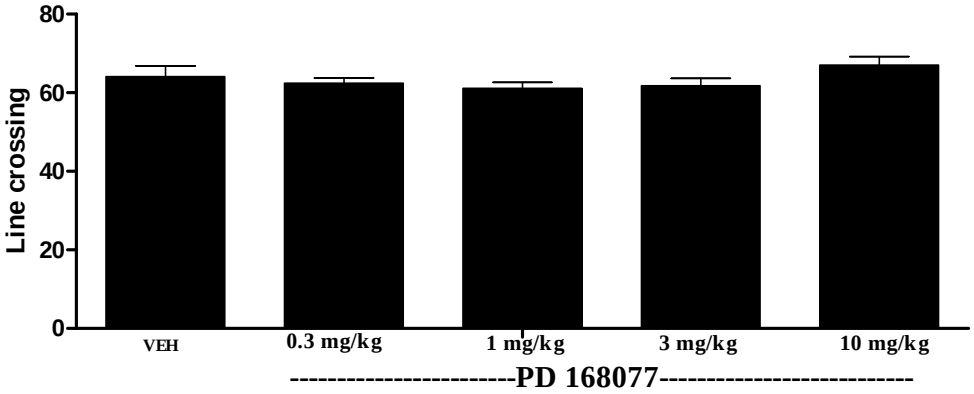

Figure 5.7: The effect of acute treatment of PD168077 (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) on locomotor activity, measured across both acquisition and retention trials. Data are expressed as mean ± SEM (n=10).

5.3.2 Experiment 2: Effect of PD168077 on NOR testing in the sub-chronic PCP pre-treated rats

Treatment with PD168077 (0.3, 1.0, 3.0 and 10.0 mg/kg., s.c.), 45 min prior to the acquisition trial had no significant effect on exploratory preference of the two familiar objects ($F[1,90] = 0.1$, $p = 0.6$; *figure 5.8*).

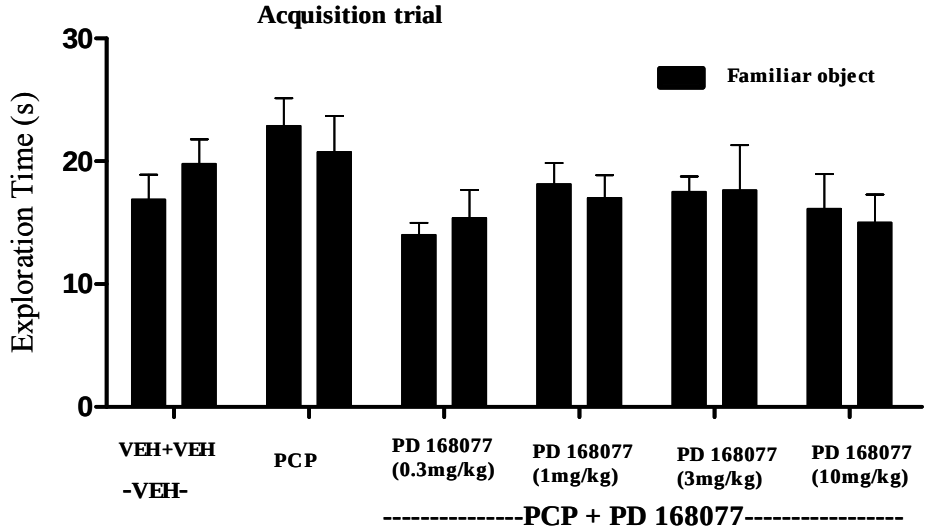

Figure 5.8: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) in sub-chronic PCP pre-treated rats on exploration time of two identical objects in the 3 min acquisition trial of the NOR task. Data are expressed as mean ± SEM (n = 8 - 9 per group).

During retention trial following a 1 min ITI, a t-test on individual groups showed a significant increase in time spent exploring the novel object compared to the familiar object ($p<0.001$) in the vehicle pre-treated group, showing intact NOR. Conversely, the animals pre-treated with PCP, and treated with vehicle on the day of test, showed impairment in NOR (*figure 5.9*) such that no significant difference was observed between exploration time of the novel and the familiar object. However, animals pre-treated with PCP, and receiving PD168077 (10 mg/kg) on the test day, showed a significant increase in exploration time of the novel object compared to the familiar object ($p<0.01$).

The data were examined further using one-way ANOVA to examine effects of the drugs on the exploration times on each object individually. There was no effect of drug treatment on exploration of the novel object ($F[5,42] = 1.4$, $p = 0.2$) , whereas there was a highly significant effect of treatment on time spent exploring the familiar object ($F[5,42] = 3.7$, $p = 0.007$), which was manifest as a significant increase in time spent exploring the familiar object in PCP pre-treated animals given 0, 0.3 or 1.0 mg/kg PD168077 (each $p < 0.05$; Dunnett's post hoc test), but not those given the higher dose of the agonist. Moreover there was a highly significant trend for increasing doses of PD168077 to reverse the effect of PCP on exploration of the familiar object ($R^2 = 0.2$; $p = 0.002$), but not the novel object ($R^2 = 0.05$, $p = 0.1$; *figure 5.9*).

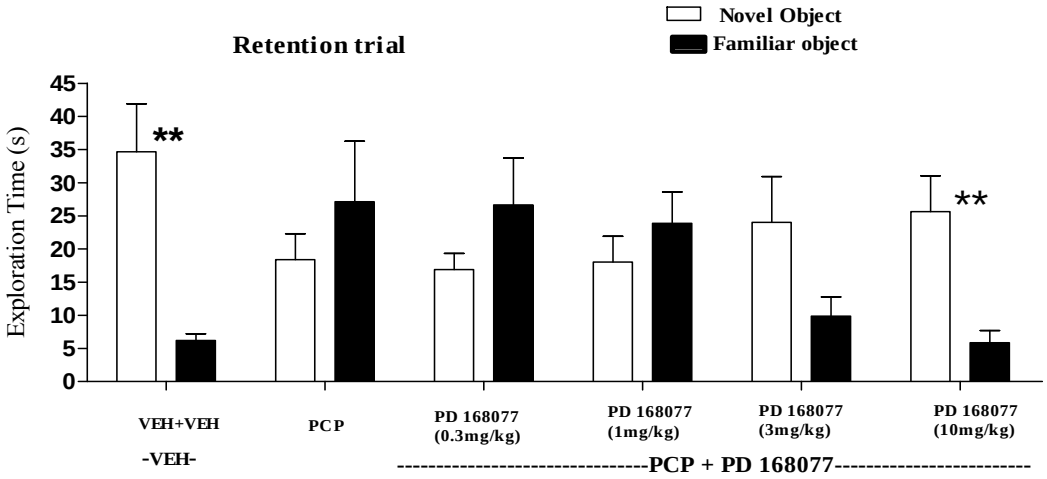

Figure 5.9: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) in sub-chronic PCP pre-treated rats on exploration time of a familiar and a novel object in the 3 min retention trial, following 1 minute ITI.

Data are expressed as mean ± SEM. (n = 8 - 9 per group). * $p < 0.05$, ** $p < 0.01$: significant difference in time spent exploring novel and familiar objects during the retention trial; paired t-test.

One-way ANOVA revealed significant difference in DI values between drug treatment groups ($F[5,43] = 6.1$, $p < 0.001$) and post hoc Dunnett's test showed a significant reduction of DI in rats treated with PCP alone. Linear trend analysis showed a significant dose-dependent reversal of this disruption with PD168077 ($R^2 = 0.1$, $p < 0.001$), leading to a significant reversal of the disruption with 10 mg/kg of the agonist ($p < 0.01$; *figure 5.10*).

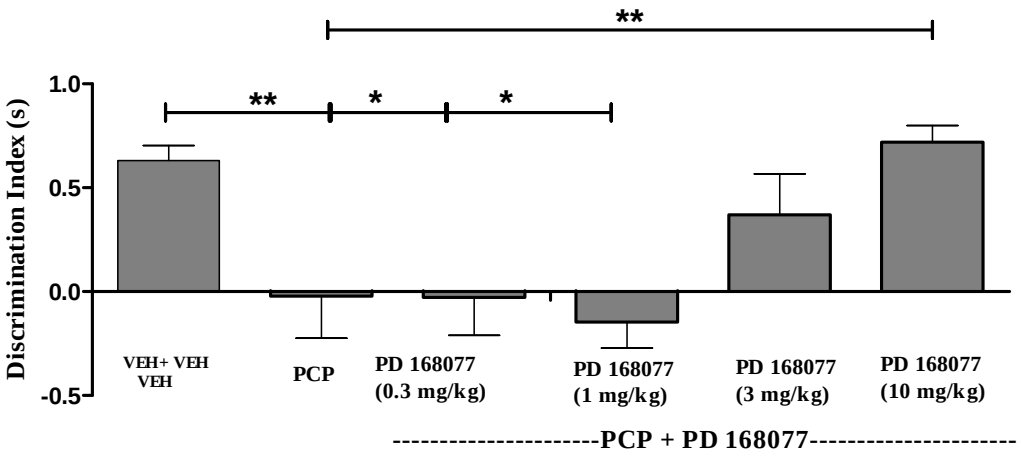

Figure 5.10: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) in sub-chronic PCP pre-treated rats on DI during the retention trial, following 1 min ITI.

Data are expressed as mean ± SEM (n=8). * p<0.05, ** p<0.01 are significant difference from DI in animals receiving vehicle pre-treatment or sub-chronic PCP pre- treatment.

One-way ANOVA revealed no significant effect on the total exploration time in acquisition and retention trials ($F[5,48] = 1.2$, $p = 0.2$; *figure 5.11*) nor on locomotor activity in either trial ($F[5,43] = 1.3$, $p = 0.2$) between the groups (*figure 5.12*).

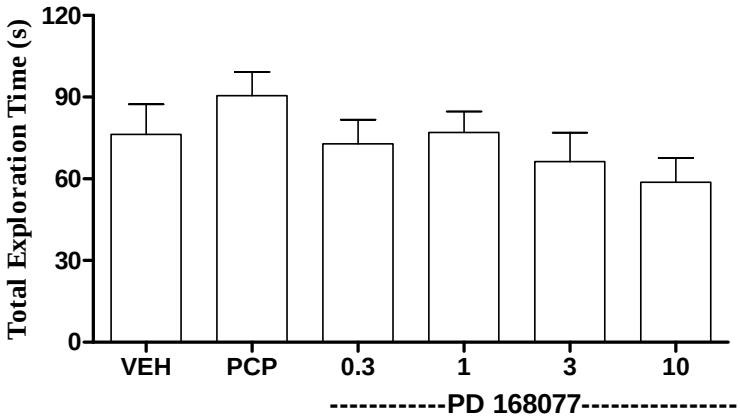

Figure 5.11: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) in sub-chronic PCP pre-treated rats on total time spent exploring objects. Data are expressed as mean ± SEM (n = 8).

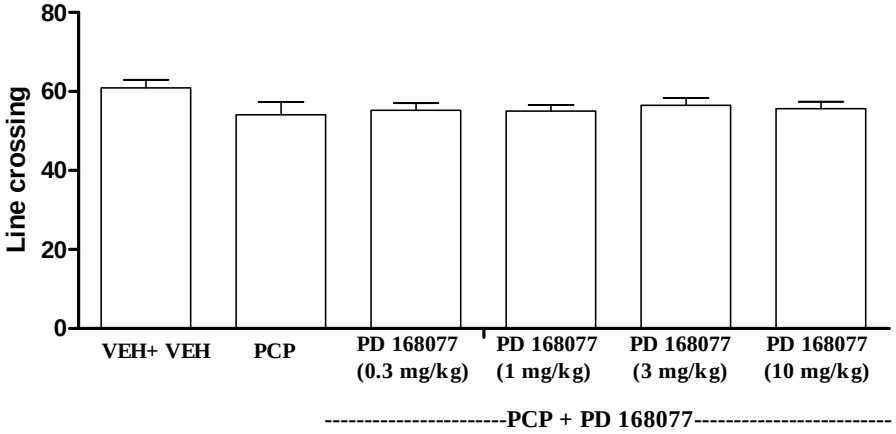

Figure 5.12: The effect of acute PD168077 treatment (0.3, 1.0, 3.0 and 10.0 mg/kg, s.c.) in sub-chronic PCP pre-treated rats on locomotor activity, measured across both acquisition and retention trials. Data are expressed as mean ± SEM (n = 8).

