Putative changes in dopaminergic neurotransmission following nicotine induced behavioural sensitisation

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Executive Summary

Behavioural sensitisation is a progressive enhancement of stereotypic or locomotor behaviour following repeated intermittent administration of a psychostimulant or stress. It is a phenomenon thought to underlie many neuropsychiatric disorders (*e.g.* schizophrenia, addiction, depressive disorders, dyskinesia, and psychosis) although its own mechanism remains contentious. In this thesis a multidisciplinary approach was used to investigate the role of dopamine in behavioural sensitisation. Different *in vivo* and *ex vivo* techniques were used to assess and elucidate putative changes in dopaminergic neurotransmission of behaviourally sensitised animals. If so, this improved understanding of behavioural sensitisation could provide a better understanding of the pathophysiologies of neuropsychiatric disorders and provide more insight into why existing pharmacotherapies for these disorders are able to confer only modest benefit. Moreover, this improved understanding can lead to development of new medication and more effective therapies to treat neuropsychiatric disorders and therapies that address the specific problems associated with them.

Previously, an oversimplified view of neurotransmitter release was used for the development of current available drugs, *i.e.* stabilising either the attenuated or increased release of neurotransmitters without considering the involvement of synaptic plasticity. Therapies being used with modest effectiveness regulate dopamine transmission levels, suggesting a putative role for dopamine. The present study used chronic intermittent nicotine administration in rodents to induce behavioural sensitisation which was monitored behaviourally by measuring locomotor activity. Further studies were performed *ex vivo* assessing receptor binding, intracellular cAMP accumulation and electrically stimulated dopamine release. Prior to the pharmacological assessments, a novel LC-MS/MS method to measure (cyclic-) nucleotides was developed and a fast cyclic voltammetry (FCV) technique was established to measure real-time neurotransmitter release. Specific pharmacological tools were used to identify the role of dopaminergic neurotransmission in behavioural sensitisation. Finally, the *ex vivo* findings using tissue from sensitised and non-sensitised animals were compared to those findings obtained *in vivo*.

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Declaration

This thesis has not been submitted before, in whole or in part, to this or any other university for any degree, and except where otherwise stated, is the original work of the author.

Signed: __________________

Wouter Goutier
June 2011

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List of Abbreviations

ATP	Adenosine Triphosphate
cAMP	3’,5’-cyclic Adenosine Monophosphate
e.g.	Latin: “*exempli gratia*”; For example
i.e.	Latin: “*id est*”; That is
ca.	Latin: *“circa”*; approximately
et al.	Latin: “*et alia*”; And others
ex vivo	Latin: “out of the living”. Means that which take place outside an organism. *Ex vivo* refers to experimentation or measurements done in or on living tissue in an artificial environment outside the organism with the minimum alteration of the natural conditions.
in vitro	Latin: “within the glass”. Refers to the technique of performing a given experiment in a controlled environment outside of a living organism; for example in a test tube or cell culturing.
in vivo	Latin: “within the living”. Means that which takes place inside an organism. In this respect animal testing is a form of *in vivo* research.
s.c.	Subcutaneous injection. Injection of a substance into the subcutis (third layer of the skin, directly below the epidermis and dermis, respectively).
i.p.	Intraperitoneal injection. Injection of a substance into the peritoneum (body cavity).
FCV	Fast cyclic voltammetry
MS	Mass spectrometry
kg	Kilo gram
mg	Milli gram
nM	Nano molar
μM	Micro molar
mM	Milli molar
μm	Micro meter

s	Second
min	Minute
h	Hour
mL	Milli liter
µL	Micro liter
A	Ampere
V	Voltage
°C	Degree Celsius
%	Percentage; indicating that the preceding number is divided by one hundred
Log	Logarithm
pEC_{50}	Negative logarithm of the concentration which produced 50% of the maximal effect
pA_2	Negative logarithm of the concentration of an antagonist which would produce a 2-fold shift in the concentration response curve for an agonist.
E_{max}	Maximal effect
F-value	F-test; Statistical test in which the test statistic has an F-distribution under the null hypothesis
P-value	The probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true.
Post hoc	Latin: "*Post hoc, ergo propter hoc*": After this, therefore because of this.
s.d.	Standard deviation
s.e.m.	Standard error of the mean
r^2	Coefficient of determination. Equals the square of the correlation coefficient between the observed and modelled data values.
Ø	Diameter

List of Publications

Part of the data in this thesis is published in the following research papers, abstracts and posters.

Peer-reviewed research papers

Goutier W, Spaans PA, van der Neut MAW, McCreary AC, Reinders JH. Development and application of a LC-MS/MS method for measuring the effect of (partial) agonists on cAMP accumulation in vitro. J Neurosci Methods, 2010:188;24-31

Brennan JA, Graf R, Grauer SM, Navarra RL, Pulicicchio CM, Hughes ZA, Lin Q, Wantuch C, Rosenzweig-Lipson S, Pruthi F, Lai M, Smith D, Goutier W, van de Neut M, Robichaud AJ, Rotella D, Feenstra RW, Kruse C, Broqua P, Beyer CE, McCreary AC, Pausch MH, Marquis KL. WS-50030 [7-{4-[3-(1H-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3H)-one]: A Novel Dopamine D2 Receptor Partial Agonist / Serotonin Reuptake Inhibitor with Preclinical Antipsychotic-Like and Antidepressant-Like Activity. J Pharmacol Exp Ther, 2010;332:190-201

Lange JH, van der Neut MA, Wals HC, Kuil GD, Borst AJ, Mulder A, den Hartog AP, Zilaout H, Goutier W, van Stuivenberg HH, van Vliet BJ. Synthesis and SAR of novel imidazoles as potent and selective cannabinoid CB2 receptor antagonists with high binding efficiencies. Bioorg Med Chem Lett, 2010;20:1084-1089

Goutier W, Bom E, Van Brussels M, O'Connor JJ. [Development and ex vivo application of a commercial fast cyclic voltammetry system for the simultaneous analysis of realtime dopamine, serotonin and histamine release].
Manuscript in preparation. To be submitted to J Neurosci Methods.

Goutier W, Kloeze BM, McCreary AC. Effect of varenicline on the development of nicotine induced locomotor sensitization in the rat.
Manuscript in preparation. To be submitted to J Neuropsychopharmacol.

Goutier W., Kloeze B.M., McCreary A.C. Effect of SCH-23390 pretreatment on in vivo nicotine induced behavioural sensitization and ex vivo cAMP accumulation.
Manuscript in preparation. To be submitted to J Neuropsychopharmacol.

Abstracts and Posters

J.A. Brennan, R.L. Navarra, S.M. Grauer, A.C. McCreary, J.H. Reinders, W. Goutier, P. vander Feer, K. Marquis, M.A.W. vander Neut, D. Rotella, R. Feenstra and M.H. Pausch. Antipsychotic-Like Profile of WS-50030, A Combined Partial D2 Receptor Agonist and Selective Serotonin Reuptake Inhibitor". Society for Neuroscience, Washington, DC, USA, 2008

Goutier W., Spaans P.A., van der Neut M.A.W., McCreary A.C., Reinders J.H. Use of LC-MS/MS for determining nanomolar levels of cyclic-AMP in Dopamine D2 cells. Solvay Science for Innovation Conference, Brussels, Belgium, Nov. 2007

Abstract

OBJECTIVES: Extensive research efforts have provided insights into understanding the aetiology of neuropsychiatric disorders, however remains still not fully understood. Herein it is hypothesised that understanding the mechanism underlying the behavioural sensitisation can help to elucidate the responsible mechanism(s) underlying certain neuropsychiatric disorders, and eventually could lead to improved (pharmaco) therapies. The present study investigated the role of the dopamine D_1 receptor in the mechanism underlying behavioural sensitisation.
METHODS: Rats were chronically (5 days) treated with nicotine to induce behavioural sensitisation. Following a withdrawal period, rats were pre-treated with SCH-23390 and/or given a final nicotine challenge. Subsequent *ex vivo* cAMP accumulation was determined using a 96-well filterplate method and LC-MS/MS analyses, and binding affinities were assessed *ex vivo* using a 96-well filterplate method and $[^3H]$-SCH-23390. Electrically stimulated dopamine release was determined using the fast cyclic voltammetry (FCV) technique. A selection of specific pharmacological tools was tested across all assays.
RESULTS: The intermittent treatment regime produced a robust expression of sensitisation, showing it is a long lasting phenomenon. The nicotine induced sensitisation was dose dependently antagonised by the dopamine D_1 receptor antagonist SCH-23390. FCV experiments showed that the frequency dependent electrical modulation of dopamine release can be altered by nicotine and moreover, for the first time, it was shown that this effect can be altered by dopamine D_1 receptor ligands. The developed LC-MS/MS method was validated and applied *in vitro* to determine cAMP accumulation in cell cultures and brain tissue samples. Behavioural sensitisation increased the efficacy of dopamine for the dopamine D_1 receptor, and decreased the potency of D_1 agonists on cAMP accumulation *ex vivo*, however, did not affect the affinity of the tested dopaminergic ligands for the dopamine D_1 receptor.
CONCLUSIONS: The present study showed that selective antagonism of the dopamine D_1 receptor blocks the expression of nicotine induced behavioural sensitisation *in vivo*, and suggests that this is a result of the enhanced response of neurones in the dorsal striatum to dopamine D_1 receptor activation. This thesis

presents a successful multidisciplinary approach which resulted in additional information about the mechanism underlying behavioural sensitisation and showed an important role for the dopamine D_1 receptor. Furthermore, the described approach provides a successful framework to study behavioural sensitisation which also can be used for future studies and ultimately the elucidation of the mechanism(s) underlying neuropsychiatric disorders.

Keywords: Behavioural sensitisation, nicotine, dopamine D_1 receptor, cAMP, neuropsychiatric disorders

Chapter 1. General Introduction

Abstract.
OBJECTIVES: Behavioural sensitisation is a phenomenon which can be developed following repeated intermittent stimulation by administration of a psychostimulant or stress. Its mechanism is suggested to play an important role in the underlying mechanism of neuropsychiatric disorders like schizophrenia, addiction, depressive disorders, dyskinesia, and psychosis. Therefore, the understanding of behavioural sensitisation could help to characterise these neuropsychiatric disorders and improve therapies to treat these major health and social problems. The objective of this chapter was to provide an introduction and literature review of the extensive research efforts into behavioural sensitisation and the current status of this research, in particular in relation to putative mechanisms underlying neuropsychiatric disorders.
CONCLUSIONS: Behavioural sensitisation is a well described and characterised phenomenon supported by preclinical studies (*i.e.* behavioural, cellular, and molecular) and clinical evidence, in control and disease models. A large number of reports suggest a common mechanism underlying neuropsychiatric disorders and point towards behavioural sensitisation as the putative mechanism. A better understanding of the mechanism(s) underlying behavioural sensitisation might give new insights to improve currently available (pharmaco-) therapies for the treatment of neuropsychiatric disorders. Preclinical studies suggest a deficiency in dopaminergic neurotransmission to be responsible for behavioural sensitisation. However, more advanced *in vivo* behavioural studies and *ex vivo* mechanistic studies are required to elucidate the exact role of dopamine in this phenomenon. In addition to the dopaminergic hypothesis, it has been shown that different aspects of the cAMP pathway are involved in behavioural sensitisation and can be used as a marker or target in the discovery of putative mechanisms underlying behavioural sensitisation.

Keywords: Behavioural sensitisation, nicotine, dopamine D_1 receptor, cAMP, neuropsychiatric disorders

1.1 INTRODUCTION

Psychostimulants administered to humans or animals can induce different behaviours including locomotor activation and stereotypic behaviours. Repeated intermittent administration of psychostimulants can develop behavioural sensitisation (Downs and Eddy, 1932), a phenomenon characterised by either a progressive enhancement in behavioural activity or an enduring behavioural hypersensitivity to these drugs (Utena, 1966; Robinson and Becker, 1986; Tadokoro and Kuribara, 1986). The origin of stimulation can vary from psychological factors such as stress, trauma or reward, and environmental stressors, or by chemical factors during prolonged drug use. The prolonged neurological stimulation can evolve in behavioural sensitisation with symptoms such as increased stereotypical behaviour or locomotor activity.