5.4 Discussion

A significant role for dopamine in the PFC orchestration of working memory was realised when depletion of dopamine in the PFC of the rhesus monkey impaired the performance of a working memory related task, as severely as surgical lesions of the same area (Brozoski *et al*., 1979). Similar results in the rodent have demonstrated an impairment of delayed

alternation response after chemical lesions of the dopamine afferents to the mPFC (Stam *et al*., 1989; Wilcott and Xuemei 1990). Another class of neurones that may play an important role in PFC function is the glutamate (excitatory amino acid) containing neurones. Morphological and biochemical studies have indicated a close interaction between excitatory amino acid and dopamine afferents in the PFC (Smiley *et al.,* 1994). Furthermore, clinical studies have reported that sub-anaesthetic doses of the non-competitive NMDA receptor antagonists (ketamine, MK-801) produce cognitive impairment and deficits in PFC sensitive tasks such as the Wisconsin Card Sorting test (Krystal *et al.*, 1994). These findings implicate excitatory amino acid neurotransmission at the NMDA receptor in the proper functioning of the PFC and suggest an interaction between glutamatergic and dopaminergic systems in the modulation of working memory. In my present study, the role of the NMDA receptor antagonist, PCP in the performance of NOR task, a episodic memory task sensitive to proper functioning of the PFC (Goldman-Rakic and Selemon 1997) was examined in the rat. Furthermore, the role of dopamine release (results from chapter 4) in this paradigm is examined by the effect of dopamine D_4 receptor agonist-PD168077 on the ability of NMDA antagonist (PCP) to alter cognitive deficit performance in NOR task.

The NOR paradigm measures non-spatial memory with the characteristics of episodic memory (Ennaceur and Delacour 1988) assessed in rodents by a visual recognition task. Importantly the task assesses the animal's behaviour during the acquisition and retention trial in terms of the time spent exploring two objects, and therefore it can be used to detect enhancement or disruption of episodic memory following experimental interventions (see reviews by Dere *et al*., 2007; Winters *et al*., 2008).

In our laboratory, control female rats recognise a familiar object when the ITI between acquisition and test is between 1 min and 3 hours, and thus, at test, show a preference to

explore the novel object over the familiar one (Sutcliffe *et al*., 2007). However, with a 24 hour ITI, the times spent in exploring the familiar and novel objects were similar, indicating that the rats no longer recognise the familiar object as familiar (Ennaceur and Delacour 1988). Hence it is the episodic memory that deteriorates as the length of time between acquisition and retention testing (ITI) increases (Bertaina-Anglade *et al*., 2006), enabling the amount of baseline memory to be manipulated within an experiment by changing the ITI between acquisition and retention test.

The current study examined the effect of the D_4 receptor agonist, PD168077 on natural episodic memory retention in two situations: first, where a decrement of memory that had occurred due to the time between acquisition and retention testing, with an ITI of 6 hours (experiment 1), and second where the memory decrement was induced by sub-chronic pre-treatment with PCP (experiment 2). Control animals which had received only vehicle injections, showed a clear and significant discrimination between the familiar object and the novel object (animals explored the novel object more than the familiar object) during the retention test phase, when testing was carried out 1 min after acquisition (*figure 5.9;* experiment 2), but not when it was carried out 6 hours after acquisition (*figure 5.4;* experiment 1). This is similar to effects previously reported, and represents the animals' memory of the familiar object, which endures for 1 min, but not for 6 hours (Ennaceur and Delacour 1988). In animals sub-chronically pre-treated with PCP, however, this discrimination after 1 min delay was abolished (*figure 5.9;* experiment 2), again replicating previous findings (Grayson *et al*., 2007). This treatment regime for sub-chronic PCP has been shown to have effects on cognitive performance which long outlast the treatment period (Abdul-Monim *et al.,* 2006 and 2007), and is believed to model some of the cognitive deficits occurring in schizophrenia (Jentsch and Roth 1999; and also see section 1.4 of introduction). In experiment 1, with ITI of 6 hours, I tested whether PD168077 alone affected retention of

episodic memory. The results from the vehicle treated animals *(figure 5.4)* confirmed that a 6 hour ITI brought about a disruption of episodic memory in the control rats (previously studied by Sutcliffe *et al.,* 2007). The results moreover showed that rats treated with doses 3 mg/kg, and 10 mg/kg of PD168077 explored the novel object more than the familiar object in the retention trial after the 6 hour ITI *(figure 5.4),* suggesting enhancement of natural episodic memory by higher doses of the drug.

The aim of experiment 2 was to examine the effect of PD168077 on the PCP-induced disruption of episodic memory (see section 1.4). Animals which were sub-chronically treated with PCP, after only a 1 min ITI, failed to discriminate between familiar and novel objects, as has previously been described (Grayson *et al.,* 2007). Thus, after a 1 min ITI, episodic memory was intact in vehicle pre-treated animals, but not in PCP treated ones *(figure 5.9).* Under these conditions PD168077 caused a dose dependent reversal of the PCP effect.

It is important to note that the sub-chronic pre-treatment with PCP was ended at least 7 days prior to behavioural testing, such that all testing was performed PCP-free. Thus it is clear that PCP treatment induced enduring memory impairments which were reversed by PD168077 and fully restored the discrimination after a dose of 10 mg/kg *(figure 5.10).* DI represents the discrimination of exploratory preference of animals to either the familiar or novel object in the retention trial and for varies from -1 to +1. A negative value indicates a familiar object preference while a positive value shows preference for the novel object. Whilst this gives a measure of the net discrimination between the novel and familiar objects, it does not necessarily indicate which aspects of the behaviour are changing. The examination of the times spent exploring the novel and familiar objects individually showed, in all cases, that the changes in DI were due to changes in time spent exploring the familiar object, and that there were no significant differences in time spent exploring the novel object. This is important

because it emphasises that the effects are indeed due to memory of the familiar object, rather than non-specific effects on exploration generally. Therefore, PD168077 improves the memory in a dose-dependent manner in the sub-chronic PCP treated rats without causing any effect on the general exploration activity or locomotor activity *(figure 5.11 and figure 5.12).*

Paradoxically, Grayson *et al.*, (2007) have previously reported that acute treatment of clozapine but not with haloperidol significantly reversed the PCP induced impairment in NOR. Clozapine is an atypical antipsychotic drug, whose effects are believed to be mediated partly through dopamine D_4 receptor blockade (Carlsson and Lindquist 1963; Seeman and Lee 1975), an effect which is difficult to reconcile with the present study where *activation* of D_4 receptors reverses the episodic memory decrement induced by either ITI or by PCP pre-treatment.

Interestingly, the results from chapter 4 show that PD168077, given either systemically or intracerebrally, increases ecf dopamine levels in the mPFC within 30-60 min post drug administration. The NOR experiments described in this chapter were assessed 45 min after the administration of PD168077 to correlate the changes with dopamine dynamics observed in chapter 4. The dopamine data from the chapter 4 *(figure 4.4 and figure 4.6)* and the NOR data *(figure 5.4 and figure 5.9)* from this chapter suggest that PD168077 induces dopamine release and enhances episodic memory. In this context it is also pertinent to note that another D_4 receptor agonist, A412997, has also been shown to improve performance in a similar NOR task, at doses which increase ecf dopamine levels in the PFC (Woolley *et al.,* 2008).

PD168077 induced dopamine release may contribute to the enhancement of episodic memory (by reducing forgetting occurring due to time in experiment 1) and the reversal of sub-chronic PCP induced cognitive deficits (experiment 2) by two possible mechanisms: First- it

enhances the PFC-hippocampus connectivity. The PFC has direct anatomical connections with many brain regions, including synergic connections with hippocampus (e.g., Suzuki and Amaral 1994; Lavenex and Amaral 2000). The role of the hippocampus in memory storage over a period of time in the mammalian brain is well studied (Suzuki and Amaral 1994; Miller 2000). Recent neuro-imaging studies also indicated that PFC neurones exhibit synchronous activity with the hippocampal neurones (Prabhakaran *et al*., 2000; Bor *et al.*, 2003) in sustained memory formation of transient events and form representational bindings between events separated in time (Asaad *et al*., 1998; Fuster 2001). In line with this functional synergy between PFC and hippocampus, it's likely that the dopamine releasing effect of PD168077 may lead to the enhancement of the PFC-hippocampus circuit, thus, resulting in sustained memory storage over a period in the NOR test.

Second, PD168077 mediates modulation of the glutamatergic system in the PFC via D_4 receptor activation (an agonist effect). It is well documented that PCP treatment causes altered levels of both dopamine and glutamate in the brain (Deutch *et al*., 1992; Moghaddam *et al*., 1993; 1997; Moghaddam and Adams 1998), and causes long term changes in these transmitters. Moreover PCP impaired performance in memory tasks is mediated by PFC function (Goldman-Rakic and Selemon 1997). Furthermore, there is a mechanistically link between the ionotropic subtypes of the glutamate receptor, (mainly the NMDA receptors) and the dopamine neurones (Sanghera *et al*., 1984) localised in the PFC region *(see figure 4.1)*. NMDA receptors can modulate the neuronal firing activity and pattern of dopamine neurones (Wang *et al*., 2002 and 2003). At basal glutamate concentrations, NMDA receptors appear to exert a tonic excitatory tone on some dopamine neurones. Under conditions of increased glutamatergic activity, the glutamate receptors (NMDA receptors and AMPA receptors) may transduce these signals into increases in dopamine release (Shepard and Bunney 1988; Shepard and Lehmann 1992). A number of studies have demonstrated that stimulation of

glutamatergic receptors on dopamine neurones, either by direct injection of glutamate agonists or stimulation of glutamatergic afferents increases the release of dopamine in many dopaminergic neurones (Shepard and Lehmann 1992). Also, there is NMDA receptor hypoactivity in the glutamatergic hypothesis of schizophrenia (Nakanishi 1992), a condition that is mimicked with sub-chronic PCP pre-treatment in rodents (see introduction section). In line with this hypothesis, PD168077 induced reversal of PCP cognitive dysfunction suggests an alleviation of the NMDA receptor hypoactivity (Wang *et al.,* 2003). The dopamine releasing characteristics of PD168077 (chapter 4) may also lead to enhancement of glutamatergic activity. The increased in dopamine levels may transduce these signals into increases in glutamate release via the close functional interaction between D_4 receptor and NMDA receptors (Wang *et al.,* 2002). Thus, PD168077 may be modulating the glutamatergic systems in the dopamine D_4 receptor mediated model of the PFC circuit *(figure 4.1).*

In summary, the D_4 receptor agonist, PD168077, reverses episodic memory decrement caused by either time or by sub-chronic PCP pre-treatment, suggesting an involvement of D_4 receptor in aspects of memory processing. Further, these results suggest that drugs acting at D_4 receptors may be useful in improving cognitive deficit symptoms of schizophrenia.

Chapter 6: Evaluation of PK/PD parameters of PD168077 (10 mg/kg) dose that possess cognitive enhancing effects: MQ microdialysis in freely moving rats

6.1 Introduction

In any pharmacological response, the key event is the formation of a complex between the ligand (or drug or molecule) and its site of action (Xie and Hammarlund-Udenaes 1998; Xie *et al.,* 1999). *In vitro* competitive binding experiments ascertain how specific the interaction is between a ligand and its binding site by examining the ability of various compounds to compete with a radiolabelled reference probe for the receptor. The more potently a drug binds to the receptor, the more effective it is at competing for the target receptors. The greater the potency a drug has for the receptor, the lower the concentration required before all available receptor sites are occupied or blocked. This affinity (termed K_d or K_i) for the receptor is quantified in *in vitro* experiments and it is empirically measured as the concentration of drug required to block half the total receptor population. The high affinity drugs have low K_d values: these drugs are better at occupying receptors. In living animals, including humans, receptor occupancy by drugs is also determined by the concentration of drug at the receptor and the concentration of endogenous neurotransmitter at the receptor (Strange 2001). These initial pharmacological concepts are important to our understanding of antipsychotic drug action, in particular the dopamine hypotheses of antipsychotic drug action, a lead candidate for antipsychotic drug discovery and relevant to the modern clinical management of schizophrenia (Goldman-Rakic 1995; Goldman-Rakic *et al.,* 2004). In this chapter I shall discuss the contribution of MQ microdialysis (as described in this thesis) in studying the drug action (in terms of dopamine release) of PD168077 in relevance, and attempt to synthesise a potent drug therapy involving D_4 receptor activity in clinical management of cognitive deficiency in schizophrenia.

Microdialysis can contribute greatly to drug development by improving the predictability of clinical outcomes from preclinical studies for a new medical entity (Hammarlund-Udenaes *et al.,* 2008). This can be achieved by better knowledge of the effect of a drug candidate at the site of action, with microdialysis sampling, that would greatly facilitate and improve selection of the best compound and optimal doses for subsequent clinical studies. By employing a quantitative microdialysis method (MQ microdialysis as described in this thesis) it is now possible to determine PK parameters, by attaining absolute *in vivo* concentration of compounds in the brain ecf space. In particular microdialysis can be employed to quantify the dynamic changes of drug levels in brain ecf (Beagles *et al.,* 1998; Hammarlund-Udenaes *et al.,* 2009), since one can measure free ecf concentrations of compounds at both sides of BBB. It can be used to assess transport across BBB which may be of interest as factor that determines drug distribution characteristics into the brain from the blood compartment (Hammarlund-Udenaes *et al.,* 1997; Wang and Welty 1996). Thus it also opened up the possibility of investigating drug equilibration across the BBB, by examining microdialysis profiles for drugs concentration in the brain ecf in relation to their profiles in plasma (Hammarlund-Udenaes *et al.*, 1997; *figure 6.1 a and b).*

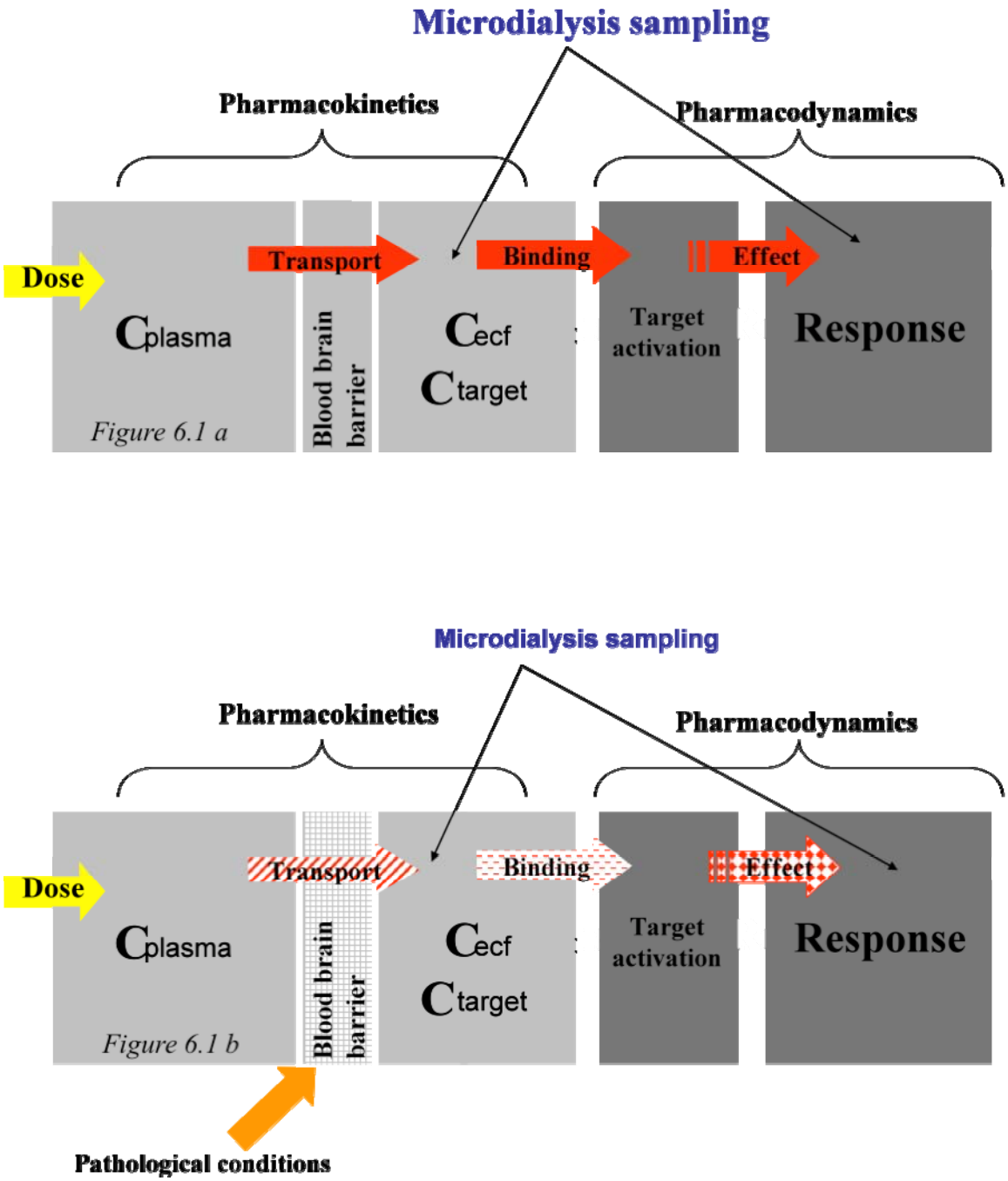

Figure 6.1: Illustration showing the interplay between the dose, and the various mechanisms that influence the delivery of a compound to its site of action, in (a) physiological or (b) pathological conditions. The resulting targeted bioavailability (Cecf) generates the observed pharmacological response (figure from Hammarlund-Udenas et al., 1997).

Since microdialysis is a labour intensive procedure and also requires specialised skills, it is not suitable for high throughput screening of large numbers of compounds in drug development. It is rather used to address specific questions from early in discovery to development (*see table 6.1*). In the early drug-target characterisation phase, microdialysis is frequently used for PK/PD correlation to establish target-response validity and provides an early proof of concept of response in vivo. As in table 6.1, in development of CNS targets, 4 stages or tiers are involved in novel drug screening. The tier 1 screening involves high throughput *in vitro* assays and *in silico* tools may be used at the onset of the lead optimisation phase to rapidly select potential candidates from a large numbers of compounds with the desired pharmacological profile for the disease target. The molecules that pass these hurdles may advance to the tier 2 screening, which generally involves *in vivo* studies. A typical tier 2 screen includes harvesting the brain to obtain the homogenate and to normalize whole brain levels to plasma levels. These data should be used with caution as they provide information on the levels that cross the BBB, but not on the free fraction of drug that is the efficacious level available to the target of interest, especially if the molecule is highly bound to brain tissue. The measurement of cerebrospinal fluid drug levels is commonly used as a surrogate for free fractions in the brain: however, this is only relevant if transporters do not play a major role in the blood brain exchange, and it is well known that there is a diverse expression and function of transporters on the BBB and the blood-CSF barrier which is not accounted for in csf measurement. While at this stage, microdialysis studies help profiling novel candidates by providing valuable information of ecf concentration to target receptor occupancy, if a molecule possesses desirable PK properties, in PK/PD studies. Moreover, transgenic or knockout models can be used to elucidate mechanisms of transport of CNS drugs across the BBB, but the data should be used with caution as compensatory pathways that do not exist in wild type animals may confound results from transgenic models. Also microdialysis in wild type animals using specific inhibitors and substrates of transporters is a

valuable alternative approach (e.g., Xie *et al.,* 2000). At tiers 3 and 4 of the development process, microdialysis is also frequently used, in particular for CNS targets, to study in detail the *in vivo* pharmacological properties and the mechanisms of action of the drug. Also the changes in PK/PD profile due to pathological conditions can be studied with microdialysis sampling of the plasma PK and biophase (ecf) PK and the resultant response. Finally, clinical observations may raise mechanistic questions, which sometimes can be addressed by preclinical microdialysis studies.

Table 6.1 Tiers (stages) of the CNS Drug Discovery and Development Process**:** (Rollema 2006):

Initial stage: Identification of novel Compounds: In Vitro Potency and Selectivity (in vitro binding affinities)

Tier 1: *In silico* :

Molecular weight, Polar Surface Area, cLogP (Liphophilicity), Permeability, Plasma protein binding

Tier 2: *In vivo:*
CSF and brain homogenate
PD models (microdialysis)

Tier 3

In vivo PK: free drug concentrations at target (microdialysis)
In vivo PD: pharmacology (microdialysis)

Tier 4

Brain-plasma ratio in Knockout mouse models (microdialysis)
Co-administration of specific transport inhibitors(microdialysis)
Pharmacology studies (microdialysis)

Near final stage: Compounds in Development or Marketed: **Special in vivo PK/PD studies (microdialysis)**

In principle the monitoring of brain ecf concentrations by microdialysis provides the opportunity to study the relationship between brain ecf drug concentrations (PK) and effects (PD). The studies by Matos *et al* in 1992 simultaneously measured the drug (the opiate, morphine) as well as the neurotransmitter (serotonin and its metabolites). Moreover, these data were related to observations on behavioural analgesia in order to identify the site of action of morphine. Stain *et al*., in 1995 studied the pharmacokinetics of morphine and its metabolite morphine-6-β-D-glucuronide together with the analgesic response, and could deduce that the metabolite had a higher analgesic potency than morphine.

The development of the quantitative MQ method described in this thesis enables absolute ecf concentrations to be measured, thus making microdialysis a valuable tool in assessing PK/PD profile of novel centrally active drugs, which was not possible with traditional quantitative microdialysis methods (Sood *et al.,* 2009). In this chapter, the pharmacological response (in terms of dopamine release, the PD parameter) and the distribution of PD168077 in the mPFC and NAc (the PK parameter) of PD168077 at the highest dose of 10 mg/kg were quantified using MQ probes. The experiments were carried out in relevance to cognitive enhancing effects of the highest dose of PD168077 in the NOR task (as shown in chapter 5). This experiment will provide useful information on synaptic concentration of dopamine and PD168077 that are crucial for the cognitive effects and the role of D_4 receptors in this study.

D_4 receptors may be critically involved in PFC functioning and neuropsychiatric disorders (Oak *et al*., 2000). Since, elevated D_4 receptors are found in the PFC patients with schizophrenia (Seeman *et al*., 1994 b) and studies show that antipsychotic drugs have high affinities for D_4 receptors (Van Tol *et al*., 1991; Kapur and Remington 2001). In agreement with this D_4 receptor antagonists ameliorate cognition deficits caused by the psychotomimetic drug PCP (Jentsch *et al*., 1997 b; Jentsch and Roth 1999). The experiments with D_4 selective pharmacological agents indicate that the receptor plays an inhibitory role on the

glutamatergic activity in the frontal cortex (Rubinstein *et al*., 2001). In addition, experiments in cellular physiology have shown that D_4 receptors play an important role in the control of excitability of pyramidal cortical neurons involving glutamatergic signalling and GABAergic signalling (Wang *et al*., 2002). Moreover mice lacking D_4 receptors exhibit super-sensitivity to psychomotor stimulants (Rubinstein *et al.,* 1997) and reduced exploration of novel stimuli (Dulawa *et al*., 1999; Harvey and Keefe 2001). This suggests that D_4 receptors are involved in PFC mediated functions and may be a useful target for developing novel antipsychotic drugs.

My previous results show that PD160877 a D_4 selective agonist, increased dopamine levels in the mPFC (see chapter 4) and enhanced novel object exploration in the NOR task in control and sub-chronic PCP pre-treated animals (see chapter 5). However the mechanism by which D_4 agonist reverses these cognitive deficits is unknown. In addition, little is known about the long-term effects of PCP on dopamine release. Jentsch *et al.,* in 1999 showed that D_4 receptor blockade increases ecf HVA (metabolite of dopamine) levels perhaps indicating enhanced dopaminergic transmission. Therefore, in the present study, we have utilized MQ microdialysis in freely moving rats to measure absolute ecf concentrations of PD168077 and its effect on dopamine release in mPFC and NAc of control and PCP treated rats. Importantly, PCP dosing ceased 7 days prior to testing such that testing was PCP-free and that the effects seen were enduring, long term changes induced by the chronic treatment rather than direct, short term PCP effect (e.g., Adams and Moghaddam 1998).

6.1.1 Aims

Experiment 1: To measure the brain distribution of PD168077 (PK parameters) and dopamine dynamics (PD parameters) before and after administration of PD168077 (10 mg/kg dose) in control rats.

Experiment 2: To measure the brain distribution of PD168077 (PK parameters) and dopamine dynamics after administration of PD168077 (10 mg/kg dose) in sub-chronic PCP pre-treated rats.

6.1 Materials and methods

6.2.1 Animals, housing, surgery and experimental procedure

For the MQ microdialysis experiments described here, female Lister Hooded rats were used to keep the strain and sex of animals consistent with the NOR task experiments as described in chapter 5. Female Lister Hooded rats (250 ±30 g; Harlan) were housed in groups of two in a temperature and humidity controlled environment and under regular lighting conditions (12 hour light/dark cycle) with food and water available *ad libitum*. Surgery was performed 7-10 days after arrival. The animals were implanted with guide cannulae aimed at mPFC and NAc, as described in chapter 4. After 7 days of recovery sub chronic pre-treatment of PCP (2 mg/kg, s.c. twice daily for 7 days) was carried out as described in chapter 5. On the day of experiment, MQ probes were inserted into the guide cannulae to lie in mPFC and NAc respectively, as described in chapter 4. The experimental time line is summarised in *figure 6.2*. The dialysate contents for dopamine, DOPAC and PD168077 was analysed by HPLC and mass spectrometry respectively as described in chapter 4.

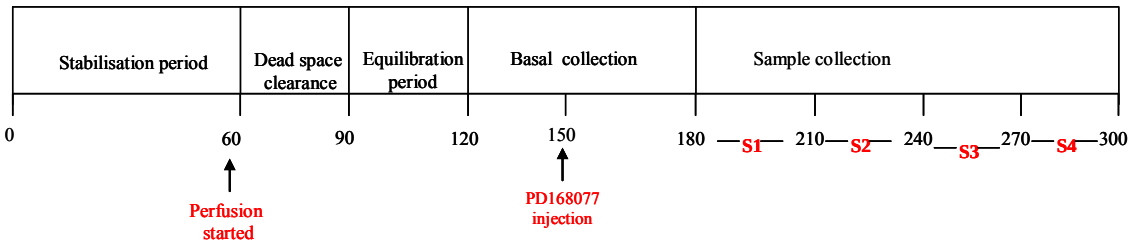

Figure 6.2: Experimental plan and sampling timeline.