Dopamine, serotonin and noradrenaline are all monoamine neurotransmitters and can be released peripherally as well as in the brain where they play an important role in emotion, cognition and motor activities. Currently, most of the available drugs to treat neuropsychiatric disorders like anxiety, depression and schizophrenia mainly target the synthesis or the rate of release of these monoaminergic neurotransmitters. Additionally, preclinical findings including neurochemical, electrophysiological, molecular and morphological changes are reported following psychostimulant induced behavioural sensitisation (Robinson and Berridge, 1993; Robinson and Becker, 1986; Sato *et al.*, 1992). Furthermore, the dopaminergic pathway is shown to be important in behavioural sensitisation (Kalivas and Stewart, 1991). The observation of symptoms following drug administration or psychosocial stressors has led to the hypothesis that processes homologous to behavioural sensitisation may underlie the development and expression of chronic and recurrent affective disorders (in animals; sensitisation to repeated stress, Doherty and Gratton, 1992). More recently, in addition to the preclinical observations, clinical evidence supports these findings and also suggests an important role for behavioural sensitisation in humans. For example, Post and colleagues described the importance of the behavioural sensitisation model to give additional rationale to the development of bipolar disorders in humans (Post *et al.*, 2001). Strakowski and colleagues reported behavioural

sensitisation following amphetamine challenges in humans (Strakowski *et al.*, 1996), and observed that patients with schizophrenic psychosis, in contrast to healthy volunteers, did not show a progressive increase in symptoms after an amphetamine challenge, meaning that they were already sensitised or do not sensitise (Strakowski *et al.*, 1997). Additionally, significantly lower dopamine D_1 receptor binding in the ventral striatum and caudate of chronic smokers was observed (Dagher *et al.*, 2001). Significantly lower striatal dopamine D_2 receptor availability has been observed in patients with cocaine (Volkow *et al.*, 1997) and methamphetamine (Volkow *et al.*, 2001) dependence. Recently, more evidence of behavioural sensitisation as a model of stress induced psychosis in humans was shown by van Os and colleagues (Myin-Germeys *et al.*, 2005; Lataster *et al.*, 2010).
Based on these findings, and the association of behavioural sensitisation with addiction, a few hypotheses to explain behavioural sensitisation are proposed and strongly point towards an important role for the neurotransmitter dopamine. Dopamine is an endogenous neurotransmitter in the brain which is involved in the neurobiology and symptoms of many neuropsychiatric disorders (for review see Marsden, 2006). In this chapter (Chapter 1) a brief overview will be presented on dopaminergic signalling. However, despite all the available information, little is known about the functionality and the exact mechanisms of how dopamine signalling is involved in behaviour. The future challenge remains to understand how dopamine is exactly involved in neuropsychiatric disorders and their behaviours.

The dopamine pathway can be triggered by binding of its endogenous ligand (*i.e.* dopamine) or synthesised ligand to its cognate receptor (Figure 1-1). This specific receptor, also called the dopamine receptor, is a member of the 7-transmembrane domain receptor family and consists of different subtypes, *i.e.* D_1 to D_5 (Neve and Neve, 1997). These subtypes can be grouped into two receptor families: the dopamine D_1-like receptors (which include the D_1 and D_5 receptors subtypes) and the dopamine D_2-like receptors (including the D_2, D_3 and D_4 receptors subtypes) (Garau *et al.*, 1978; Kebabian and Calne, 1979). The dopamine receptor subtypes can enhance (D_1-like) or inhibit (D_2-like) through activity of adenylate cyclase, coupling to stimulatory (Gas) or inhibitory (Gai) G proteins, respectively. Upon the binding of a ligand to the corresponding receptor

protein, the conformation of this protein will change and activate intracellular guanine nucleotide binding proteins, and therefore the dopamine receptor is also a member of the G-protein coupled receptor (GPCR) family. The G-protein (guanine nucleotide-binding protein) is a heterotrimer with an alpha (α), beta (β) and gamma (γ) subunit. Receptor activation will displace the guanosine diphosphate (GDP) from the alpha subunit by guanosine triphosphate (GTP) and subsequently release the alpha-subunit and beta-gamma dimmer from the G-protein heterotrimer. GTP is catalysed back to GDP via an enzymatic autoregulation process, by the alpha-subunit itself and re-joined by the beta-gamma dimer. There are 4 main subtypes known for the alpha unit of the G-protein: Gs, Gi, Gq and Go. There are also distinct families for the beta and gamma subunits, but they will not be discussed here. Receptors are not specifically coupled to a single G-protein subtype and the wide variety of receptor subtypes and G-protein subtypes can result in many possible pathways. Moreover, not only the alpha subunit but also the beta-gamma dimer can affect enzymes like adenylate cyclase (Bayewitch *et al.*, 1998) and, in addition, several families of proteins have been characterised that act as “accessory” G-protein signal regulators (AGS: Berman and Gilman, 1998; Blumer and Lanier, 2003), revealing even more possible pathways for controlling downstream GPCR effects. Previous studies have shown that behavioural sensitisation can affect G-proteins (Nestler *et al.*, 1990; Terwilliger *et al.*, 1991; Striplin and Kalivas, 1993; for review see Nestler, 2001a), and Bowers and colleagues showed that the regulatory protein AGS3 was strongly elevated in PFC and nucleus accumbens following chronic cocaine treatment (Bowers *et al.*, 2004) and involved in addiction associated behaviours (Bowers *et al.*, 2003).

G-proteins can also affect ion-channels directly or by targeting one of the following three enzymes: Adenylate Cyclase, Phospholipase C and Phospholipase A (Figure 1-1). To add to the complexity of GPCR signalling, these specific enzymes and their subtypes can also differ widely among cells. These enzymes typically activate a diffusible second messenger which can trigger a cascade of biochemical reactions that ultimately can alter neuronal function. Theoretically, the actions of neurotransmitters (including dopamine) are mainly mediated through the activation or inhibition of the catalytic unit of adenylate cyclase by Gαs or Gαi, respectively (Gilman, 1989; Neer, 1995). The adenylate cyclase (AC; EC 4.6.1.1) family consists of at least 10 distinct

adenylate cyclase isoforms (see Patel *et al.*, 2001 for review). All of these isoforms are integral transmembrane proteins (except for one that is found to be soluble, sAC) and consist of two sets of six transmembrane domains. In the last few decades, Vogel's laboratory has performed extensive studies to adenylate cyclase and shown a distinct role for the specific isozymes of adenylate cyclase in the effects of chronic treatment with opioids (Avidor-Reiss *et al.*, 1995a; 1995b; 1996; 1997; Kato *et al.*, 1998) or cannabinoids (Rhee *et al.*, 2000) in transfected cells.

An important biological function of adenylate cyclase is the cyclisation of adenosine triphosphate (ATP) to adenosine-3',5'-cyclic monophosphate (cAMP) (Sutherland *et al.*, 1962). cAMP, discovered by Rall and Sutherland (Rall *et al.*, 1958; Sutherland and Rall, 1958) is one of the most important second messengers in biology. The second domain of adenylate cyclase contains two catalytic domains which can form an intramolecular hetrodimer which activates the formation of cAMP (Krupinski *et al.*, 1989). Subsequently, adenylate cyclase will attack the 3'-OH group of the α-phosphate of ATP which will result in the release of cAMP and pyrophosphate (Tesmer *et al.*, 1997; Zhang *et al.*, 1997).

Following its synthesis and release, cAMP can target different effectors (Figure 1-1). A key signalling pathway is activation of protein kinases. cAMP can bind to the homodimerised regulatory subunits of the cAMP-dependent protein kinase (PKA; EC 2.7.1.37) and phosphorylates serine/threonine (Meinkoth *et al.*, 1993) which will cause dissociation from the catalytic subunit homodimer and results in phosphorylation of substrate proteins including enzymes, ion channels and transcription regulators. The key effector protein, protein kinase A (PKA), is also found downstream of the dopamine D_1-like and D_2-like receptors intracellular pathways and is essential for their effects (Flores-Hernandez, *et al.*, 2000). Psychostimulants are also able to activate the cAMP-pathway which subsequently activates a wide variety of downstream signalling pathways (Svenningsson *et al.*, 2003). Activation of PKA can further modulate the phosphorylation state of DARPP-32. It has been shown that dopamine D_1 and D_2 receptor activation may result in opposite and phosphorylation site specific regulation of DARPP-32 levels (Hamada *et al.*, 2004; Svenningsson *et al.*, 2004). The effect of systemic nicotine on dopamine levels in the nucleus accumbens is also mediated through PKA (Inoue *et al.*, 2007) and increases phosphorylation of DARPP-32 at both Thr34 and Thr75 (Zhu *et al.*, 2005), which could be a direct

effect on DARPP-32 signalling through α4 nAChRs (Hamada *et al.*, 2005) or through nicotine induced dopamine release, as activation of nAChRs can result in the release of dopamine, particularly in the striatum (Westfall, 1974). Furthermore, nicotine can stimulate release of serotonin (5HT) in the striatum (Reuben and Clarke, 2000) and subsequently produce an increase in phospho-Thr34-DARPP-32 and a decrease in phospho-Thr75-DARPP-32 (Svenningsson *et al.*, 2002), similar to dopamine D_1 receptor mediated activation.

Long term changes in the cAMP pathway (for example increase in PKA activity) have been reported following chronic cocaine exposure (Terwilliger *et al.*, 1991; Unterwald *et al.*, 1996; Miserendino and Nestler, 1995). cAMP can directly bind to and regulate a subtype of ion channel and early physiological studies have shown that cyclic nucleotides can directly control their opening (Fesenko *et al.*, 1985). It became evident that these channels contain an intracellular cyclic nucleotide binding domain that gated the opening of the channels upon binding cAMP or cGMP (reviewed by Zagotta and Seigelbaum, 1996; Zufall *et al.*, 1997; Kaupp and Seifert, 2002; Mansuy, 2004; Dremier *et al.*, 2003). These channels are now known as cyclic nucleotide gated (CNG) channels.

cAMP is removed by a group of enzymes called phosphodiesterases (PDE; EC 3.1.4.17) (Butcher *et al.*, 1962). There are at least 19 subtypes of phosphodiesterase (Conti *et al.*, 1999) but only a limited number are known to specifically catalyze the hydrolysis of the 3'-carbon phosphoester bond of cAMP to the corresponding 5'-monophosphate, AMP (*e.g.* PDE4). Using selective PDE inhibitors it has been shown that PDEs are also involved in behavioural sensitisation (*e.g.* PDE4, Knapp *et al.*, 2001; Thompson *et al.*, 2004).

Guanosine-3',5'-cyclic monophosphate (cGMP) together with cAMP are the two most important cyclic nucleotide second messengers in biology. cGMP was originally isolated from urine (Price *et al.*, 1967) but later also found in many tissues including brain tissue (Goldberg *et al.*, 1969; Ishikawa *et al.*, 1969). Like cAMP, cGMP is synthesised by a specific enzyme, in this case guanylate cyclase (GC; EC 4.6.1.2) (Hardman *et al.*, 1969; Schultz *et al.*, 1969) and like adenylate cyclase is found in different subtypes (*i.e.* soluble forms (sGC) and transmembrane isoforms (tmGC)).