6.2.2 Statistical analyses

Two-way ANOVA was used to evaluate differences between concentrations of PD168077 and dopamine measured over the duration of the experiment in the two brain areas. Results showing significant overall changes were subjected to Dunnett's post hoc test in order to identify the samples which differed significantly from the basal levels. All statistical tests were carried out by using Graphpad Prism v5. Data are presented as mean ± SEM.

6.3 Results

6.3.1 Experiment 1: Systemic administration of PD168077 (10 mg/kg) in the control rats

Sub cutaneous administration of PD168077 (10 mg/kg) caused an increase in the ecf concentration of the drug in both mPFC and NAc, which reached a maximum in the sample immediately following the drug injection in each case (in the samples labelled as S1 in the *figure 6.3*). Two-way ANOVA revealed a significant main effect of sample ($F[4, 64] = 58.2$, $p < 0.001$), indicating significant increases in the drug levels over the duration of the experiment, and also there was significant main effect of brain region ($F[1, 64] = 13.3$), $p = 0.002$). However, there was no sample x brain region interaction ($F[4, 64] = 0.8$), $p = 0.4$)

indicating that the concentrations of the drug was similar in both brain regions across the duration of the experiment. Post hoc analysis using Dunnett's test showed significant increases in drug concentrations in mPFC in the samples (S1 to S4) and in NAc in the samples (S1 to S3) immediately following injection of the dose of PD168077 (10 mg/kg; *figure 6.3*).

The maximum absolute ecf concentrations of PD168077 measured in mPFC and NAc were 2371.8 ±168.3 nM and 2491.8 ±573.8 nM respectively, which were not statistically different from each other ($t(10) = 0.2$, $p = 0.8$; paired t-test).

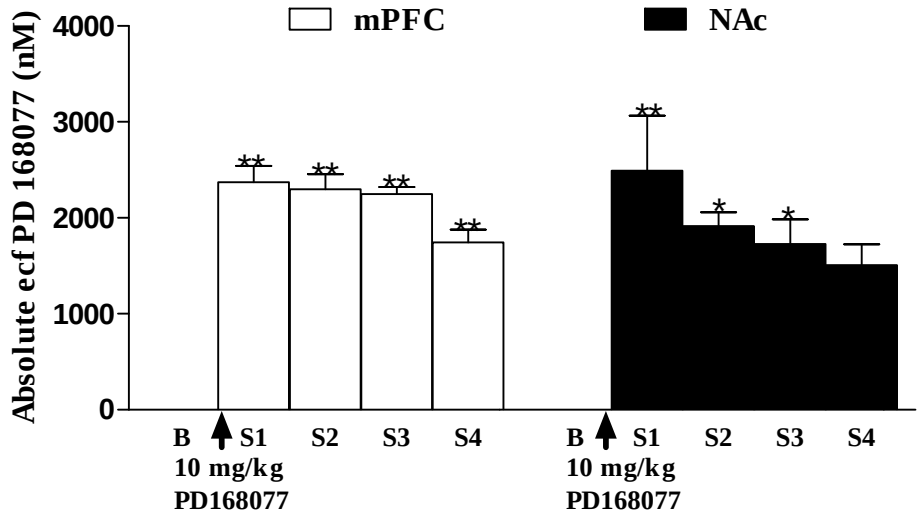

Figure 6.3: The absolute ecf concentration of PD168077 (nM) in mPFC and NAc after the s.c. dose of 10 mg/kg PD168077 (n=9).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10 mg/kg; s.c. injection) was administered as indicated by the arrows, and four consecutive 30 min samples were collected (S1, S2, S3 and S4). For clarity samples following different brain regions are shaded differently. * $p < 0.05$, ** $p < 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

In the same experiment, the absolute ecf levels of dopamine were also measured. The absolute basal levels of dopamine measured in the mPFC and NAc were 8.2 ±0.5 nM and

25.4 ±2.1 nM, respectively, a difference which was statistically significant ($t(10) = 10.97$, $p < 0.001$; paired t-test). PD168077 evoked an increase in ecf levels of dopamine in both brain regions. Two-way ANOVA revealed a significant main effect of sample ($F[4, 64] = 395.2$, $p < 0.001$; *figure 6.4*) indicating a change in dopamine over the course of the experiment and a significant main effect of brain region ($F[1, 64] = 909.4$, $p < 0.001$), reflecting the difference in overall levels of dopamine in the two brain regions. Also there was a significant sample x brain region interaction ($F[4, 64] = 51.3$, $p < 0.001$; *figure 6.4)* indicating that the response profiles in the two brain regions were different. Post hoc analysis (Dunnett's test) showed a significant elevation of dopamine (to 38.0 ±2.1 nM) in the mPFC and (to 83.4 ±2.2) in NAc in sample S1 after the dose *(figure 6.4)*.

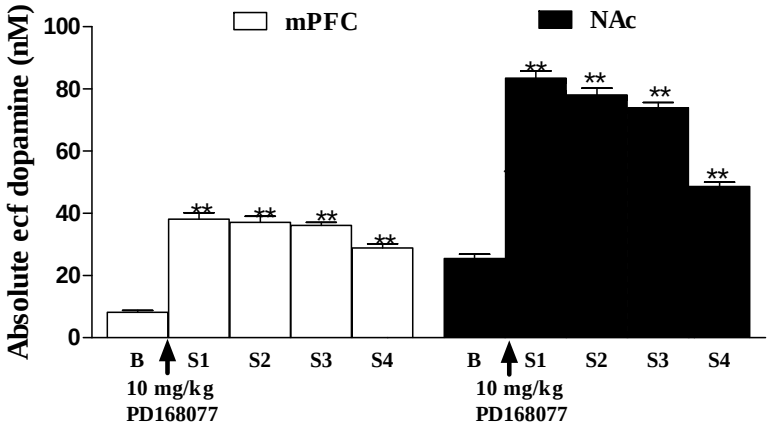

Figure 6.4: The absolute ecf concentration (nM) of dopamine in mPFC and NAc (n=9).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10 mg/kg; s.c. injection) was administered as indicated by the arrows, and four consecutive 30 min samples were collected (S1, S2, S3 and S4). For clarity samples following different brain regions are shaded differently. ** $p < 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

In the same experiment, the absolute ecf levels of DOPAC were also measured. DOPAC levels showed a similar pattern to the dopamine levels in response to the PD168077 challenge. The absolute basal levels of DOPAC measured in the mPFC and NAc were 330.8 ±14 nM and 1143.1 ±42.2 nM, respectively, a difference which was statistically significant ($t(10) = 16.8$, $p < 0.001$; paired t-test). Two-way ANOVA revealed a significant main effect of sample ($F[4, 64] = 292.2$; $p < 0.001$; *figure 6.5*) indicating a change in DOPAC over the course of the experiment and a significant main effect of brain region ($F[1, 64] = 599.4$, $p < 0.001$), reflecting the difference in overall levels of DOPAC in the two brain regions. Also there was a significant sample x brain region interaction ($F[4, 64] = 232$; $p < 0.001$; *figure 6.5)* indicating that the response profiles in the two brain regions were different. Post hoc analysis (Dunnett's test) showed a significant elevation of DOPAC (to 1212.4 ±25.4 nM) in the mPFC and (to 3286.1 ±72.1 nM) in the NAc in sample S1 after the dose *(figure 6.5)*.

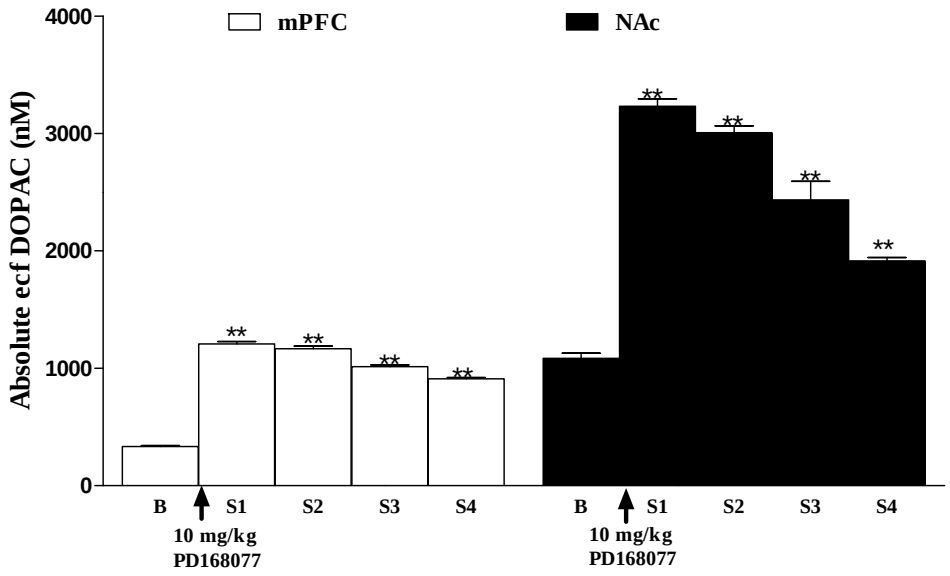

Figure 6.5: The absolute ecf DOPAC concentrations (nM) in mPFC and NAc (n=9).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10 mg/kg; s.c. injection) was then administered as indicated by the arrows and four consecutive 30 min samples were collected (S1, S2, S3 and S4) were collected for 10 mg/kg s.c. dose of PD168077. For clarity samples following different brain regions are shaded differently. ** $p< 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

6.3.2 Experiment 2: Systemic administration of PD168077 (10 mg/kg) in sub-chronic PCP pre-treated rats

Sub cutaneous administration of PD168077 (10 mg/kg) caused an increase in the ecf concentration of the drug in both mPFC and NAc, which reached a maximum in the sample immediately following the drug injection in each case (in the samples labelled as S1 in the *figure 6.6*). Two-way ANOVA revealed a significant main effect of sample ($F[4,56] = 57.9$, $p <0.001$), indicating significant increases in the drug levels over the duration of the experiment. However there was no significant main effect of brain region ($F[1, 56] = 0.6$; $p = 0.4$) and there was no sample x brain region interaction ($F[4, 56] = 0.6$, $p = 0.6$) indicating that the concentrations of the drug was similar in both brain regions across the duration of the

experiment. Post hoc analysis using Dunnett's test showed significant increases in drug concentrations in mPFC and in NAc in the samples (S1 to S4) immediately following injection of the dose of PD168077 (10 mg/kg; *figure 6.6*). The maximum absolute ecf concentrations of PD168077 measured in mPFC and NAc were 2384.6 ±143.2 nM and 2231.8 ±373.6 nM respectively, which were not statistically different from each other ($t(10) = 0.3$, $p = 0.7$; paired t-test).

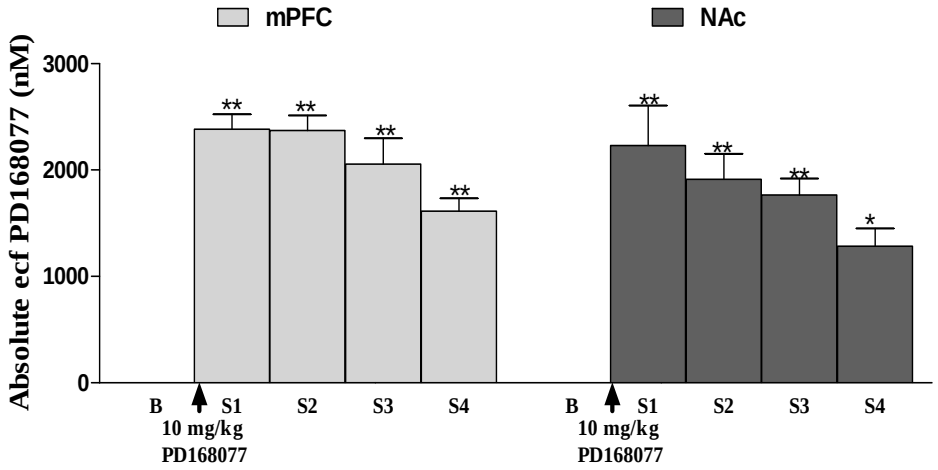

Figure 6.6: The absolute ecf concentration of PD168077 (nM) in mPFC and NAc in sub-chronic PCP pre-treated rats after the s.c. dose of 10 mg/kg PD168077 (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10 mg/kg; s.c. injection) was administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes), and four consecutive 30 min samples were collected (S1, S2, S3 and S4). For clarity samples following different brain regions are shaded differently. * $p < 0.05$, ** $p < 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

In the same experiment, the absolute ecf levels of dopamine were also measured. The absolute basal levels of dopamine measured in the mPFC and NAc were 3.8 ±0.2 nM and 19.3 ±1.2 nM, respectively, a difference which was statistically significant ($t(10) = 12.5$, $p <$

0.001; paired t-test). PD168077 evoked an increase in ecf levels of dopamine in both brain regions. Two-way ANOVA revealed a significant main effect of sample ($F[4, 56] = 40.9$, $p < 0.001$; *figure 6.7*) indicating a change in dopamine over the course of the experiment and a significant main effect of brain region ($F[1, 56] = 284.7$, $p < 0.001$) reflecting the difference in overall levels of dopamine in the two brain regions. Also there was a significant sample x brain region interaction ($F[4, 56] = 7.4$, $p < 0.001$; *figure 6.7)*indicating that the response profiles in the two brain regions were different. Post hoc analysis (Dunnett's test) showed a significant elevation of dopamine (to 10.2 ±1.1 nM) in the mPFC and (to 34.5 ±1.9 nM) in NAc in sample S1 after the dose *(figure 6.7)*.

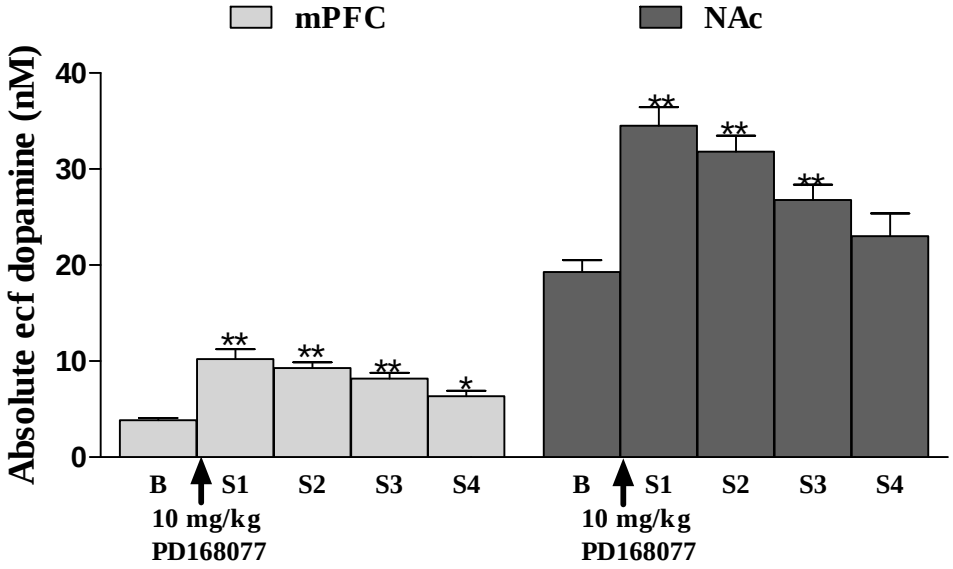

Figure 6.7: The absolute ecf concentration (nM) of dopamine in mPFC and NAc in sub-chronic PCP pre-treated rats after the s.c. dose of 10 mg/kg PD168077 (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10mg/kg; s.c. injection) was administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes), and four consecutive 30 min samples were collected (S1, S2, S3 and S4). For clarity samples following different brain regions are shaded differently. * $p < 0.05$, ** $p < 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

In the same experiment, the absolute ecf levels of DOPAC were also measured. DOPAC levels showed a similar pattern to the dopamine levels in response to the PD168077 challenge. The absolute basal levels of DOPAC measured in the mPFC and NAc were 164.8 ±9 nM and 872.2 ±15.5 nM, respectively, a difference which was statistically significant ($t(10) = 52.6$, $p < 0.001$; paired t-test). Two-way ANOVA revealed a significant main effect of sample ($F[4, 56] = 29.3$, $p < 0.001$; *figure 6.8*) indicating a change in DOPAC over the course of the experiment and a significant main effect of brain region ($F[1, 56] = 464.7$, $p < 0.001$), reflecting the difference in overall levels of DOPAC in the two brain regions. Also there was a significant sample x brain region interaction indicating that the response profiles in the two brain regions were different ($F[4, 56] = 252.0$, $p < 0.001$; *figure 6.8)*. Post hoc analysis (Dunnett's test) showed a significant elevation of DOPAC (to 317.1 ±5.5 nM) in the mPFC and (to 996.5 ±2.0 nM) in the NAc in sample S1 after the dose *(figure 6.8)*.

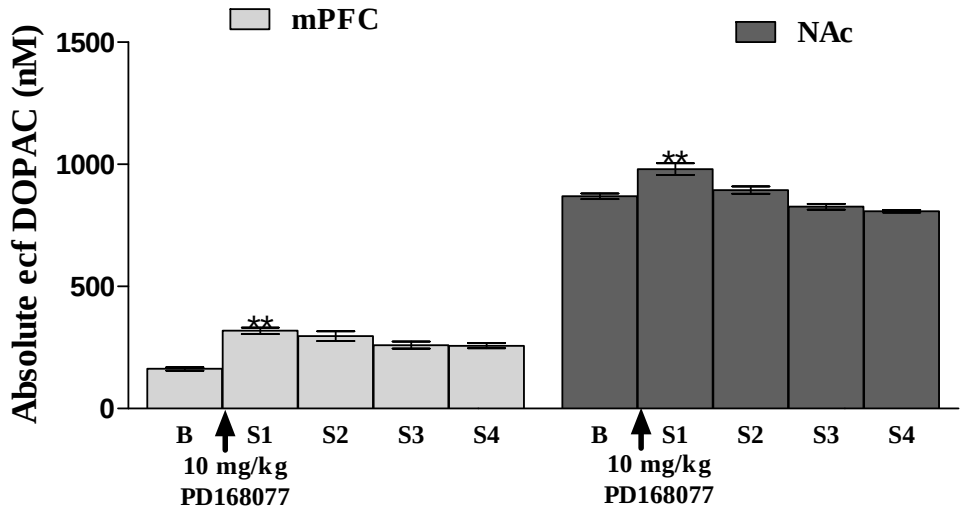

Figure 6:8: The absolute ecf DOPAC concentrations (nM) in mPFC and NAc in sub- chronic PCP pre-treated rats after the s.c. dose of 10 mg/kg PD168077 (n=8).

Consecutive 30 min dialysate samples were collected from mPFC and NAc over the duration of the experiment. After 60 min basal collection (B: mean of two samples), PD168077 (10 mg/kg; s.c. injection) was then administered as indicated by the arrows (the timings shown take into account the dead time of the outlet tubes), and four consecutive 30 min samples were collected (S1, S2, S3 and S4). For clarity samples following different brain regions are shaded differently. ** $p < 0.01$ indicate samples which were significantly different from the basal levels (Dunnett's post hoc test).

Table 6.2: Summary of the results from experiment 1 and experiment 2:

Control rats (experiment 1)

Dose (mg/kg)	Samples	mPFC PD168077 concentration (nM) (average, n= 9)	Dopamine (nM) (average, n= 9)	Dopamine release (% of basal)
10.00	B	0	8.2	100
10.00	S1	2371.8	38.0	464.6
10.00	S2	2297.2	37.1	452.8
10.00	S3	2248.6	36.0	440.2
10.00	S4	1746.6	28.8	351.9

Sub-chronic PCP pre-treated rats (experiment 2)

Dose (mg/kg)	Samples	mPFC PD168077 concentration (nM) (average, n= 8)	Dopamine (nM) (average, n= 8)	Dopamine release (% of basal)
10.00	B	0	3.8	100
10.00	S1	2384.6	10.2	266.4
10.00	S2	2370.3	9.2	242.1
10.00	S3	2057.2	8.2	213.7
10.00	S4	1614.4	6.3	165.9

Control rats (experiment 1)

Dose (mg/kg)	Samples	NAc PD168077 concentration (nM) (average, n= 9)	Dopamine (nM) (average, n= 9)	Dopamine release (% of basal)
10.00	B	0	25.4	100
10.00	S1	2491.8	83.4	328.1
10.00	S2	1915.9	78.0	306.6
10.00	S3	1728.8	73.9	290.8
10.00	S4	1509.7	48.6	191.2

Sub-chronic PCP pre-treated rats (experiment 2)

Dose (mg/kg)	Samples	NAc PD168077 concentration (nM) (average, n= 8)	Dopamine (nM) (average, n= 8)	Dopamine release (% of basal)
10.00	B	0	19.3	100
10.00	S1	2231.8	34.5	178.8
10.00	S2	1914.2	31.8	164.9
10.00	S3	1765.0	26.7	138.7
10.00	S4	1284.7	23.0	119.3

6.3.2 Comparison of absolute ecf concentrations of PD168077 (nM) and dopamine (nM) in the mPFC in the control rats and the sub-chronic PCP pre-treated rats

The absolute ecf concentration of PD168077 in the mPFC was not statistically different in the control rats and the sub-chronic PCP pre-treated ($t(8) = 0.07$, $p = 0.9$; paired t-test); indicating that sub-chronic PCP pre-treatment did not alter the distribution of PD168077 in the mPFC. However, the absolute ecf concentration of basal dopamine was significantly reduced in the sub-chronic PCP pre-treated rats ($t(10) = 6.8$, $p < 0.001$; paired t-test); indicating that sub-chronic PCP pre-treatment significantly reduced the basal dopamine levels in the mPFC.

6.3.2 Comparison of absolute ecf concentrations of PD168077 (nM) and dopamine (nM) in the NAc in the control rats and the sub-chronic PCP pre-treated rats

The absolute ecf concentration of PD168077 in the NAc was not statistically different in the control rats and the sub-chronic PCP pre-treated ($t(8) = 0.1$, $p = 0.8$; paired t-test); indicating that sub-chronic PCP pre-treatment did not alter the distribution of PD168077 in the NAc. However, the absolute ecf concentration of basal dopamine was significantly reduced in the sub-chronic PCP pre-treated rats ($t(10) = 3.2$, $p < 0.001$; paired t-test); indicating that sub-chronic PCP pre-treatment significantly reduced the basal dopamine levels in the NAc.

6.4 Discussion

One of the main objectives of this study was to evaluate the distribution of PD168077 in two brain areas, mPFC and NAc, after 10 mg/kg s.c. injection. The distribution was measured in control rats and rats sub chronically pre-treated with PCP. The results show that the overall absolute ecf concentrations of PD168077 were significantly different in these two brain regions *(figure 6.3)*. In control rats, administration of PD168077, 10 mg/kg s.c. injection resulted in increased dopamine levels in both mPFC and NAc compared to basal levels. Again, the PD168077 induced increases in dopamine levels were higher in mPFC: 464.6% of basal levels than NAc: 328.1% of basal levels (*table 6.2).* This predominant effect of PD168077 in mPFC is again is due to attribution of higher D_4 receptor density in the mPFC compared to NAc. Moreover, the increase in the dopamine levels was sustained until the end of the experiments, 120 min after drug administration. Also a sustained increase in DOPAC levels in mPFC and NAc was seen concomitant with the increase in dopamine release. The increase in DOPAC suggests that the increase in dopamine is a result of stimulated release rather than mechanisms that affect the uptake and breakdown of dopamine as seen with drugs such as amphetamine (see chapter 3). These data strongly suggest that PD168077 at high dose of 10 mg/kg, stimulates dopamine synthesis and or release of dopamine into the synaptic cleft. The increase in dopamine levels induced by PD168077 may have implications in physiological functions such as in cognition and memory (see chapter 5). The release of dopamine is also a characteristic of many antipsychotic drug actions. Most antipsychotic drugs exert their therapeutic effect by acting on the central dopaminergic neurons and biochemical findings indicate that antipsychotic drugs alter the turnover and release of dopamine by blocking central dopamine receptors, as discussed below (Carlsson 1963; Seeman and Lee 1975). Antipsychotic drugs not only exert different effect on the different dopamine pathways but also have differential effects at different doses at the dopaminergic

neurones. This aspect of antipsychotic drugs haloperidol and (-)-sulpiride (both dopamine D_2-like receptor blockers) and clozapine (more selective at D_4 receptors) was studied using microdialysis by Moghaddam and colleagues (Moghaddam and Bunney 1990 a and b; Moghaddam *et al.,* 1990). Their studies revealed that haloperidol (0.1 mg/kg., i.v.) elevated the ecf dopamine level in caudate putamen, 40 min after injection and elevated ecf dopamine in the NAc shell, 60 min after the injection, whereas it had no effect on mPFC ecf dopamine levels. Interestingly at a higher dose of 0.5 mg/kg, haloperidol enhanced the outflow of dopamine in all three regions. (-)-Sulpiride at 20, 50, 100 mg/kg increased the ecf level in dopamine in the caudate putamen. On the other hand, at a dose of 20 mg/kg sulpiride did not increase dopamine levels in NAc shell whereas the higher dose of 50 mg/kg was effective in inducing dopamine outflow in NAc shell. Moreover, even at the very high dose of 100 mg/kg, sulpiride had no effect on dopamine outflow in mPFC. In contrast, clozapine at 5 mg/kg increased dopamine outflow in all three brain areas: caudate putamen, NAc and mPFC (Imperato and Angelucci 1989). Also there was a trend for variations in the duration of effect in the following order: mPFC > NAc > caudate putamen. The main conclusion is that the dopamine innervations of the mPFC may respond differently to acute typical (haloperidol and sulpiride) or atypical (clozapine) antipsychotic treatment from that of the NAc and caudate putamen. The result, however, demonstrated that all antipsychotic drugs increase ecf dopamine levels (Gessa *et al.,* 2000). In general the dopamine release enhancing properties of antipsychotic drugs are thought to be mediated by two mechanisms: (1) activation of a neuronal feedback loop on blockade of dopamine postsynaptic receptors and/or (2) increase of synthesis and release by interaction with the presynaptic receptor on the terminals on the dopamine neurons (Carlsson and Lindqvist 1963; Bunney *et al*., 1973; Gessa *et al.,* 2000).