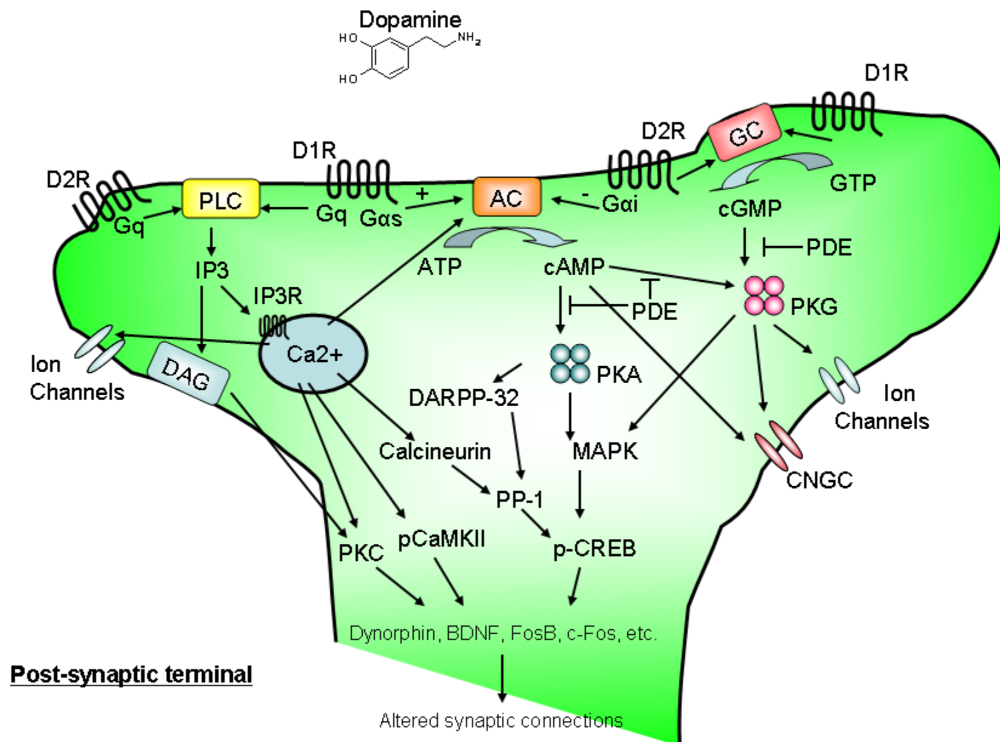


Figure 1-1 Signalling pathways of the dopamine D_1 and D_2 receptors

Schematic representations of hypothesized dopamine D_1 and D_2 receptor activation-induced signalling pathways. Dopamine, or dopamine receptor ligands, can activate either or both D_1 or D_2 receptors and subsequently activate AC-cAMP-PKA-CREB signaling and CRE-mediated gene expression. D1R/D2R, dopamine receptor 1/2; AC, adenylate cyclase; PLC, phospholipase C; DAG, diacylglycerol; GC, guanylate cyclase; PDE, phosphodiesterase; PKA/C/G, phosphokinase A/C/G; MAPK, mitogen activated protein kinase; PP-1, protein phosphatase-1; IP3, inositolphosphate3; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; GTP, guanosine triphosphate; cGTP, cyclic guanosine monophosphate; CREB, cAMP response element-binding; CNGC, cyclic nucleotide gated channels. References used, Hopf et al., 2003; Neve et al., 2004; Hyman et al., 2006; McClung and Nestler, 2008; Nishi et al., 2011, and references herein.

Psychostimulants like cocaine or amphetamine affect dopamine release directly by dopamine receptor activation or dopamine uptake transporters. However, systemically administered nicotine will activate nicotinic acetylcholinergic receptors (nAChRs) prior to other systems. The acetylcholinergic receptor family (AChRs) are transmembrane receptors which can be divided into two sub-classes: the nicotinic (ionotropic, nAChRs) and muscarinic (metabotropic, mAChRs) acetylcholinergic receptors. The nAChR's are members of the superfamily of ligand-gated ion channels and are composed of a diversity of subtypes (Dani, 2001). To date eight alpha subunits (α_2 – α_9) and three beta subunits (β_2 - β_4) are known and are found in many different combination (Conroy and Berg, 1995; Colquhoun and Patrick, 1997; Lukas, 1998; Lukas *et al.*, 1999). The three properties of these receptors that contribute to their physiological effects include activation, desensitisation, and upregulation following activation. Each of these phenomena is likely to be involved in behavioural sensitisation to nicotine, but the relative importance of each is still unknown (Picciotto et al., 2008). Different subtypes of the nAChR are reported to be involved in the nicotine elicited effect on locomotor activity (*e.g.* α_6, Le Novere *et al.*, 1999; $\alpha_4\beta_2$, Goutier et al., manuscript in preparation). nAChR antagonists are potential antidepressants (a hypercholinergic hypothesis, Shytle *et al.*, 2002) and, moreover, nAChR (partial-) antagonists also have been shown to attenuate behavioural sensitisation, for example varenicline blocks nicotine induced sensitisation (Goutier *et al.*, manuscript in preparation).

nAChRs are widely expressed on dopaminergic cell bodies and terminals, neighbouring afferents and interneurones (Gotti *et al.*, 2007; 2009; 2010). nAChRs are specifically located in midbrain dopaminergic neurones that project to the dorsal striatum, nucleus accumbens and prefrontal cortex. These projections are correlated to specific behaviours, notably motor control, 'reward' and executive function, respectively. Dopamine is an important neurotransmitter and once released by the cell, and extracellularly available in the synapse, it can bind and activate dopamine receptors. Therefore, the actual synaptic concentration of dopamine is of great importance. In the dorsal striatum, a brain area with relatively high dopamine levels, synaptic dopamine levels are regulated by i) the efflux of dopamine from intra-cellular vesicles, and ii) the function of uptake transporters like the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT). Dopaminergic neurotransmission in subcortical

regions like the basal ganglia, including the striatum, is synaptic and the uptake transporters are of great importance. In cortical regions there are only a few transporters and dopamine is allowed to diffuse away from the synapse, a phenomenon called "volume transmission". The mechanisms through which nAChRs modulate the activity of dopaminergic neurones and ascending pathways, is extensively reviewed by Livingstone and Wonnacott (2009).

Specific reuptake inhibitors, including psychostimulants (*e.g.* cocaine and amphetamine) and antidepressants have their mode of action by inhibition of reuptake thus increasing the extracellular dopamine levels. It is also suggested that the DAT is involved in cocaine sensitisation (Cass *et al.*, 1993; Alburges *et al.*, 1993; Zahniser *et al.*, 1995) and the DAT mRNA levels are upregulated following amphetamine sensitisation (Shilling *et al.*, 1997). Daily administration of cocaine decreased [^{3}H]-dopamine uptake (24 h after last injection), increased receptor affinity (K_m) and had no change on the maximum binding rate (V_{max}) in rat nucleus accumbens, although it had no effect on uptake in the striatum (Izenwasser and Cox, 1990). This study also showed that the potency of cocaine to inhibit [^{3}H]-dopamine uptake was increased following chronic cocaine treatment compared to saline. In contrast, it was shown that after 3 days of withdrawal following chronic cocaine administration (14 days) DAT density (B_{max}) or affinity (K_d) were not affected in the nucleus accumbens, using [^{3}H]-GBR-12935 as the radioligand, however the density of the DAT was increased in the striatum (Claye *et al.*, 1995).
Moreover, previous results suggests that nicotine can inhibit DAT indirectly through nAChR (Wonnacott, 1997), although contradictory results are reported on the effect of nicotine on the mRNA expression of DAT following chronic nicotine administration *in vivo* (Ferrari *et al.*, 2002; Li *et al.*, 2004). In humans, striatal uptake of [^{18}F]-fluoro-dopa (fluoro-3,4-dihydroxyphenylalanine, a dopamine precursor) was 16-29% higher in regular smokers compared to non-smoking subjects (Salokangas *et al.*, 2000), which also supports a role for nicotine in dopamine uptake.

The basal ganglia circuit plays a key role in the regulation of voluntary movements as well as in behavioural control and cognitive functions (Graybiel *et al.*, 1994). The mesocorticolimbic dopamine pathway, which arises in the ventral

tegmental area and innervates the nucleus accumbens among other regions, has been implicated in mechanisms underlying neuropsychiatric disorders and behavioural sensitisation. The basal ganglia contains many different types of neurones which are modulated by numerous neurotransmitter systems including gamma-aminobutyric acidergic (GABAergic), dopaminergic, noradrenergic, glutamatergic, cholinergic, and peptidergic afferents. Changes or dysfunction between these different neurotransmitter systems in the basal ganglia can result in changes in behavioural responses. Previous reports have shown that nAChRs are located at neurones facilitating GABA and dopamine transmission within different brain areas, *e.g.* prefrontal cortex, thalamus, ventral tegmental area, substantia nigra, nucleus accumbens, and striatum (Clarke *et al.*, 1984; Clark and Pert, 1985; Picciotto and Corrigall, 2002; Wonnacott *et al.*, 2005). Like other psychostimulants, nicotine can also induce the release of dopamine in these areas (Clarke and Pert, 1985; Grady *et al.*, 1992; Pontieri et al, 1996). Evidence from several studies suggests that dopamine D_1 receptors in the cerebral cortex are primarily localised on GABAergic interneurones, and to a lesser degree on pyramidal excitatory amino acid neurones (Al-Tikriti et al, 1992). The dopamine D_2 receptor is primarily localised on pyramidal cells and to a lesser degree on GABAergic interneurones (Al-Tikriti et al, 1992; Vincent *et al.*, 1995).
The striatum is known to be involved in inhibitory as well as excitatory neurotransmission pathways, *i.e.* indirect inhibitory: Cx (D_2 MSN) → STr → GPe → STN → GPi → Thal → Cx, and direct excitatory: Cx (D_1 MSN) → STr → GPi → Thal → Cx, respectively. The striatum is important in linking the neuronal pathways of different brain areas and 'filters' these signals as shown in Figure 1-2, although, it is important to recognise that this is an extremely oversimplified view of the anatomy and function of the basal ganglia and other neurotransmitters and neuromodulators (*e.g.* choline, histamine, serotonin) are involved as well.

Nicotine is one of the most extensively abused drug in society and yet despite the enormous amount of literature about nicotine, only a limited number of studies on nicotine induced sensitisation are documented. Most studies report on the addictive properties, socioeconomic and health problem of nicotine. However, as behavioural sensitisation is considered a putative mechanism underlying addiction it is important to understand its own mechanism of action. Nicotine is of particular interest for studying dopaminergic mechanisms putatively underlying

behavioural sensitisation. Most studies to date have investigated the effect of direct acting dopaminergic drugs (*e.g.* cocaine and amphetamine) on behavioural sensitisation. However, the ability of indirect dopaminergic drugs to induce sensitisation is also of great interest for the development of novel pharmacotherapies.

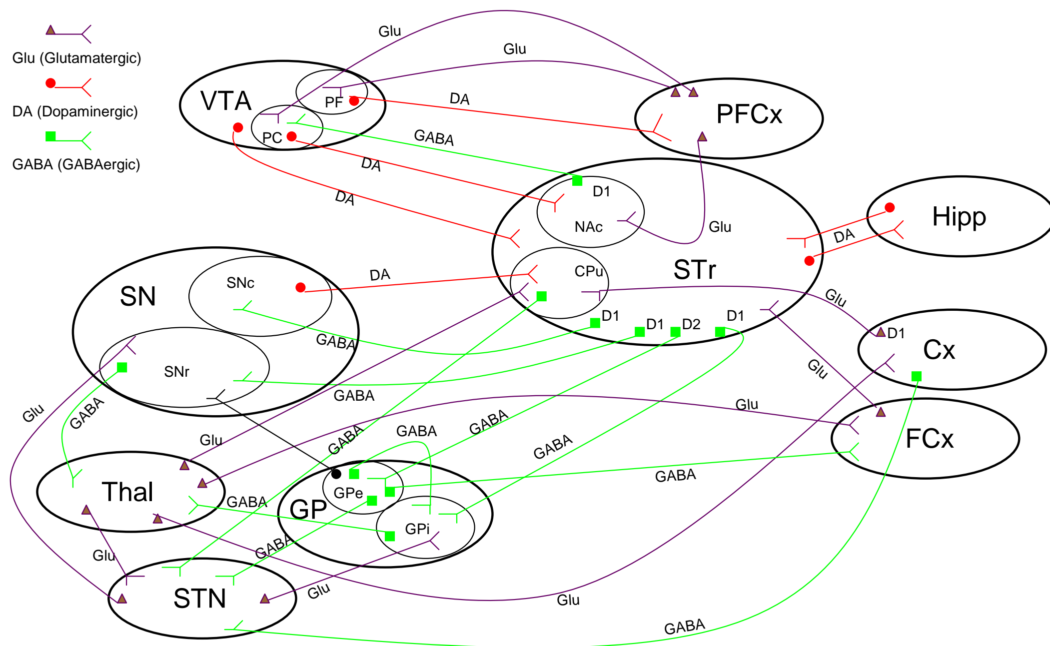


Figure 1-2 Dopaminergic signalling complex of the basal ganglia

Schematic showing the signalling complex of the basal ganglia including the established dopaminergic pathways: Mesolimbic (VTA (pc) → Nac); Mesocortical (VTA (pf) → PFCx); Nigrostriatal (SNc → CPu); Striatonigral (STr → SNc/SNr); and Striatopallidal (STr → GPe/GPi). VTA, ventral tegmental area; including PF, parafascicular; and PC, paracentral; STr, striatum; including NAc, nucleus cccumbens; and CPu, caudate putamen; GP; globus pallidus; including GPe, globus pallidus-external; and GPi, globus pallidus-internal; STN, subthalamic nucleus; SN, substantia nigra; including SNc, substantia nigra pars compacta; and SNr, substantia nigra pars reticulata; Thal, Thalamus. References used, Fallon and Moore, 1978; Johnson and North, 1992; Koob, 1992; DeLong, 2000; Hummel and Unterwald, 2002; Marsden, 2006; and Vezina *et al.*, 2007.

Nicotine administered intra-nucleus accumbens induced behavioural sensitisation in rat (Kita *et al.*, 1992). The nicotine induced sensitisation could be antagonised by systemic administration of SCH-23390 (Chapter 2). However, in addition intra-accumbens infusion of SCH-23390 did not alter cocaine induced locomotor activity, despite occupying 40-60% of the dopamine D_1-like receptors in the anterior nucleus accumbens core and shell (Neisewander *et al.*, 1998). This suggests that regions other than the nucleus accumbens are involved in locomotor activity produced by intra-accumbens infusion of cocaine and that stimulation of dopamine D_1 receptors in the nucleus accumbens is possibly important but not necessary for this behaviour.

On the basis of previous findings, the present study investigated the role of dopamine in nicotine induced behavioural sensitisation at a behavioural, cellular, and molecular level in the rat dorsal striatum. With regards to the scope of the performed research, this thesis distinguishes five result chapters based on the techniques used.

HYPOTHESES AND AIMS

Hypothesis: Alterations in dopaminergic neurotransmission, particularly dopamine D_1 receptor mediated, plays an important role in the mechanism underlying the expression of nicotine induced behavioural (i.e. locomotor) sensitisation.

Aims:

- Investigate the involvement of the dopamine pathway in nicotine induced locomotor sensitisation.
- Characterize the interaction of the dopamine D_1 with D_2 receptors and nicotine on dopamine release.
- Examine putative changes in dopamine D_1 and D_2 mediated cAMP accumulation and receptor binding pharmacology following chronic nicotine induced locomotor sensitisation.

Chapter 2. The effect of dopamine D_1 receptor blockade on nicotine induced behavioural sensitisation

Abstract.

OBJECTIVES: Behavioural sensitisation is a phenomenon which can occur after repeated intermittent administration of a drug. It is also suggested as a putative mechanism underlying many neuropsychiatric disorders and therefore, understanding of its mechanism is of great importance. Intermittent chronic psychostimulant administration has been shown to induce behavioural sensitisation in the rat. This study investigated whether the expression of nicotine induced sensitisation could be blocked by a dopamine D_1 receptor antagonist.

METHODS: Rats were chronically (5 days), intermittently treated with vehicle or nicotine and ambulatory locomotor activity was measured using a photobeam system. Following different withdrawal periods (5 or 17 days), chronic nicotine treated animals were pre-treated with SCH-23390 (a dopamine D_1 receptor antagonist) or vehicle and 30 min later challenged with nicotine.

RESULTS: Acute nicotine (0.4 mg/kg, s.c.) significantly increased locomotor activity. Subsequently, intermittent chronic nicotine administration for 5 days induced the development of locomotor sensitisation. Moreover, a nicotine challenge given after 5 or 17 days of withdrawal produced a robust sensitised response (*i.e.* expression of sensitisation) showing that sensitisation is a long lasting phenomenon. The expression of nicotine induced sensitisation was dose dependently antagonised by the dopamine D_1 receptor antagonist SCH-23390.

CONCLUSIONS: An *in vivo* locomotor activity paradigm was successfully validated to assess nicotine induced behavioural sensitisation in the rat. The present findings suggest a role for the dopaminergic D_1 receptor in the underlying mechanism of the expression of nicotine induced behavioural sensitisation. This model can now be used for future *ex vivo* experiments investigating molecular mechanisms of behavioural sensitisation.

Keywords: Locomotor activity, behavioural sensitisation, nicotine, SCH-23390

2.1 INTRODUCTION

Impairment of dopaminergic neurotransmission in the mesolimbic, mesocortical and nigrostriatal pathways has been suggested to contribute to the pathogenesis of *e.g.* schizophrenia, tardive dyskinesia, psychoses, Parkinson's disease including L-DOPA (L-3,4,-dihydroxy phenylalanine) induced dyskinesia, and (drug) addiction (see Chapter 1). Each of these pathologies has their own hypothesis for underlying molecular mechanisms, although, they share one specific hypothesis explaining the pathologies by dysfunction of dopaminergic neurotransmission. Altered dopaminergic neurotransmission can be studied in the laboratory by using several different animal models, where the pathology can be (partially) mimicked by using different pharmacological tools. Psychostimulants are known to alter dopaminergic neurotransmission, and psychomotor stimulant drugs have been associated with different behavioural effects in rats depending on the administration regimen. With respect to locomotor behaviour, repeated intermittent daily injections of psychostimulants produces behavioural sensitisation, a gradual increase in drug induced locomotor activity or other behaviours (*e.g.* conditioned place preferences). In contrast to continuous infusion using a minipump leads to the development of behavioural tolerance, in which the locomotor behaviour is suppressed over time (Post, 1980; Burger *et al.*, 1994). Given this fact, one is able to study reversal, attenuation or blockade of the induced pathogenesis by examining novel hypotheses or drugs in these models.

The first report to describe behavioural sensitisation was that of Tatum and colleagues (Tatum *et al.*, 1929). However, behavioural sensitisation using psychostimulants was first described by Downs and Eddy (1932). Around the same time it was shown that daily cortical stimulation kindling showed progressive development of motor seizures (Watanabe, 1936), which was later found to induce long-term sensitisation (Goddard, 1969). Many years later behavioural sensitisation was proposed as a model for stimulant induced psychosis (Segal *et al.*, 1983; Post *et al.*, 1984; Robinson and Becker, 1986) and has been extensively linked to drug addiction (Robinson and Berridge, 1993).

Behavioural sensitisation is a significant focus of research in behavioural pharmacology. Sensitisation can translate in many different responses, although, the most commonly studied drug responses showing sensitisation are different forms of motor activation, *i.e.* locomotor activity, stereotyped and rotational behaviours (Post and Rose, 1976; Kalivas and Stewart, 1991; Robinson and Becker, 1986; Wise and Leeb, 1993). Sensitisation can be induced by stress, punishment/reward, chemically (by local potassium administration), by administration of a drug (systemically or locally through a cannula), or by electrical stimulation of specific brain regions (e.g. Le Merrer and Stephens, 2006, Vezina *et al.*, 2007).

Behavioural locomotor activity sensitisation is the enhanced motor-stimulant response that occurs with repeated exposure to psychostimulants. To develop sensitisation, it is crucial to administer the challenge intermittently (Volkow, 2006). For example, daily intermittent injections of cocaine produced behavioural sensitisation to the locomotor response in rats, whereas continuous cocaine infusions produced behavioural tolerance (Burger *et al.*, 1994). The development of sensitisation can also be dependent on the environment, which might depend, at least to some extent, on the fact that different drug evoke behaviours depending on the environment (Einat and Szechtman, 1993a, 1993b). Furthermore, extensive research has shown that behavioural sensitisation can occur and be studied in several different stages, *i.e.* the acquisition (or development) of sensitisation, a withdrawal period, reversal treatment period, and an (re-) expression phase of sensitisation following a challenge. Previous reports showed that these stages are temporally, anatomically, and neurochemically distinct, suggesting they consist of different pharmacology (*e.g.* Kalivas *et al.*, 1993). These conclusions are made on the basis that some drugs selectively target (*i.e.* induce, block, stimulate or reverse) the development or expression phase of behavioural sensitisation. Reversal treatment is studied by a regime consisting of the development of sensitisation by psychostimulants, followed by chronic treatment with a drug (reversal treatment) and a withdrawal period after which a challenge with the psychostimulant only was given (*e.g.* Li *et al.*, 2000; Moro *et al.*, 2007). Furthermore, it has been shown that after the development of behavioural sensitisation with a certain drug, the same subject can express behavioural sensitisation following a withdrawal period and exposure to another

drug. This phenomenon is called cross-sensitisation (DiFranza and Wellman, 2007).

For this reason numerous studies have been performed which assessed the role of dopamine in behavioural sensitisation over the last few decades. In keeping with its ability to modulate dopaminergic neuronal function in the brain, behavioural sensitisation is currently considered as a behavioural model to improve treatments of neuropsychiatric disorders related to dopaminergic neuronal dysfunction, such as depression, schizophrenia, Parkinson's disease, anxiety, obsessive compulsive disorder, dyskinesia, bipolar disorder (Post *et al.*, 2001), and drug addiction. Behavioural sensitisation can be induced by psychostimulants which share the ability to increase synaptic dopamine levels directly (*e.g.* cocaine or amphetamine) or indirectly (*e.g.* nicotine or PCP). Although the exact mechanism underlying behavioural sensitisation is not fully understood yet, several mechanisms are proposed. Most of these include the dopaminergic system, *i.e.* the mesolimbic and/or nigrostriatal dopaminergic systems (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Wolf *et al.*, 1994). Behavioural sensitisation to psychostimulants (*e.g.* cocaine or amphetamine) involves changes in dopaminergic transmission, particularly in the mesolimbic dopamine system. These include augmentation of nucleus accumbus and striatal extracellular dopamine levels in response to a challenge with the sensitising drug (Kalivas and Duffy, 1993; Wolf *et al.*, 1993), a transient increase in the basal activity, or supersensitivity, of dopaminergic neurones in the VTA, and a subsensitivity of VTA dopamine D_2 autoreceptors (Henry *et al.*, 1998). More enduring cellular changes include supersensitivity of nucleus accumbens dopamine D_1 receptors, an effect that can persist for at least 1 month following withdrawal from cocaine (Henry and White, 1995). Over time, repeated exposure to drug that alters the activity and metabolism of cells, can alter the properties of individual cells and their circuitries. Subsequently, this can result in complex and changed behaviours (*e.g.* sensitisation, tolerance, dependence, craving: Koob 2000; Nestler and Aghajanian, 1997; Vezina, 2004).

Previous studies have suggested a role for the dopamine D_1 receptor in the striatal brain region. Increased inhibitory response of neurones in the nucleus accumbens to D_1 receptor agonists following chronic cocaine (Henry and White,

1991), and repeated administration of SKF-38393 enhanced the sensitivity of neurones in the nucleus accumbens and caudate putamen to D_1 receptor agonists (White *et al.*, 1990; Henry and White, 1991; and other work by White's laboratory). Like other psychostimulants, daily systemic administration of nicotine is known to sensitise ambulatory stimulant effect in rats (Clarke, 1987).

However considered as a psychostimulant, nicotine does differ in mechanism of action at a molecular level compared to other psychostimulants. Cocaine and amphetamine act directly on dopaminergic neurotransmission by blocking dopamine uptake and stimulate release, respectively, whereas nicotine binds to nAChR's and indirectly affects dopaminergic neurones (see for review Mash and Staley, 1997). Moreover, nicotine effects on behaviour are often much weaker compared to cocaine or amphetamine. Earlier studies indirectly implicated a role for dopamine D_1 receptors in psychostimulant induced locomotor sensitisation as it has been shown that systemic SCH-23390 can block, or at least attenuate, sensitisation to amphetamine (*e.g.* Vezina and Stewart, 1989; Drew and Glick, 1990), cocaine (*e.g.* McCreary and Marsden, 1993; Kuribara and Uchihashi, 1993; Mattingly *et al.*, 1996), 3,4-methylenedioxymethylamphetamine (MDMA: *e.g.* Ramos *et al.*, 2004), and other dopaminergic agonists. However, the effect of nicotine on behavioural sensitisation is less well understood and the effect of SCH-23390 on nicotine induced behavioural sensitisation has not been investigated yet. Therefore present study investigates the effect of the dopamine D_1 antagonist SCH-23390 on the expression of nicotine induced sensitisation.