In experiment 2, the distribution of PD168077 and its effect on dopamine dynamics in mPFC and NAc, were studied in PCP pre-treated rats. The difference in distribution of PD168077

between brain regions (mPFC and NAc) which was seen in control rats *(figure 6.3)* was abolished in sub chronic PCP pre-treated rats *(figure 6.6)*. There is evidence that the neuroanatomical changes that occur after exposure to PCP have impact on brain penetration and distribution of exogenous compounds in the brain. There is a reduction in overall blood flow and glucose utilisation in the brain after PCP administration (Hertzmann *et al.*, 1990). In addition, PCP toxicity has also been associated with signs of apoptosis (programmed cell death) as well as necrosis (Fix *et al.*, 1993). Further evidence suggests that PCP induced neuronal injury can be blocked with antipsychotic drugs such as clozapine and haloperidol (Sharp *et al*., 1994).

The acute effects of PCP on dopamine release are well studied and evidence show that acute PCP administration induces stimulation of dopamine release within PFC and NAc (Adams and Moghaddam 1998; Adams *et al*., 2002). However, the duration over which these elevated levels remain sustained during continuous, sub chronic treatment, remains unknown. However, Balla *et al* in 2001 using microdialysis showed no significant sustained elevations in basal dopamine release following either 3-day or 2-week PCP treatment at any of the doses tested (5, 10, 15 and 20 mg/kg/day) in either mPFC or NAc. It appears that tolerance is reached to the acute dopamine releasing effects of PCP within a 3 day period. Interestingly, I found that in sub-chronic PCP pre-treated rats, the basal levels of dopamine were reduced in both mPFC and NAc compared to non PCP pre-treated rats. These data suggest that sub-chronic treatment (2 mg/kg dose twice a day for 7 days, followed by 7 days wash out) has long term effects on dopaminergic systems. In line with these findings, other microdialysis study showed that sub-chronic exposure (5 mg/kg dose for 7 days followed by 7 days wash out) reduced basal ecf levels of dopamine in the mPFC (Jentsch *et al*., 1998 a), in line with my findings.

My study extended the previous studies by showing for the first time that the reduction in the basal dopamine levels in mPFC was more pronounced than the reduction in the NAc (53% in mPFC and 24% in NAc). However, the studies in the literature have widely stated that the sub-chronic (repeated or intermittent) treatment of PCP to rats can lead in PCP induced cognitive impairments associated with frontal cortical dysfunction (Jentsch 1997; Jentsch *et al.,* 1998 b). The studies by Jentsch *et al.,* 1998 b have shown that sub-chronic exposure of PCP had a prolonged effect of hypoactivity of mesocortical dopamine neurones. My finding of reduced basal levels of dopamine in the mPFC adds to the dopaminergic dysfunction effects of PCP predominantly in frontal cortical dysfunction and than in the subcortical region (NAc) after sub-chronic PCP administration. Thus, this is analogous to PCP induced cognitive and dopamine deficits in mPFC.

Furthermore, from my study, the administration of 10 mg/kg PD168077 induced increases in dopamine levels in mPFC and NAc in sub-chronic PCP pre-treated rats. The increases in dopamine levels were 266% of basal in mPFC and 178% of basal in NAc in these rats *(table 6.2).* From the current results, I suggest that PD168077 enhances the reduced basal dopamine levels in the PCP pre-treated rats, and this may contribute to the cognitive enhancing effect in NOR task (see chapter 5). Also, another important finding of this study is that PD168077 not only induced an increase in basal levels of the dopamine after its acute administration, it also increased DOPAC levels in the mPFC which were also reduced in the sub-chronic PCP pre-treated animals. This suggests that PD168077 repairs the reduced utilisation of the dopamine in sub-chronic PCP animals, which is essential for cognitive functioning of the mPFC. It has been hypothesized that decreased dopaminergic neurotransmission in the PFC contributes to the cognitive impairment and that excessive prefrontal dopamine may be deleterious to cognition (Goldman-Rakic 1998; Goldman-Rakic *et al.,* 2004). Therefore it seems that precise regulation of prefrontal dopaminergic tone is essential. It has been suggested that

dopamine function in the PFC follows an inverted-U shaped dose response curve whereby increases or decreases from an optimal level result in cognitive impairment (Goldman-Rakic 1998). I suggest from the current data that PD168077 establishes the dopamine level needed for a normal cognitive function in the PFC in the sub-chronic PCP pre-treated rats. At acute dose of 10 mg/kg, PD168077 induces dopamine increase in the sub-chronic PCP pre-treated rats where the basal dopamine levels are reduced and brings the dopamine levels back to normal levels.

Wang *et al.,* in 2003 reported a mechanistic link between D_4 receptor and NMDA receptors in the prefrontal pyramidal neurons *(figure 4.1)* and results showed that PD168077 modulated NMDA receptor function by Ca^{2+}- calmodulin – dependent kinase II (CaMKII) and their results show that PD168077 reversibly decreased NMDA receptors induced currents and also reduced surface expression of NMDA receptors effects which were blocked by D_4 receptor antagonists in cultured cells. However, further studies by Gu and Yan in 2004 showed that PD168077 has a bidirectional regulation of Ca^{2+}/calmodulin- dependent protein kinase II activity in PFC. In PFC slices with high neuronal activity PD168077 produced potent reduction of CaMKII activity whereas in PFC slices with low neuronal activity, PD168077 caused remarked increase of the CaMKII activity by acting at D_4 receptors. Thus here I suggest that PD168077 increases NMDA receptors function in the PFC by increasing dopamine levels that were depleted due to PCP. PD168077 has a modulatory effect on NMDA receptors *in vivo*, thus alleviating PCP caused cognitive deficits (see proposed model; *figure 4.1*).

The other main goal here was to predict CNS drug effects in terms of time course, following a single dose regimen under normal physiological and pathological condition (*figure 6.1 a and b*). In general, for PK/PD modelling, only plasma PK data are used. However for CNS

drugs, biophase PK may differ significantly from the plasma PK, because BBB transport and brain distribution often do not occur instantaneously and to a full extent. Therefore the ecf concentration of PD168077 obtained from MQ probe measurements gave direct brain distribution of PD168077 that determined its effect on dopamine changes. Moreover, these data also showed that under pathological conditions as modelled in PCP pre-treated rats, the distribution of PD168077 within the brain (between mPFC and NAc; *figure 6.3*) was similar whereas in the control rats, the distribution of PD168077 was different between mPFC and NAc *(figure 6.6)*. However, the absolute ecf concentrations of PD168077 within individual brain regions (mPFC or NAc), was not altered due to PCP (section 6.3.3 and 6.3.4). The added value of using microdialysis in PK/PD research is that there is an opportunity for direct measurement of drug concentrations close to the site of action and its PD effects, thus furthering our understanding of relationships at local target site. It allows measurement of unbound drug at sites that have not previously been possible to measure such as local sites of brain. Further drug transport processes can be separated from PD processes; pathological influences on drug distribution or PD biomarkers can be mapped and sites of drug action can be distinguished after drug delivery to different locations. There is much to gain from this approach for example, in early drug development, the distribution and PD properties of new drug candidates can be distinguished. This is especially valuable in brain research where it is essential either to obtain the best possible drug candidates for brain penetration or CNS drug action. Moreover, MQ adds value by quantifying both drug and neurotransmitter concentrations simultaneously obtained from the same animals. Furthermore, MQ probes can provide numerous data points from a relatively small number of individual animals to determine the PK/PD profile with less variability in the samples in a dynamic system.

Chapter 7: General Discussion

7.1 MQ probes: A novel approach to measure absolute ecf concentrations of exogenous and endogenous compounds in a dynamic system to yield PK/PD parameters

This chapter discusses the main findings of the current project in utilising microdialysis in evaluation of PK/PD profile of a novel drug and thus contributing to select high quality potential novel compounds for drug development. Microdialysis data are likely to become an important part of new drug submissions, and thus may potentially contribute to the FDA Critical Path Initiative to facilitate innovation in drug development. Recent calculations, which take into account the costs to discover, develop, and launch a single new drug, that is, the investment per successful compound, is currently nearly US$ 1.7 billion, a more than 50% increase over the estimated average costs over the 1995-2000 period (DiMasi *et al*., 2003). One of the major challenges facing the pharmaceutical industry is thus to analyse why attrition occurs and how to reduce attrition to an acceptable rate. Early identification of compounds or chemical series that are likely to have good biopharmaceutical properties has thus a significant impact on the attrition rate, since this selection process determines the potential success of a drug for a larger part (Lipinski 2001). With the regard to its impact on the three main attrition factors, that is, toxicity, poor bioavailability, and lack of efficacy, microdialysis holds greatest potential in the assessment of *in vivo* bioavailability and *in vivo* activity, and thus proof of concept, at any point in the discovery and development process.

The rapid synthesis and characterisation of a large number of compounds are made possible by tremendous progress in combinatorial chemistry, computational methods, and high throughput screening techniques focussing on the identification of highly potent and selective

compounds based on *in vitro* bindings for the target receptors. However increased *in vitro* potencies of the candidates can be offset by poor drug like properties such as high lipophilicity, low solubility, low permeability, and inadequate absorption, which together result in poor bioavailability and unacceptably low in vivo potency, ultimately leading to compound attrition. Therefore, extending predictions beyond *in vitro* bioavailability and especially efficacy estimates by taking into account actual *in vivo* measures early in the discovery stage would obviously greatly facilitate the selection process. An early assessment of actual in vivo activities by including PK or PD properties of representatives of a chemical class can thus be extremely advantageous and cost effective, since compounds with optimal potency, selective, and PK profile will greatly reduce the attrition risk. Microdialysis is a useful tool for the PK profiling of library templates, with the goal of selecting the best chemical series to design around or the candidate to move forward into developmental stage. The concentration of drug obtained from microdialysis is the unbound fraction of the drug in the ecf, which is in equilibrium with biophase membrane, BBB barriers and receptors. This provides the link between *in vivo* PK/PD analysis from microdialysis studies to *in vitro* transport and biology models. As pharmacologically active biophase concentrations and the distribution relationship between blood and biophase can be ascertained by microdialysis in preclinical models, it becomes possible to predict clinical doses designed to achieve therapeutically relevant concentrations in the biophase. This approach may importantly expedite the "whether to proceed" decisions in clinical development and improve the efficiency of the drug development process.

PK is the study of the absorption, distribution, metabolism and excretion of a drug. Out of these four areas, microdialysis sampling of tissues has been most widely used in the determination and understanding of drug distribution and metabolism. One of the earliest applications of microdialysis in PK was to study of drug distribution in the brain. In CNS

drug discovery and development in particular, a key question is whether a compound reaches the intended site of action and at what concentration pharmacological responses occur. There are two advantages of microdialysis for answering these questions. First, the free ecf concentration measured by microdialysis is comparable to the unbound concentration used for *in vitro* transport and efficacy models, thus making the *in vitro-in vivo* correlation with microdialysis more relevant. Second, microdialysis sampling provides the ecf concentrations of exogenous drugs in different regions of the brain which can be measured continuously with its pharmacodynamics response that cannot be done *in vitro*. Since the effect on transmitters is one of the key *in vivo* endpoints in the evaluation of compounds acting on the CNS, intracerebral microdialysis is increasingly used in the pharmaceutical industry for the *in vivo* evaluation of novel CNS drugs, mainly to study the mechanism of action and to estimate *in vivo* potency and selectivity in comparison with pharmacological standards or marketed compounds. A prerequisite for its application is that the BBB transport characteristics will not be significantly influenced by microdialysis probe implantation and presence in the brain. This prerequisite appears to hold based on a series of studies performed to validate the usefulness of intracerebral microdialysis in measurements of passive, as well as, active, BBB transport (e.g., Xie and Hammarlund-Udenaes 1998; Xie *et al*., 1999). The biophase kinetics of a CNS drug is an important determinant in the time course and intensity of its CNS effects. Apart from plasma PK mechanisms that govern CNS biophase kinetics include the rate and extent of BBB transport and the kinetics of processes of distribution and elimination within the brain. BBB transport occurs by passive diffusion as well as active transport. Active transport occurs by many membrane transporters such as the P-glycoprotein and the multi drug resistance associated proteins. BBB functionality is dynamically controlled by blood components and the surrounding brain cells by direct contact or indirectly by their extracellular products. Thus, BBB functionality may vary among different physiological, pathological and chronic drug treatment conditions. It is anticipated that such variations in

BBB functionality will ultimately affect the biophase kinetics of CNS drugs (Brunner *et al.,* 2006).