HYPOTHESES AND AIMS

Hypothesis:

The dopamine D_1 receptor is suggested to be involved in the underlying mechanism of behavioural sensitisation. Herein it is hypothesised that a dopamine D_1 receptor antagonist will block the expression of nicotine induced locomotor sensitisation.

Chapter 2 The effect of dopamine D_1 receptor blockade on nicotine induced behavioural sensitisation

Aims:

- Induce behavioural locomotor activity sensitisation by chronic intermittent nicotine administration.
- Show that the sensitised response is a relatively long lasting phenomenon by examination of sensitisation following a relative short and relative long withdrawal period.
- Investigate if a dopamine D_1 antagonist blocks the expression of nicotine induced locomotor sensitisation.
- Establish a robust *in vivo* behavioural (locomotor) sensitisation model which can be used in future studies investigating putative molecular changes which occur as a result of locomotor sensitisation (see Chapter 5, cAMP accumulation and Chapter 6, receptor binding).

2.2 MATERIALS AND METHODS

The described *in vivo* behavioural experiments were performed at Abbott Healthcare Products, the Netherlands.

2.2.1. Drugs

(-)-Nicotine dihydrate ditartrate and saline were purchased from Acros Organics (Geel, Belgium). (-)-Nicotine dehydrate tartrate (1.228 mg/mL) was dissolved in 0.9% sterile saline and adjusted to pH 5.0 with NaOH/HCl. The final dose for administration was 0.4 mg/kg (-)-nicotine (measured as the freebase). SCH-23390 was purchased from Sigma-Aldrich (Amsterdam, the Netherlands), dissolved in saline and adjusted to pH 6.6 with NaOH/HCl.

(-)-Nicotine and saline were administered subcutaneously (s.c.) and SCH-23390 and corresponding vehicle were given by intraperitoneal (i.p.) administration. All nicotine injections were in volumes of 1 mL/kg body weight, drugs and corresponding vehicles were administered in volumes of 2 mL/kg.

2.2.2 Animals

Subjects were naïve adult male Wistar rats (HsdCpb:WU, Harlan, Horst, the Netherlands) weighing between 250-270 g at delivery. Animals were housed 2 rats/cage in a temperature (21 ± 1 °C) and humidity (40-50 %) controlled environment, and were habituated for 1 week prior to experimentation. The rats had ad libitum access to food (RM1 (E) pellets, SDS Special Diet Service, England) and tap-water, except during experimental sessions. Lighting was maintained under a 12 h light-dark cycle (lights on 06:00-18:00 h). All experimental procedures were performed between 08:00 and 16:00 h. The methodology was approved by the animal ethics committee of Abbott Healthcare Products, the Netherlands, and is in accordance with all local laws.

2.2.3 Locomotor activity measurements

Animals were matched for body weight and randomly assigned to treatment groups. Locomotor activity measurements were performed using 24 transparent perspex cages (21 x 36 x 18 cm) which were placed between 7 photobeams. Locomotor activity (ambulatory movement) was monitored and quantified using "Photobeam Activity System" software (PAS; San Diego Instruments, San Diego, CA, USA).

2.2.4. Experimental designs

All experiments in this study used a dose of 0.4 mg/kg nicotine (s.c.) as previous studies in this laboratory and previous literature have shown that this dose is optimal to induce behavioural sensitisation (*e.g.* Domino, 2001). One day prior to each experiment (day 0), all animals were habituated to the activity cages for one h followed by a saline injection and locomotor activity was recorded.

Experiment 1. – Effect of acute and chronic nicotine administration on locomotor activity.

To assess the ability of nicotine to induce development of behavioural sensitisation, three groups of naïve rats (*n* = 8 rats/group) were habituated as described above. On the testing days (day 1 and 5) the rats were habituated for 60 min to the test apparatus, before administration of either saline (s.c., 2 groups) or (-)-nicotine (0.4 mg/kg, s.c., 1 group). After receiving the injections, the rats were directly placed back in the test apparatus and locomotor activity was measured for 45 min, in 5 min time epochs. This first time dose of nicotine was considered the acute dose. On day 2, 3 and 4 all groups received the same treatment as the day before and were directly placed back in their home cage.

After a 16 day withdrawal period (day 22), all groups received a saline injection (s.c.) and locomotor activity was measured to assess if the response was context-dependent. On day 23 (*i.e.* after a 17 day withdrawal period), rats were habituated for 60 min to the test apparatus prior to administration of either a saline (s.c., 1 vehicle group) or (-)-nicotine challenge (0.4 mg/kg, s.c., 1 vehicle group and 1 nicotine group). The rats were directly placed back in the test

apparatus and locomotor activity was measured for 45 min, in 5 min time epochs. See Table 2-1 for study design.

Table 2-1 Study design chronic nicotine administration

Group	Pre-treatment (development)	Pre-challenge	Challenge (expression)
VEH – VEH	5 Saline injections	1 Saline injection	7th dose of Saline
VEH – NIC	5 Saline injections	1 Saline injection	1st dose of Nicotine
NIC – NIC	5 Nicotine injections	1 Saline injection	6th dose of Nicotine

VEH, vehicle; NIC, nicotine

Experiment 2. – Effect of a nicotine challenge after chronic nicotine followed by a 5 days withdrawal period.

In a second experiment, three groups of naïve rats (*n* = 12 rats/group) rats were treated following the same protocol as described in 'Experiment 1'. However, in this experiment a shorter withdrawal period was investigated and rats received a saline injection at day 9, and the nicotine challenge at day 10 (*i.e.* after 5 days of withdrawal). See Figure 2-1 for the treatment scheme.

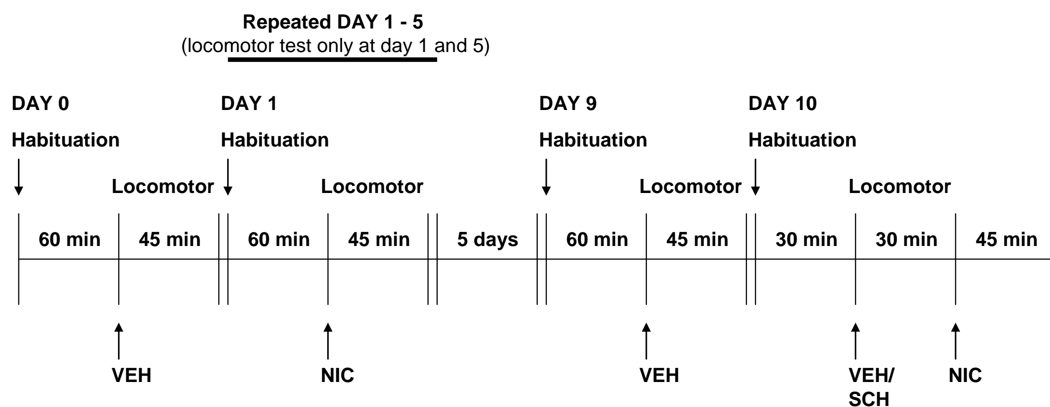


Figure 2-1 Treatment scheme to induced locomotor sensitisation

To study habituation, development and expression (including pretreatment) of locomotor sensitisation the following paradigm was used as described in this figure. VEH, vehicle; NIC, nicotine; SCH, SCH-23390.

Experiment 3. – Effect of SCH-23390 on spontaneous locomotor activity and the expression of nicotine induced locomotor sensitisation.

Six groups of naïve rats (*n* = 8 rats/group) were habituated to the activity cages one day prior to the experiment (day 0). On the testing days (day 1 and 5) the rats were habituated for 60 min to the test apparatus, before administration of either saline (s.c., 2 groups) or (-)-nicotine (0.4 mg/kg, s.c., 4 groups). After the injections, the rats were directly placed back in the test apparatus and locomotor activity was measured for 45 min. On day 2, 3 and 4 all groups received the same injections as given on day 1 and were directly placed back in the home cage.

After a 16 day withdrawal period (day 22), all groups received a saline injection (s.c.) and locomotor activity was measured. After a 17 day withdrawal period (day 23), rats were habituated for 60 min to the test apparatus before administration of a pre-challenge of either saline (s.c., 2 vehicle groups and 1 nicotine group) or SCH-23390 (0.003, 0.01 or 0.03 mg/kg, i.p., to the 3 nicotine groups respectively). The rats were directly placed back in the test apparatus and locomotor activity was measured for 30 min. After 30 min of either saline or SCH-23390 administration, one of the saline groups received another saline challenge and all other groups were challenged with 0.4 mg/kg (-)-nicotine (s.c.). Locomotor activity was measured for another 45 min, as previously described.

Experiment 4. – Effect of SCH-23390 (0.03 mg/kg) on the expression of nicotine induced sensitisation.

In this experiment, four groups of naïve rats (*n* = 12 rats/group) were treated following the same protocol as described in “Experiment 2.” to develop nicotine sensitisation. After 4 days of withdrawal, rats were given a saline injection and locomotor activity was assessed as previously described. On day 10, rats were habituated for 60 min to the test apparatus before administration of a pre-challenge of either saline (s.c., 1 vehicle group and 2 nicotine groups) or SCH-23390 (0.03 mg/kg, i.p., third nicotine group). The rats were directly placed back in the test apparatus and locomotor activity was measured for 30 min. After 30 min of either saline or SCH-23390 administration, the Vehicle - Vehicle group was challenged with saline and all other groups were challenged with 0.4 mg/kg

(-)-nicotine (s.c.). Locomotor activity was measured for another 45 min, as previously described. See Table 2-2 for study design.

Table 2-2 Study design chronic nicotine with SCH-23390 pretreatment

Group	Pre-treatment (development)	Pre-challenge	Challenge (expression)
VEH - VEH	5 Saline injections	1 Saline injection	7th dose of Saline
VEH - NIC	5 Saline injections	1 Saline injection	1st dose of Nicotine
NIC - NIC	5 Nicotine injections	1 Saline injection	6th dose of Nicotine
NIC - SCH/NIC	5 Nicotine injections	1 SCH injection	6th dose of Nicotine

VEH, vehicle; NIC, nicotine; SCH, SCH-23390

2.2.5. Data analyses

Data were analysed and presented as mean total amount of ambulant movements (beam breaks) over a period of 30 min (pretreatment) or 45 min (challenge) after injection ± standard error of the mean (s.e.m.). Data was taken from all animals regardless of their behavioural (sensitised) response and mean values were calculated. Graphs and statistical analyses were performed using Prism graphing software (GraphPad, USA). The main effects of drug treatments were analysed using one-way analyses of variance (ANOVA): [F (df_b, df_w) = x; P = x]. When a significant difference among the treatments was obtained using an ANOVA, this was followed by a *post hoc* Bonferroni's multiple comparison test analyses where appropriate. Differences between day 1 vs. day 5 were analysed using an unpaired two-tailed t-test. Data with $P < 0.05$ were considered as significant.

Methodological considerations: Statistics

The experimental design of pre-treatment of animals to induce sensitisation before administration of a challenge was considered as a 2 factor (*i.e.* a within-subjects factor of day and between-subjects factor of treatment) and would require a two-way ANOVA. However, the factor of day was not quantitative and therefore a one-way ANOVA was performed.

2.3 RESULTS

One day prior to each experiment (day 0), all animals were given a subcutaneous (s.c.) saline injection to assess the basal response to injection in addition to habituate the rats to the experimental conditions (*e.g.* test apparatus, handling and injections). There was no significant difference found in locomotor activity between the experimental and control groups in response to saline on day 0 ([F (2, 20) = 1.175; P = 0.3293]), and therefore all animals were used to perform this study.

Experiment 1. – Effect of acute and chronic nicotine administration on locomotor activity.

The effect of acute nicotine on locomotor activity in Wistar rats was investigated. Two groups received saline (s.c.) and one group nicotine (0.4 mg/kg, s.c.), and locomotor activity was measured for 45 min (Figure 2-2A). There was a significant treatment effect ([F (2, 20) = 12.46; P = 0.0003]), and *post hoc* testing demonstrated that acute nicotine produced a significant (P < 0.01), but modest, increase in locomotor response compared to vehicle groups (see Figure 2-2B).

The two saline groups and the nicotine group all received their treatment intermittently, ones a day for five consecutive days. Statistical analysis using ANOVA showed a significant treatment effect ([F (2, 20) = 46.12; P < 0.0001]). *Post hoc* testing demonstrated that the groups which received chronic saline did not showed any change in locomotor activity after 5 days of treatment, however, the animals which received chronic nicotine (0.4 mg/kg, s.c.) for five days showed an increased locomotor response which is consistent with the development of behavioural sensitisation (Figure 2-2C and D). This effect was only observed in the nicotine group and was well established by the 5th (challenge) dose.

To demonstrate that behavioural sensitisation was not conditioned to the test apparatus, all groups (both vehicle and nicotine groups) were given a saline injection (s.c.) after a 16 days withdrawal period (day 22). There was no significant difference in locomotor activity between the treatment groups after the

saline injection [*F* (2, 20) = 0.09662; *P* = 0.9083], see Figure 2-3A and B. This suggests that there was no evidence of a conditioned response (*i.e.* it was a context-independent response) from the repeated pairing of nicotine with the injection or test cage.

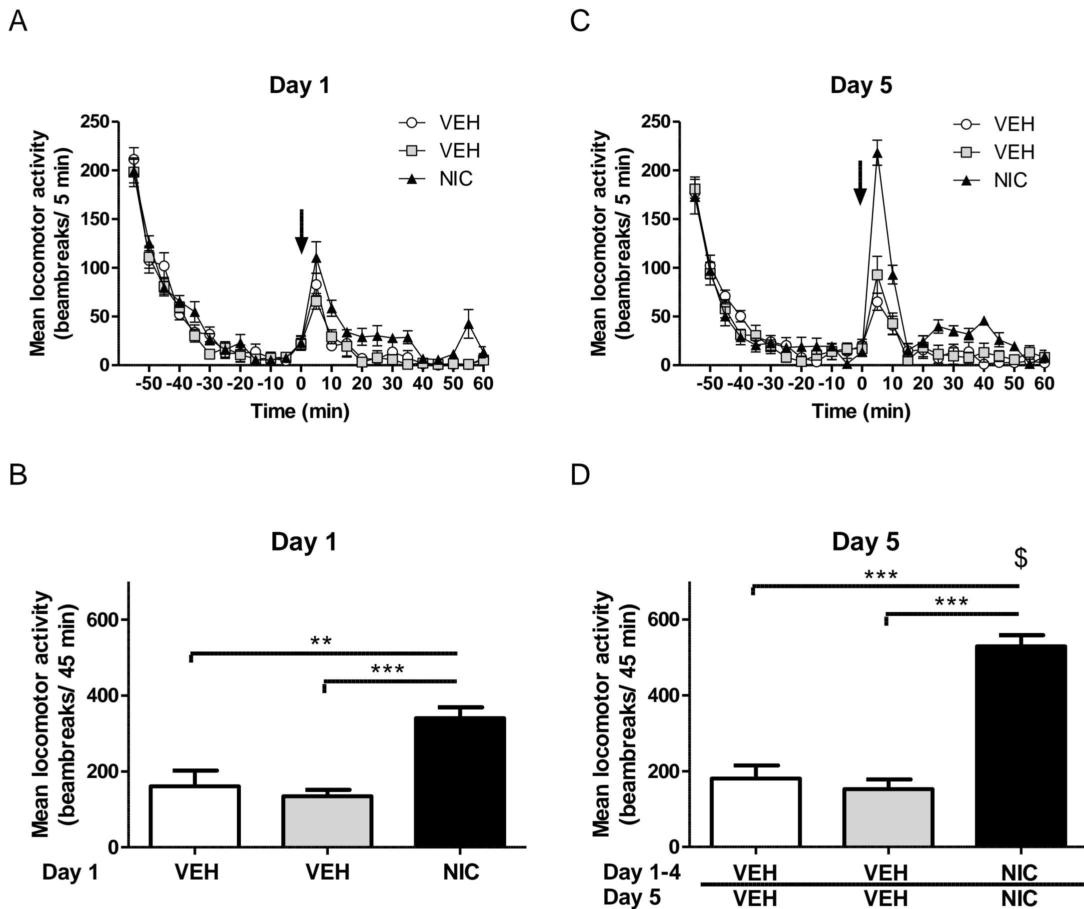


Figure 2-2 Effect of acute and chronic nicotine on locomotor activity in rats

A. Effect of acute nicotine administration in naïve rats. Changes in locomotor activity over time (min) in response to an acute dose of nicotine (see Table 2-1 for study design). Data represent mean ± s.e.m., *n* = 8 animals per group. B. Mean locomotor activity ± s.e.m. of 45 min after acute nicotine administration (0.4 mg/kg, s.c.). C. Effect of a nicotine challenge after chronic nicotine pretreatment on locomotor activity. D. Chronic nicotine administration significantly increased locomotor activity compared to vehicle ($P < 0.001$). Moreover, the response after chronic nicotine treatment was increased (*i.e.* sensitisation) compared to acute nicotine (unpaired t-test day 1 vs day 5, $ $P < 0.001$). *Post hoc* Bonferroni's, ** $P < 0.01$, *** $P < 0.001$. $n = 8$ animals/group.

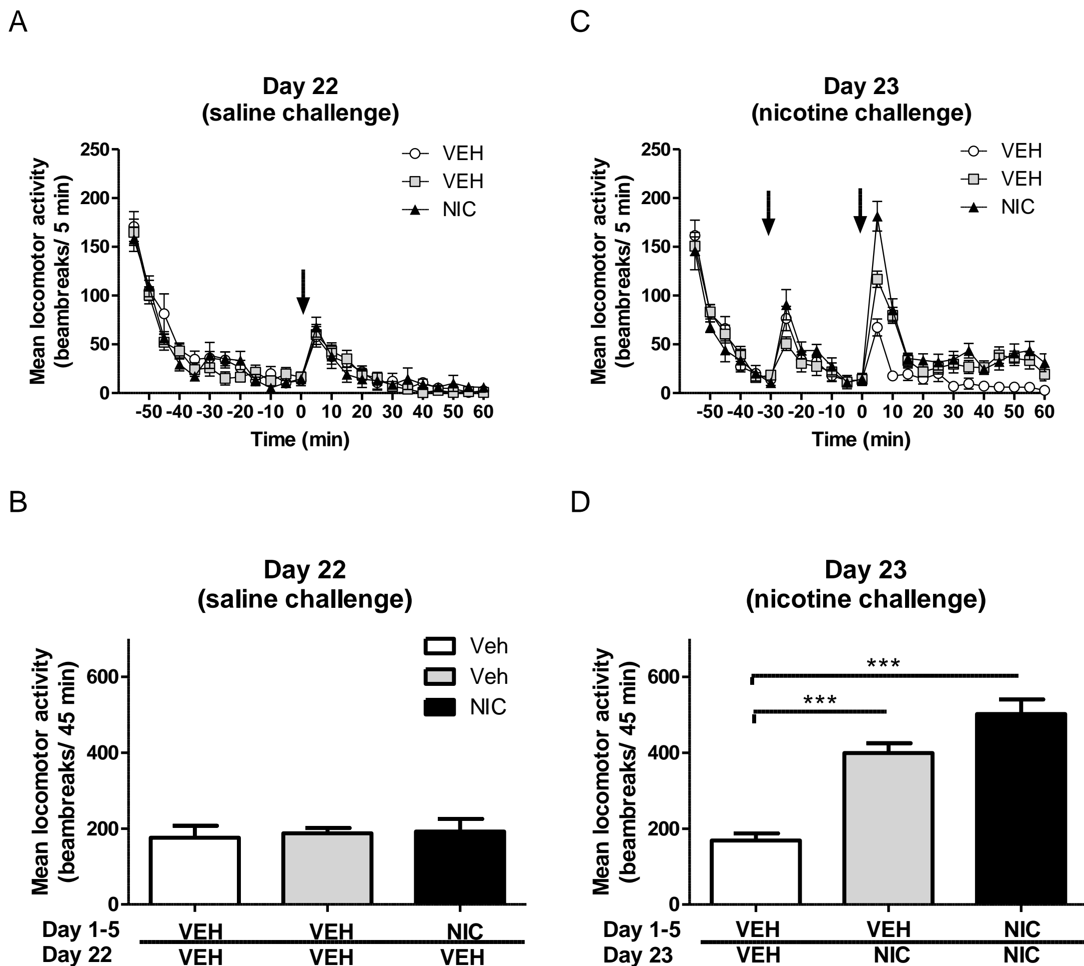


Figure 2-3 Effect of a challenge injection after a 17 days withdrawal period

A. Effect of a saline challenge on locomotor activity after chronic nicotine treatment followed by a 17 day withdrawal period. B. Mean locomotor activity ± s.e.m. of 45 min after a saline challenge. No significant difference between the groups was observed. C. Locomotor activity of a saline injection followed by a nicotine challenge. D. Effect of a nicotine challenge after chronic nicotine treatment followed by a 17 day withdrawal period [$F\,(2, 20) = 37.86$; $P < 0.0001$]. *Post hoc* Bonferroni's, ** $P < 0.01$, *** $P < 0.001$. $n = 8$ animals/group.

After the development of nicotine sensitisation, followed by a 17 day withdrawal period, all groups received a challenge: The chronic vehicle group received saline (s.c.), the second vehicle group received for the first time nicotine (0.4 mg/kg, s.c.), and the chronic nicotine group received a nicotine challenge (0.4 mg/kg,

s.c.). The vehicle and acute nicotine groups gave equal responses compared to vehicle and acute nicotine at day 1 (Figure 2-3D vs. Figure 2-2B). The chronic nicotine treated group showed equal response compared to day 5 (Figure 2-3D vs. Figure 2-2D); *i.e.* expression of behavioural sensitisation. These data show that the behavioural sensitised response is a long lasting phenomenon.

Experiment 2. – Effect of a nicotine challenge after chronic nicotine followed by a 5 days withdrawal period.

A second experiment was performed using the exact same treatment as described in "Experiment 1.", but this time using a shorter (*i.e.* 5 days) withdrawal period. Thirty six rats were randomly divided into three groups of 12 rats each. Locomotor sensitisation was developed by daily nicotine administration for 5 consecutive days and was comparable to previous findings ('Experiment 1'). The development of sensitisation was followed by a relative short withdrawal period, and all groups received a saline injection at day 9. One-way ANOVA showed a significant effect [F (2, 33) = 7.342; $P < 0.0023$] and *post hoc* Bonferroni's analysis revealed that the chronic nicotine group was modest but significantly increased compared to only one of the vehicle groups ($P < 0.01$, Figure 2-4A). After 5 days of withdrawal (*i.e.* day 10), one vehicle group received saline and the other vehicle and nicotine groups received a nicotine challenge (0.4 mg/kg, s.c.). One-way ANOVA analysis showed that there was a statistical difference [F (2, 33) = 37.74; $P < 0.0001$] and *post hoc* Bonferroni's showed that all groups were significantly different to each other Figure 2-4B. Locomotor activity was significantly enhanced after acute nicotine administration ($P < 0.001$ vs vehicle) and after chronic nicotine treatment ($P < 0.001$ vs vehicle) (Figure 2-4B). Furthermore, the nicotine challenge showed a larger response in rats sub-chronically preatreated with nicotine, compared to rats which received nicotine for the first time (pretreated vs. acute, $P < 0.01$), which represents the expression of nicotine sensitisation.