This current research project tested novel methods to measure absolute/true free concentration of psychoactive drugs to determine the PK/PD profile. In chapter 1 I have highlighted the importance of measuring absolute concentration in the brain and why it is important in PK/PD profiling of novel CNS drugs. For quantitative microdialysis studies on a new compound, it is advisable to perform *in vitro* experiments before animal use, to check for *in vitro* adsorption to tubing and time delays in solute movement, and to compare solute gain and loss, all in order to obtain basic information on the feasibility of the method *in vivo*. The *in vitro* testing of compounds highlighted the main advantages and disadvantages in the use of traditional quantitative microdialysis methods such as varied flow rate method and retrodialysis method, which has been well discussed in literature. The main problem that I have addressed in my initial studies (Stahl *et al.,* 1991; chapter 2 and 3) is the need of a novel quantitative microdialysis method that can eliminate the drawbacks in the traditional methods. In brief, the traditional quantitative methods cannot be used to measure absolute ecf concentrations in a dynamic system; they do not give changes in ecf absolute/true concentrations for a single dose systemic administration. The dynamic NNF method (Olsen and Justice 1993) uses the same principle as NNF method, but instead of serial perfusion of individual animals with different concentrations (C_{in}) via the probe, different groups of animals are treated identically in terms of drug administration while each group receive different perfusion concentration (C_{in}). The results are combined as each point yielding actual C_{ecf} associated at that time point. Although this provides a method for measuring NNF in a dynamic environment, being based on between-subject measurements, it inevitably shows more variability and requires relatively large numbers of animals.

To address this problem, I developed a novel method based on the principle of NNF method. The dual probe NNF method enables measurement of absolute/true ecf concentrations of compounds in a dynamic system: this is achieved by having the dual probes in the same external (brain) concentration as opposed to traditional NNF method were the probe are employed in different experimental subjects and therefore needs a steady state to keep the external concentrations the same. Thus, the dual probe NNF method provides within subject measures of C_{ecf} at each time point in a dynamic system. Another novel method that was validated to measure absolute/true ecf concentration of compounds in a dynamic system was the MQ probes (see introduction for details). This method fulfils all the criteria of an quantitative method to study PK/PD profile of drug of interest: 1) assessment of absolute concentrations; 2) in dynamic (as opposed to steady-state) situation; 3) measurement of brain drug concentrations, and neurotransmitter levels simultaneously, and 4) thus yielded PK/PD characteristics for both experimental drug (amphetamine) and novel CNS agent (PD168077).

My initial studies with amphetamine (chapter 3) in anaesthetised rats validated the use of dual probe NNF in measuring absolute concentration of compounds as the brain ecf concentrations obtained were similar to MQ measurements. These two novel methods based on two different traditional dialysis principles allow measurement absolute/true levels of exogenous compounds in a dynamic system. The aim was not only to measure absolute/true concentrations of exogenous compounds but was also to measure their effects on neurotransmitters systems; I was particularly interested in the dopaminergic systems and in measuring changes in ecf dopamine levels. The dual probe NNF was limited in this aspect as it was not possible to measure the changes in dopamine levels in challenge to amphetamine, as the nature of this method incorporates brain perfusion with drug of interest. This interferes with the measurement of the drugs effect, given systemically. On the other hand, MQ probes not only can measure absolute levels of exogenous compounds but can also measure the

changes in the neurotransmitter of interest at the same time. Moreover, use of dual probe NNF is limited in terms of this poor spatial resolution as dual probes cannot be implanted in smaller brain regions such as mPFC or NAc and only feasible in larger brain regions such as caudate nucleus: even then this relies on the assumptions that the area is homogenous. This limits its application in studies that focus on monitoring regional differences in drug distribution.

My data with MQ probes for the first time gave a single dose-response profile of amphetamine in the caudate nucleus. The brain PK parameter followed similar profile as in the blood PK parameters. Another PK parameter that can be obtained with these data is the half-life of compounds of interest in brain and can be compared to its half-life in the blood. Previous microdialysis studies have revealed that half-lives of several unbound drugs are longer in the brain than in the blood. The half-life of a drug is mostly important as the half-life of a drug in the tissue will have an impact on the timing of drug effect in that tissue, and may explain time delays in PD results compared with results using plasma concentrations. For example, half-lives are longer in the brain ecf than in blood for morphine in rats, pigs, and human (Bouw *et al*., 2000; Bouw *et al*., 2001 a and b). The half-lives of some other drugs studied e.g., AZT (Wong *et al*., 1992) and norfloxacin (Chenel *et al*., 2004) are similar in spite of differing absolute unbound concentrations in brain and blood. This is due to the fact that brain has various efflux systems and the local brain half-life is mainly determined by both the efflux clearance and brain distribution volume (Syvanen *et al*., 2006). My MQ data revealed that amphetamine has similar PK (distribution) in the blood and in the brain. Moreover, the timing of drug effect: PD (response in terms of dopamine release) did not show a delay in compared with blood or brain amphetamine concentrations. MQ probes were then employed to characterise a novel compound: PD168077 a dopamine D_4 receptor agonist in freely moving animals, to evaluate its PK/PD profile.

7.2 Characterisation of PD168077 by using MQ probes

In this present study, I was interested in studying the biophase kinetics of CNS drug using MQ probes. The *in vitro* studies (chapter 2) showed that at very slow flow rate around the MQ dialysis tip the recovery was near 100% for the compounds tested. In my *in vivo* studies, I evaluated the brain distribution (PK) of a novel dopamine D_4 selective compound: PD168077 in two brain regions, mPFC and NAc. In addition to its PK parameter, the PD parameter, in terms of changes seen in dopamine levels were also studied. This chapter discusses the evaluation of the PK/PD profile of PD168077 simultaneously, that was made feasible by measurements obtained by MQ probes (See Chapter 4 and 6). The main objective in using MQ probes was to determine the pharmacologically active unbound concentration of drugs close to the site of action in the rat CNS. In addition to the unbound ecf concentration of PD168077, the absolute/true concentration of dopamine was obtained to evaluate the PD parameters of PD168077 action. The MQ measurements of absolute PD168077 from the respective brain region of interest provides a useful insight into the PK/PD parameters of the novel compound. There are many reasons for studying the free concentrations of drugs in ecf rather than in the blood. First, the time course (drug concentration over time) for the ecf may differ substantially from the time-course in blood; second, the ecf is the biophase for the multitude of drugs acting on cell surface bound receptors and for CNS active drugs, the unbound concentrations in the target tissue will ultimately determine the PD response over time. This is very critical in respective to PK/PD studies of novel compounds.

7.2.1 Role of dopamine D_4 receptor in cognition and in antipsychotic effect

Many pharmaceutics are designed to act in the brain. The true validation of potential drugs requires a comprehensive understanding of the relationship between brain effects and local drug concentrations. With this knowledge, the effective dose to man can be predicted, thus minimising expense in clinical trials and failure in phase II. My laboratory is mainly interested in how neuroactive drugs, especially those affecting the dopamine system, affect neurotransmitter release and behaviour. The characteristics of a novel drug, focussing on modulation of the dopamine function in the NAc, area which is of interest to our group (e.g., Young 1992; Young 1993; Young *et al*., 1998). For this current project I was also interested in the brain region, mPFC and to study the role of dopamine D_4 receptors (sub type of D_2-like receptors) cognitive dysfunction (as in schizophrenia) and the role of dopamine D_4 receptor selective novel compounds in antipsychotic action. This is mainly due to the anatomical localization of the dopamine D_4 receptor in the PFC, which is involved in cognition, compared with little or no expression in the basal ganglia, this also hints that a dopamine D_4 receptor selective drug may possess antipsychotic actions with a low propensity for extrapyramidal side effects.

The dopamine D_2-like receptors (D_2, D_3 and D_4 receptors) have been a primary target of drug development efforts for treating disorders of dopamine neurotransmission such as schizophrenia. The functional effects of dopamine D_2 like receptor antipsychotic ligands in brain arise from their interactions with dopamine D_2 presynaptic receptors expressed on dopamine neurones and with D_2 hetero-receptors on target cells (Lahti *et al.,* 1995). The D_2 and D_3 receptors are characterised as presynaptic (autoreceptors) in their expression however the identification of dopamine D_4 receptors has been more difficult due to the absence of adequate radio-ligands. The mechanism by which dopamine D_4 receptor expression is regulated is unclear and whether they solely mediate tissue specific expression is not yet understood (Okuyama *et al*., 1999 a and b). There is also little known about *in vivo*

regulation of dopamine D_4 receptor density, through desensitization and down-regulation, if at all (khan *et al.,* 1998). The experimental data derived from heterologous expression in human embryonic kidney HEK-293 cells suggests that this receptor type is not strongly regulated through desensitization or internalisation mechanisms (Watts *et al*., 1999). In general, the dopamine D_4 receptor displays a very comparable pharmacological profile to that of the dopamine D_2 and D_3 receptors. The D_2 presynaptic receptors regulate the firing of dopamine neurones, as well as the synthesis and release of dopamine.

My ultimate goal was to develop PK/PD model to characterise and to predict CNS drug responses in both physiological and pathological conditions. There was no difference in distribution of PD168077 between these two regions, for both 0.1 mg/kg and 0.5 mg/kg injection of PD168077. However, there was a regional difference in response to the ecf PD168077 concentration. There was a significant increase in dopamine levels which was only seen in mPFC (see chapter 4) and not in NAc. These data emphasise two main points in terms of PD168077 induced dopamine release at low doses of 0.1 and 0.5 mg/kg: first, even though PD168077 was uniformly distributed in both regions it was only pharmacologically active in the mPFC and second; PD168077 predominantly exerts its pharmacological activity at a high receptor density area such as mPFC rather than NAc.

At a higher dose of 10 mg/kg injection, PD168077 showed similar brain (mPFC and NAc) distribution and induced dopamine release in both control rats and sub-chronic PCP pre-treated rats. There is some indication of the dopamine response reaching a maximum at 2-3 μM brain concentrations of PD168077 but more data at higher concentrations would be required to confirm this. This concentration is much higher than the *in vitro* potency against the D_4 receptor (*in vitro* affinity = 5 nM; Pfizer in house data) but does not reach concentrations required for activity at the D_2 and D_3 receptor (>10 μM; Pfizer in house data). The brain is not a homogenous tissue, because it consists of many anatomical structures with

different compartments such as the brain ecf, the brain intracellular space and the CSF. The brain intracellular distribution is in general more quantitatively more profound for the liphophilic drugs; however drug transport across the BBB is determined both by blood brain barrier characteristics and by the physiochemical properties of the drug. The BBB situated at the brain capillary endothelium is characterised by the presence of narrow tight junctions. Moreover the brain endothelial cells express numerous influx and efflux transporters, such that the drug transport across the BBB occurs through both passive and active transports. The passive diffusion can be either permeability limited or cerebral blood flow limited and depends on the size, charge and lipid solubility of the drug. The permeability limited BBB transport is applicable for more hydrophilic drugs whereas liphophilic drugs easily diffuse transcellularly and for these drugs the cerebral blood flow may become the main determinant in the rate of transport across the BBB. The active transport across the BBB occurs by transport and endocytosis systems (Xie *et al*., 2000). PD168077 is a moderately liphophilic drug and the ecf concentration obtained form 0.1, 0.5 and 10 mg/kg dose suggest that it is transported across the BBB via passive transport. Moreover, the reduced ecf concentrations of PD168077 in the PCP pre-treated rats compared to control rats suggest that the reduced cerebral blood flow induced due to PCP neurotoxicity may have resulted in this change in brain transport of PD168077.

7.2.2 Neurochemical effect of PD168077

Antipsychotic drugs such as haloperidol and *S*- (-) sulpiride increase dopamine release in the striatum and the NAc, most likely due to the blockade of presynaptic dopamine D_2-like autoreceptors (Westerink and DeVries 1989). The atypical antipsychotic clozapine has been reported to produce greater increases in ecf dopamine levels in the mPFC compared with the striatum and the NAc (Moghaddam and Bunney 1990). By contrast, haloperidol has a limited

effect on ecf dopamine levels in the mPFC. Therefore, it is a hypothesis that all antipsychotic drugs of the D_2 receptor type act preferentially in the striatum, and D_4 type act preferentially in the mPFC. In accordance with the hypothesis my microdialysis data showed that, PD168077 reported as a D_4 agonist, increase dopamine levels in the mPFC compared to NAc at low dose of 0.5 mg/kg (chapter 4). Furthermore, it was interesting to investigate antipsychotic effects of PD168077, to characterise its dopamine release effects.