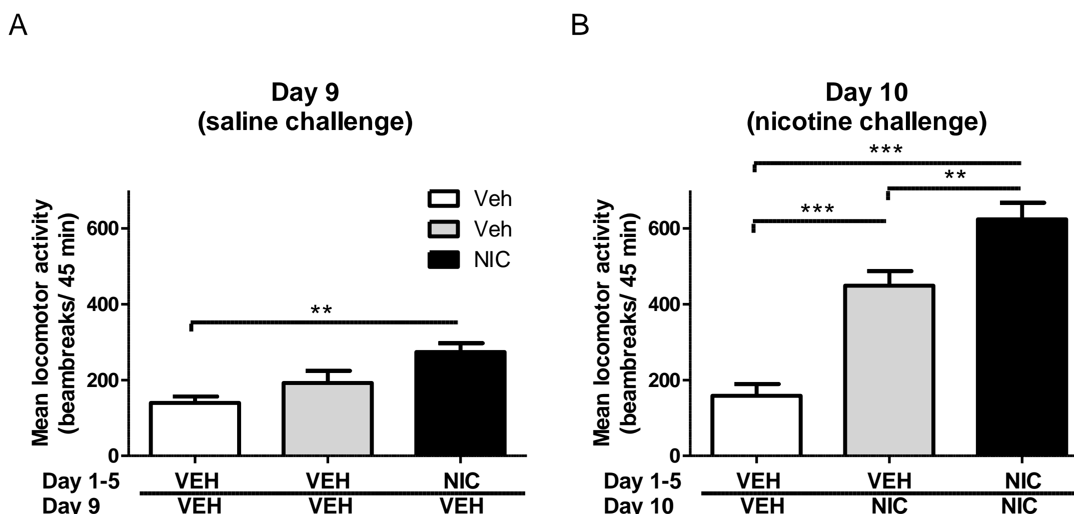


Figure 2-4 Effect of acute and chronic nicotine on locomotor activity

A. Effect of a saline injection to all treatment groups after a 4 day withdrawal period. The nicotine pretreated group showed a significant increase compared to only 1 vehicle group. B. Effect of a nicotine challenge after 5 days of withdrawal. *Post hoc* Bonferroni's, ** $P < 0.01$, *** $P < 0.001$. $n = 12$ animals/group.

Statistical comparison of the nicotine sensitised responses after 5 and 17 days of withdrawal showed that the responses were not different to each other (unpaired t-test, $P > 0.05$). The observed effects of nicotine induced sensitisation were found very robust during this study where the experiment was repeated 13 times (for behavioural characterisation and in order to obtain tissue for *ex vivo* studies, see Table 5-1 and Chapter 5) and showed high reproducibility.

Experiment 3. – Effect of SCH-23390 on spontaneous locomotor activity and the expression of nicotine induced locomotor sensitisation.

Nicotine sensitisation was developed with the same results as described in "Experiment 1.", $P < 0.001$ (data not shown).

On day 23, *i.e.* after 17 days of withdrawal, the three additional chronicnicotine treated groups were pretreated with different doses of SCH-23390 (0.003, 0.01 and 0.03 mg/kg, i.p.) and the effect of SCH-23390 on spontaneous activity was investigated. No significant treatment effect of SCH-23390 was observed using one-way ANOVA [F (5, 41) = 0.7219; P = 0.6108], see Figure 2-5A and Figure

2-5B. These results show that SCH-23390 does not cross-sensitise with nicotine. Furthermore, the effect of SCH-23390 pretreatment on the expression of nicotine induced sensitisation after 17 days (day 23) of withdrawal was studied. When SCH-23390 was pre-administered 30 min prior to the nicotine challenge, SCH-23390 induced a dose dependent attenuation of nicotine sensitisation (Figure 2-5B).

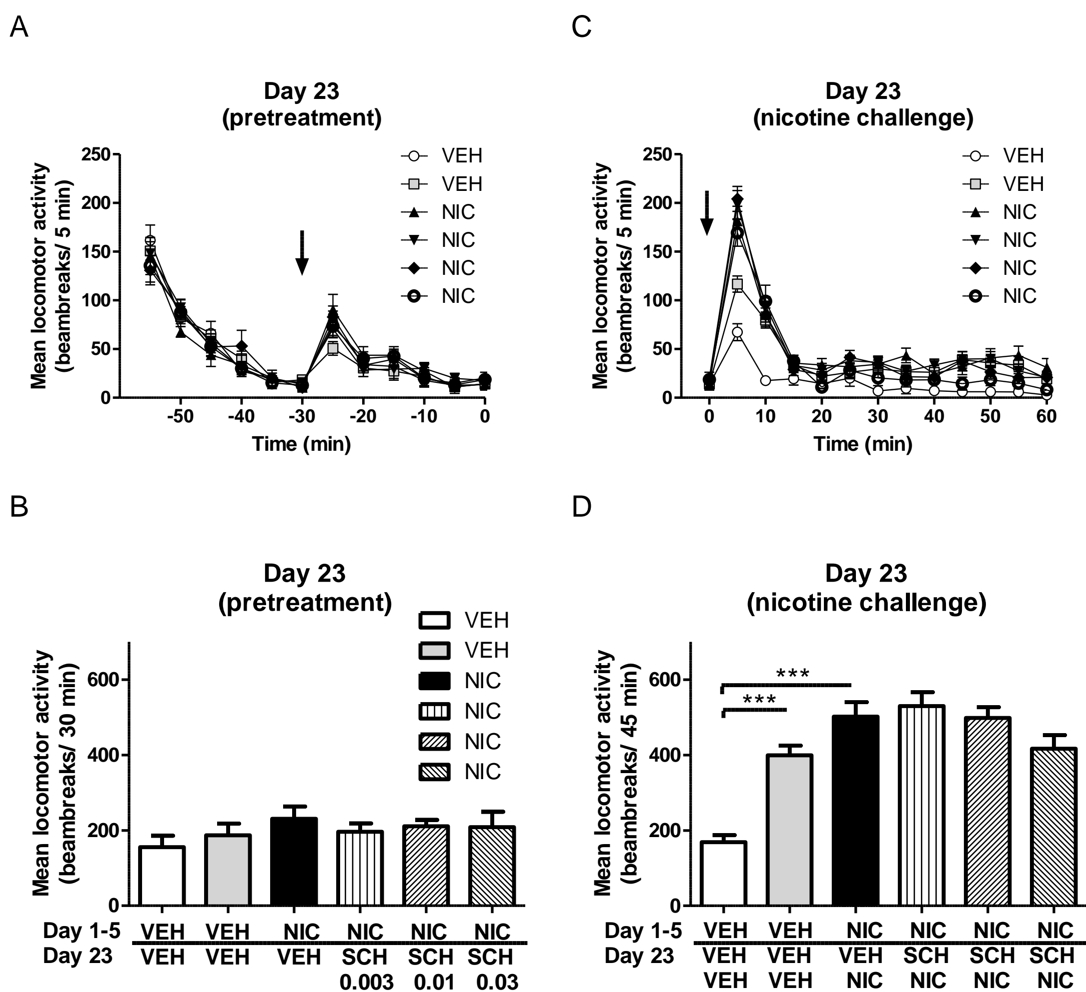


Figure 2-5 Effect of SCH-23390 pretreatment on the expression of nicotine induced sensitisation after 17 days (day 23) of withdrawal

A/B. The first three groups were given saline and the last three groups SCH-23390 (0.003, 0.01, 0.03 mg/kg) was given 30 min prior to nicotine. One-way ANOVA showed no significant effect of either saline or the three tested dose of SCH-23390. C/D. All groups were given a nicotine challenge, except from one

vehicle group [F (5, 41) = 18.27; $P < 0.0001$]. Acute and sub-chronic nicotine control groups showed a significant increase and sensitised response, respectively. The three SCH-23390 pre-treated groups were significantly different compared to vehicle ($P < 0.001$), but were not significantly different compared to acute or chronic nicotine. *Post hoc* Bonferroni's, *** $P < 0.001$. $n = 8$ animals/group.

Experiment 4. – Effect of SCH-23390 (0.03 mg/kg) on the expression of nicotine induced sensitisation.

In a separate experiment, the effect of SCH-23390 pretreatment on the expression of nicotine induced sensitisation after 5 days of withdrawal was studied. 48 animals were randomly assigned to four groups: vehicle - vehicle (pre-treated and challenged with saline); vehicle - nicotine (pre-treated with saline and challenged with nicotine); nicotine - nicotine (pre-treated and challenged with nicotine) and, nicotine – SCH/NIC (pre-treated with nicotine and challenged with SCH-23390 followed by nicotine challenge), see Table 2-2 for study design.

Nicotine induced locomotor sensitisation was developed following 5 days of chronic nicotine treatment with similar results compared to previous experiments ("Experiment 1."). After a withdrawal period of 5 days, rats were given a pre-challenge of saline (vehicle) or SCH-23390 30 min prior to the nicotine challenge. After the nicotine challenge to the nicotine - nicotine group, a statistically significant increase in locomotor activity was observed [F (3, 44) = 34.15; $P < 0.0001$]. Furthermore, it was found that SCH-23390 pretreatment (0.03 mg/kg, i.p.) significantly blocked the expression of nicotine sensitisation (*post hoc* Bonferroni's, ** $P < 0.01$), see Figure 2-6.

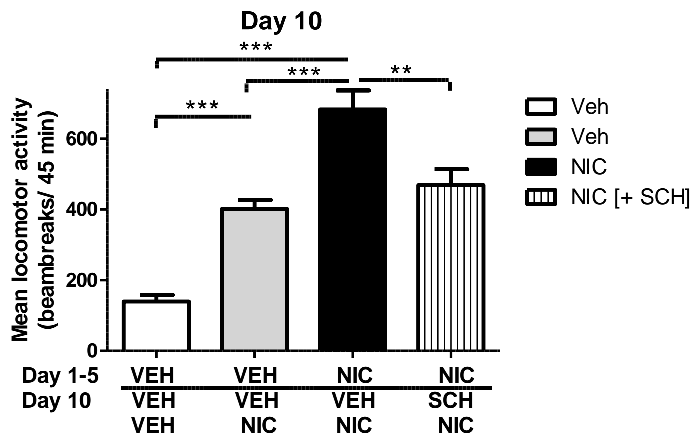


Figure 2-6 Effect of SCH-23390 pretreatment on the expression of nicotine induced sensitisation after 5 days of withdrawal

Effect of SCH-23390 (0.03 mg/kg, i.p.) pretreatment on the expression of nicotine induced locomotor sensitisation after 5 days of withdrawal (*i.e.* day 10). SCH-23390 pre-treated group was significantly different to vehicle *** $P < 0.001$. *Post hoc* Bonferroni's, ** $P < 0.01$, *** $P < 0.001$. $n = 12$ animals/group.

2.4 DISCUSSION

This study investigated the effect of repeated intermittent nicotine administration on locomotor activity and the effect a dopamine D_1 antagonist on the expression of nicotine induced behavioural sensitisation. This study demonstrated for the first time that SCH-23390 (a selective dopamine D_1 receptor antagonist) dose dependently blocked the expression of nicotine induced behavioural locomotor sensitisation in the rat.

Nicotine is a non-specific agonist of nicotinic acetylcholinergic receptors (nAChR; see Chapter 6). In rodents, nicotine alters locomotor activity (Clarke and Kumar, 1983; Ksir *et al.*, 1985). The present results demonstrated that acute nicotine increased locomotor activity. However, some previous studies also did report opposing effects, *i.e.* increasing, decreasing, or no effect on locomotor activity after acute nicotine (Domino, 2001; Palmatier and Bevins, 2002). These contradictory results may be due to methodological differences between studies. Different environmental experience (*i.e.* habituation) to the animal prior to nicotine injection can give opposing observations. For example, nicotine induced highly reproducible, but modest, hyperactivity in rats that had been habituated to the test cage; however, it produced only a small increase in locomotion in animals not previously exposed to the test environment (O'Neill *et al.*, 1991). In the present study, the animals were habituated (including saline injection) one day before acute nicotine treatment (*i.e.* Day 0) and at the beginning of each experimental day. The animals showed an increased locomotor activity in the first 30 min, this is probably due exploratory behaviour of a novel environment. Chronic nicotine and saline treated animals showed equal exploratory activity in the first 30 min when exposed to the test cage and was the same for all days (*i.e.* day 1, day 5 or after withdrawal), indicating no significant effect on habituation or no conditioning effect of the test environment. On treatment days 2, 3, and 4 the animals received the treatment in their home cage instead of in a test cage. As discussed before, the development of sensitisation can be dependent on the test environment. Therefore, it should be noted that, in this case, both the test cages and homecages were of the same design. It is therefore unlikely that appreciable

differences in the test and home cage environment contribute tot “differential conditioning” of the two environments. Nonetheless, behavioural sensitisation following repeated intermittent nicotine administration was observed regardless of the observed first-time (acute) effect of nicotine (Domino, 2001).