Dopaminergic hypofunction in the PFC has been suggested to be related to the aetiology of cognitive dysfunction of schizophrenia (Sawaguchi and Goldman-Rakic 1991). The relationship between cognitive deficits and schizophrenia was characterised nearly a century ago, however the effective treatments for these deficits have not been developed. Until recently, antipsychotic drugs has been mainly known to reduce the positive symptoms of schizophrenia, (Miyamoto *et al*., 2005) and indeed, all the current medications appear to be similar in efficacy for reducing positive symptoms in schizophrenic patients (Lieberman *et al*., 2005; Meyer *et al*., 2005). Recent meta-analysis also shown that patients treated with typical antipsychotics actually have small but detectable improvements in several cognitive domains. This is however, overshadowed due to extrapyramidal side effects. In addition, there is some evidence for the few atypical antipsychotics, such as olanzapine and risperidone, over typical antipsychotics in improving cognitive performance (Purdon 2000; Purdon *et al*., 2000 a and b). The concern is that the benefits are relatively small and have not been consistently reproduced. The widespread use of the atypical antipsychotics overall has likely offered some cognitive benefit for schizophrenic patients, however, the persistence of significant deficits, suggests a need for directive treatments for enhancing cognition. In addition the MATRICS conference identified pharmacologic strategies that hold promise for the treatment of impaired cognition in schizophrenia. The primary molecular targets identified to have an effect included dopamine receptors in the PFC (Moghaddam and Bunney 1990 a and b; Moghaddam *et al.,* 1990), the glutamatergic excitatory synapse, the GABA system,

nicotinic and muscarinic acetylcholine receptors, and various serotonin receptors (Moghaddam and Bunney 1994).

Studies also suggest that brain dopamine synapses are considered to be overactive in schizophrenics (Seeman 1992 a). This may be due to either an excess release of dopamine or overactivity of dopamine receptors. The evidence for the dopamine hypothesis in schizophrenics relies on findings that antipsychotics block dopamine D_2 receptors in direct relation to their clinical antipsychotic potencies (Seeman *et al*., 1995; Seeman and Van Tol 1995). The high affinity of clozapine for dopamine D_4 receptors led to speculation that D_4 receptors may be the main factor to clozapine's efficacy (Van Tol *et al*., 1991; Seeman *et al*., 1992 b*)* in the treatment of acute schizophrenia and a very low incidence of extrapyramidal motor side effects (Volavka *et al.,* 2005). The action of clozapine has been suggested via blocking D_4 receptors. However, the mechanism by which D_4 blockade could improve cognition is not fully known and though D_4 receptors are present on both pyramidal neurones and GABA producing interneurones in the PFC (see *figure 4.1*) and hippocampus, areas important for cognitive function. Moreover, increasing evidence suggests that the dopamine D_4 receptor may represent a selective dopamine target that could mediate cognitive processes (Wong and Van Tol 2003 a). I was mainly interested in studying the dopamine D_4 receptor as a target and to monitor the neurochemical and cognitive effect of PD168077. The main was to characterise a potential drug development strategy aimed at enhancing cognition process.

One of the main findings of my study was that PD168077, improved cognitive (episodic memory) function in preclinical NOR model of cognition deficit in rodents (see chapter 5). The test was carried out to investigate the consequences of increase in ecf dopamine levels in mPFC in challenge to low dose of systemic PD168077 administration (see chapter 4). Before the current findings on PD168077 (induced dopamine release), PD168077 was only

characterised in cultured prefrontal cortical neurones, where it activated dopamine D_4 receptors inducing synaptic translocation of Ca^{2+}/calmodulin-dependent protein kinase II. Thus, PD168077 was pharmacologically classified as a D_4 receptor agonist. The action of PD168077 centrally *in vivo* was not studied before. I showed that PD168077 at pharmacologically active ecf concentrations, induced increase in dopamine, in the mPFC at dose of 0.5 mg/kg; a physiological effect. This suggests that PD168077 mediated activation of the dopamine D_4 receptors in the mPFC and NAc causes synaptic overflow of dopamine. This finding adds to its pharmacological profile *in vivo* as an agonist. These findings suggest that the release in dopamine maybe either due to D_4 receptor mediated increase in the synthesis of dopamine or blockade of inhibition of dopamine synthesis in the PFC dopaminergic neurones. The increase in ecf dopamine levels also raised the question whether PD168077 may have influences on the behavioural functions that involve the utilisation of dopamine in the mPFC. The functions of PFC in the rat are often discussed in the context of learning and memory. In order to compare the direct outcome of the PD168077 induced dopamine release in mPFC and its cognitive effects as in memory, the present study investigated the performance of the rats in a task in which an exploration based test of object recognition (Ennaceur and Delacour 1988).

7.2.3 Behavioural effect of PD168077 in NOR task

The NOR test is evaluated entirely on the basis of spontaneous exploration and neither acquisition nor retention of an experimental rule is required. The test utilises the tendency of rats to primarily explore novel rather than previously encountered familiar objects. The level of recognition of an object to which the animal has previously been exposed is therefore reflected in the degree of exploration of such an object relative to the exploration of a novel one (Ennaceur and Delacour 1988). This gives a measure of episodic memory (see chapter 5).

My data revealed that PD168077 at 10 mg/kg dose reversed the cognition deficit in sub-chronic PCP pre-treated rats (see chapter 5). This suggests that PD168077 at high dose, enhanced disrupted episodic memory in PCP pre-treated rats in NOR paradigm.

The question addressed here was whether activation of D_4 receptors in dysfunctional NMDA system (caused due to sub-chronic PCP pre-treatment) may have a reversal effect on the dysregulation of the NMDA system. Thus may contribute to the improvement of cognition deficit. To address this, I further investigated the brain distribution and changes in ecf dopamine levels at higher dose of 10 mg/kg of PD168077 (that showed cognition enhancing effects) in the mPFC and NAc, by employing MQ probes (chapter 6). Jentsch *et al* in 1999 b, showed that a potent and selective D_4 receptor antagonist NGD94-1 reversed cognitive deficits in monkey that were pre-treated with PCP, whereas haloperidol (non specific, all D_2 receptor antagonist) exacerbated PCP induced cognitive impairment. My current study looked into an agonist effect at the D_4 receptor in reversal of cognition deficit. These findings are in line with a previous study, where A412997, a D_4 agonist improved cognitive performance in rodents in a similar NOR task (Woolley *et al.,* 2008). Thus compounds targeted at D_4 receptors may play a therapeutic role in treating cognitive deficit.

7.2.4 Mechanism of PD168077 action *in vivo*

Further microdialysis data revealed a higher increase in ecf dopamine levels in the mPFC compared to NAc after administration of 10 mg/kg PD168077 in control and PCP pre-treated rats in comparison to their respective basal levels. Also, in sub-chronic PCP pre-treated rats, I found that the basal levels of dopamine were remarkably reduced and the reduced levels were much lower in the mPFC than in the NAc. Furthermore, administration of 10 mg/kg PD168077, induced increase in dopamine levels in mPFC and NAc from the basal levels in

sub-chronic PCP pre-treated rats. The increase in dopamine levels was higher in mPFC than compared to in the NAc. This increase in dopamine plays an important role in D_4 mediated modulation of NMDA receptors; putative mechanism can be postulated as it is well known that PCP induces its cognitive disruptive effects by suppression of NMDA receptor activity. Previous studies have shown that D_4 receptors work in synergy with NMDA receptors in the PFC (Wang *et al*; 2003). In addition, recent study with whole cell recording showed activation of dopamine D_4 receptors produced a significant suppression of NMDA receptor transmission in PFC pyramidal neurones, which was dependent on the inhibition of active CaMKII in control rats. However, in PCP pre-treated rats, the D_4 modulation of NMDA receptors was significantly impaired, with the concomitant loss of D_4 regulation of CaMKII activity. Furthermore, treatment with the antipsychotic drug clozapine restored the D_4 regulation of NMDA receptors in PCP pre-treated rats (Wilson *et al*., 1998). This may suggest that the selective disruption of the interaction between D_4 and NMDA receptors in the PCP model, attributable to the impaired D_4 mediated downstream signalling, may contribute to the aberrant PFC neuronal activity in cognitive deficit models. Extending from the above discussed studies, I suggest that PD168077 in my study, repairs the PCP induced impairment of D_4 modulation of NMDA receptors, with the concomitant gain of D_4 regulation of CaMKII activity (Gu and Yan 2004). This may be of relevance in reversal of cognition deficit symptoms. I have also suggested other mechanisms by which PD168077 may possess antipsychotic characteristics (see chapter 6).

Furthermore, my findings lead to the speculation that PD168077 may possess antipsychotic action in an animal model of cognitive deficit. Moreover, the main outcome of the present results suggests that development of novel antipsychotic drugs may be classified on the basis of their ability to increase ecf dopamine levels in the mPFC versus the NAc. Although the clinical relevance of such a classification is not clear at present, the assumption that the preferential activation of prefrontal dopaminergic neurotransmission may contribute, at least

in part, to the efficacy in treating negative symptoms and cognitive impairment leads to testable hypotheses for developing new antipsychotic drugs. Therefore my findings may have clinical and pharmacological implications in developing new therapeutics in treating cognitive deficits as seen in schizophrenia.

7.3 Future directions

The physiological role of D_3 and D_4 receptors in the dopaminergic system is still mostly unknown. They are specifically localised in limbic and cortical regions and these regions are involved in the control of cognition and emotion which makes them attractive and promising targets for new generations of antipsychotic drugs. Moreover, the expression of the receptor subtypes, to a lesser extent in the dorsal striatum, with low incidence of extrapyramidal side effects. It is believed that the antipsychotic effects of are due to the action on the dopaminergic receptors in the mesolimbic system, whereas the extrapyramidal side effects are thought to result from blockade of D_2 receptors in the striatum (e.g., O'Connor *et al.,* 1989). From this perspective, the discovery of multiple dopamine receptors compound, with differential expression in the brain and with different affinities for antipsychotic drugs is of great interest. The low level of expression of the D_3 and D_4 receptors in the striatum and their relatively high expression in limbic and cortical areas led to the suggestion that the antipsychotic actions may be mainly mediated through D_3 and D_4 receptors, whereas the side effects may be mediated through D_2 receptors. Of particular interest is the high affinity of atypical antipsychotics, such as sulpiride and its derivatives and clozapine, for D_3 and D_4 receptors, respectively (e.g., Pillai *et al.,* 1998; Sanner *et al.,* 1998).

Additional work will be required to confirm the role of D_3 and D_4 receptor in antipsychotic actions, since many of these observations came from indirect measurements with partially

selective ligands. The development of specific antipsychotics targeting a single dopamine receptor subtype should shed more light on the role of each of the dopamine receptors in schizophrenia. The antipsychotic effects of dopamine blockers (see chapter 5 and 6) are believed to exert their effects on PFC dopamine release. It is very important, in the development of new antipsychotic drugs, whether these compounds exert a selective action on the release of dopamine in the PFC, for this purpose a comparison with other dopaminergic forebrain structures, such as NAc and striatum, is crucial.

To meet this end, MQ measurements will be valuable in measuring absolute brain concentrations of drugs and the neurotransmitter dopamine simultaneously, following systemic administration of the drugs will provide useful information on brain distribution, and neurochemical effects of novel atypical drugs. Moreover studies can be carried out in combination with their behavioural effects.

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Appendix

List of publications and conference proceedings

Sood P, Cole S, Fraier D, Young A. 2009. Evaluation of Metaquant microdialysis for measurement of absolute concentrations of amphetamine and dopamine in brain: a viable method for assessing pharmacokinetic profiles of drugs in the brain; Journal of Neuroscience Methods. 185 (1). 39-44.

Sood P, Idris N, Cole S, Neill J, Young A. 2009. PD 168077, a D4 receptor selective; enhances natural episodic memory and reverses sub chronic PCP induced cognitive deficits in the novel object recognition (NOR) task in rat: Potential role for D4 receptor mechanisms in improving cognitive deficit symptoms in schizophrenia, Journal of Psychopharmacology; 2009 (*Paper submitted*).

Sood P, Cole S, Fraier D, Young A. *2008.* Comparison of methods for measuring absolute brain concentrations of drugs, following systemic administration; *12th International Conference on In Vivo Methods August 10 – 14.*

Sood P, Cole S, Fraier D, Young A. 2009. Behavioural and neurochemical effects of D4 receptor agonist; PD 168077: measurement of absolute brain concentrations of dopamine using microdialysis*. BNA 20th National Meeting; April-19-22.*

Sood P, Idris N, Cole S, Neill J, Young A. 2009. PD 168077, a D4 receptor agonist; reverses PCP induced cognitive deficits in the novel object recognition (NOR) task in rats: Potential role for D4 receptor mechanisms in improving cognitive deficit symptoms in schizophrenia; *British Association of Psychopharmacology, summer meeting; 26th-29th July.*