In the present study, nicotine induced locomotor activity sensitisation was used as a model for behavioural sensitisation. Chronic (*i.e.* 5 days intermittent) nicotine administration increased ambulatory locomotor activity in rats in a time-dependent manner. After 5 days of treatment a plateau of locomotor sensitisation development was achieved. Another aspect of behavioural sensitisation is the expression phase. A phenomenon that is characterised by expression of the same sensitised response to a challenge after a certain withdrawal period. Behavioural sensitisation is known to be a long lasting phenomenon and present study examined the expression of sensitisation after two withdrawal periods, *i.e.* a relative long 17-days and relative short 5-days period. The first expression experiments performed in this study used a 17 day withdrawal period and nicotine sensitisation was shown long lasting (*e.g.* as shown in “Experiment 1.”). Although the chronic nicotine administration was shown to be a robust paradigm, a shorter withdrawal period was assessed and as expected nicotine sensitisation was also expressed after 5 days of withdrawal (as shown in “Experiment 2.”). Moreover, the 5 days paradigm showed an even greater magnitude (*i.e.* more locomotor activity counts) of the expression phase of sensitisation, showing that the induced sensitisation is a long lasting phenomenon however is attenuating over time.

This study investigated the hypothesis that the dopamine D_1 receptor is involved in expression of behavioural sensitisation. First, it was shown that SCH-23390 at different doses did not alter basal locomotor activity (Figure 2-5B) which was in agreement with previous publications (O’Neill *et al.*, 1991). However, it should be noted that present data did not include the effect of SCH-23390 on nicotine induced locomotor activity in experimentally naïve animals, but only chronic nicotine treated animals. Previous literature, however, showed that SCH-23390 (0.001, 0.01 and 0.1 mg/kg) dose dependently blocked the effect of acute nicotine administration on locomotor activity (O’Neill *et al.*, 1991). Furthermore, it

has been reported that SCH-23390 blocks the acquisition of amphetamine induced locomotor sensitisation (e.g. Vezina and Stewart, 1989) but not cocaine induced locomotor sensitisation (e.g. Mattingly et al., 1996) in the rat. No data is available for nicotine induced sensitisation. Together with other examples not mentioned inhere, the effect of SCH-23390 on the development of behavioural sensitisation is not consistent and therefore its effect on nicotine induced sensitisation should be determined empirically. Although SCH-23390 blocks the effect of acute nicotine, it is hypothesised that SCH-23390 will also block the development of nicotine induced locomotor sensitisation.
The effect of the dopamine D_1 receptor antagonist SCH-23390 on the expression of nicotine sensitisation after different periods of withdrawal was examined. After a relative long withdrawal period of 17-days, SCH-23390 pretreatment did not significantly blocked the expression of nicotine induced sensitisation, although it was shown that SCH-23390 attenuated the expression of sensitisation ("Experiment 3."). This could be explained by the chronic nicotine treated group which was not significant different from the acute nicotine group, which is normally significant as showed in "Experiment 1" and "Experiment 2". There was only a small pharmacological window to significantly block the sensitised nicotine response by SCH-23390, and the expression of nicotine sensitisation was more evident after 5 days of withdrawal compared to 17 days ("Experiment 1 and 2"). Therefore the expression study using SCH-23390 was repeated with a high dose of SCH-23390 (0.03 mg/kg) and assessed after 5 days of withdrawal. After a shorter withdrawal period of 5 days, SCH-23390 (0.03 mg/kg, i.p.) did significantly block the expression of nicotine sensitisation ("Experiment 4.").

This study describes SCH-23390 as a selective dopamine D_1 antagonist although *in vitro* SCH-23390 also showed high affinity for the serotonin $5HT_2$ receptor subtype (Millan *et al.*, 2001; see Chapter 7 for more discussion). However, the doses required to induce a similar response *in vivo* are greater than 10-fold higher than those required to induce a dopamine D_1 mediated response (for review see Bourne, 2001). Interestingly, $5HT_2$ receptor agonists do also block nicotine induced sensitisation. For example, 2,5-dimethoxy-(4-chlorophenyl)-isopropylamine (DOI: a selective $5HT_2$ agonist) blocks the expression but not the acquisition of nicotine sensitisation (Olausson *et al.*, 2001) and Ro-60-0175 (a

$5HT_{2C}$ agonist) blocks the acquisition of nicotine induced sensitisation (Grottick *et al.*, 2001). And $5HT_{2C}$ agonists Ro-60-0175 and WAY-163,909, but not $5HT_{2C}$ antagonist SB-242,084, attenuated the expression of nicotine induced sensitisation after a 5 day withdrawal period following a 5 day intermittent nicotine administration (Zaniewska *et al.*, 2007). Although unlikely, it should be kept in mind that activation of $5HT_2$ receptors by SCH-23390 may contribute to its behavioural properties both in animals as well as in human.

Conclusions

In conclusion, the present study showed that repeated nicotine administration can develope sensitisation to behavioural locomotor activity in the rat and is a long lasting phenomenon. The present results provide evidence for the pharmacological attenuation of the expression of nicotine induced behavioural sensitisation by SCH-23390, therefore suggesting a role for the dopamine D_1 receptor and potential dopamine D_1 ligand pharmacotherapies. However, due to the complexity of brain signalling it is likely that other neurotransmitter systems (*e.g.* serotonergic, noradrenergic, and cholinergic) can play a role as well, and other classes of ligands may also be considered as potential pharmacotherapies. Nonetheless, it is clear from this study and supportive available literature that dopamine and dopamine D_1 receptors play a particularly important role in behavioural sensitisation. This model is now ready for further studies investigating the molecular mechanisms of behavioural sensitisation.

Chapter 3. The effect of nicotine and dopamine D_1 ligands on ex vivo dopamine release in the dorsal striatum

Abstract.

OBJECTIVES: The exact molecular mechanism of behavioural sensitisation is still yet unknown, however it is suggested that dopamine release plays an important role. Extensive research have been carried out to investigate the role of dopamine D_2 receptor mediated dopamine release, however the role of the dopamine D_1 receptor is less well studied. As shown in Chapter 2, nicotine induced behavioural sensitisation can be blocked by a dopamine D_1 receptor antagonist. This study investigated the effect of nicotine and dopamine D_1 receptor ligands on electrically evoked dopamine release *ex vivo* in corticostriatal slices.

METHODS: In this study, *ex vivo* fast cyclic voltammetry (FCV) was used to examine the effect of nicotine, SCH-23390 (D_1 antagonist) and SKF-38393 (D_1 agonist) on electrically evoked dopamine release. The release of dopamine was measured following stimulations at different frequencies, in dorsal striatal slices.

RESULTS: Nicotine induced a decrease in dopamine efflux at low frequency and an increase at high frequency electrical field stimulation. Neither SCH-23390 nor SKF-38393 altered single pulse or multiple pulse stimulated dopamine release on their own. However, both D_1 ligands blocked the nicotine induced decrease in dopamine release at single pulse, but not multiple pulse electrical stimulation.

CONCLUSIONS: These results showed that the frequency dependent electrical modulation of dopamine release can be altered by nicotine and moreover, for the first time, showed that this effect can be altered by dopamine D_1 ligands. These findings suggest a potential role of frequency dependent signalling by nicotine, which may contribute to identify the mechanism of behavioural sensitisation.

Keywords: FCV, fast cyclic voltammetry, dopamine release, nicotine, dopamine D_1 receptor, SCH-23390, SKF-38393, dorsal striatum, *ex vivo*

3.1 INTRODUCTION

Nicotine, like other psychostimulants, can induce behavioural sensitisation as previously discussed in Chapters 1 and 2. Moreover, nicotine, like other psychostimulants, is able to increase extracellular dopamine levels in the brain of rats after systemic administration, as measured by *in vivo* microdialysis (Anderson *et al.*, 1981; Imperato *et al.*, 1986; Robinson *et al.*, 1988; Brazell *et al.*, 1990) and by *in vivo* electrochemistry (Stamford *et al.*, 1984; Stamford, 1985; Robinson *et al.*, 2003; Robinson and Wightman, 2007). There is also evidence for increased extracellular dopamine levels in rats sensitised to amphetamine as measured *in vivo* and *ex vivo* (Jones *et al.*, 1996; Domino and Tsukada, 2009). It is suggested that repeated administration of a psychostimulant or contextual stimuli exposure (for example in the case of stress) affects brain neurochemistry and is mediated by increased dopamine release (Martin-Iverson *et al.*, 1993). More specifically, *ex vivo* studies have shown that nicotine, like other psychostimulants, can also induce dopamine release in striatal brain slices (*i.e.* superfusion using [^{3}H]-dopamine (Rowell *et al.*, 1987; Grady *et al.*, 1992; el-Bizri and Clarke, 1994), or by using fast cyclic voltammetry, FCV (Zhou *et al.*, 2001; Zhang *et al.*, 2009).

Dopamine transmission in the brain has an autoregulatory system by which dopamine D_2-like receptor activation will inhibit the further release of dopamine from the presynaptic cell. The effect of dopamine D_2 receptor stimulation or inhibition is well described as it is functionally coupled to presynaptic dopamine release and uptake. In contrast to the dopamine D_2-like receptors, the post synaptically located dopamine D_1-like receptors are not autoreceptors and therefore activation of the dopamine D_1 receptor will not directly regulate the release of dopamine. For example, neither the dopamine D_1 agonist 6-Cl-APB nor D_1 antagonist SCH-23390 altered single pulse dopamine release on their own, using *ex vivo* striatal slices (O'Neill *et al.*, 2007). Neither could the inhibitory effect of quinpirole (a selective D_2-like receptor agonist) on electrically stimulated dopamine release be antagonised by SCH-23390 (Palij *et al.*, 1990). Another study using electrically stimulated [^{3}H]-dopamine release from guinea pig brain

slices showed that the dopamine D_1 agonist SKF-38393 did not affect dopamine release. (-)-Sulpiride (a selective dopamine D_2-like receptor antagonist) reversed the inhibition induced by quinpirole, while SKF-38393 did not have any effect on quinpirole (Kondo *et al.*, 1986). A more detailed description of the dopamine D_1 receptor and intracellular signalling is given in Chapter 1.

As previously shown, psychostimulants are able to increase dopamine levels and induce behavioural sensitisation. However, only a limited number of studies investigated the putative correlation between these two phenomena *ex vivo*. One example is the examination of cocaine induced behavioural sensitisation on dopamine release, studied *ex vivo* in brain slices using superfusion and radiolabeled ligands. Bowyer and colleagues showed that in striatal slices from native animals, bath applied amphetamine evoked the release of [^{3}H]-dopamine and inhibited subsequent K^+-stimulated [^{3}H]-dopamine release (Bowyer *et al.*, 1987). A study by Yi and Johnson showed that chronic administration of cocaine abolished the stimulatory effect that amphetamine normally has on the efflux of [^{3}H]-dopamine from striatal synaptosomes, but K^+-stimulated release was not affected (Yi and Johnson, 1990). However, chronically administered cocaine, plus acute challenge with cocaine potentiated the effect of amphetamine on the K^+ induced release of [^{3}H]-dopamine (Yi and Johnson, 1990). In a study reported by Risso and colleagues, it was shown that chronic (-)-nicotine continuously administered through osmotic mini-pumps for 10 days did not affect K^+ (15 mM) stimulated [^{3}H]-dopamine release in rat striatum (Risso *et al.*, 2004) which is in consensus with behavioural data (as discussed in Chapter 2). In another study it was shown that chronic nicotine treatment (0.4 mg/kg, 14 days) neither affected basal nor electrically stimulated (20 mA, 2 Hz, 4 min) [^{3}H]-dopamine release in rat VTA slices (Amantea, 2004). However, the inhibitory effect of baclofen ($GABA_B$ agonist) on [^{3}H]-release was abolished after chronic nicotine treatment. These studies have shown that dopaminergic neurotransmission is affected after chronic psychostimulant administration. However, more evidence is needed to prove the importance of dopamine release in chronic psychostimulant induced behavioural sensitisation.

Stimulation of neurones by depolarisation using a high concentration potassium (K^+) as a stimulus is a common method to induce neurotransmitter release for *in vivo* and *ex vivo* studies (van der Werf *et al.*, 1987; Schlicker *et al.*, 1989; Hoffman *et al.*, 1998; Hoffman and Gerhardt, 1999; Molchanova *et al.*, 2004; Nickell *et al.*, 2005). However, this method does not represent the physiological temporal resolution of physiological stimulations in the intact brain. Neurotransmission is changed by normal experience or neuropathology and the actual neurotransmission operates on a millisecond timescale, and it is suggested that higher cognitive function and motor movement are regulated by the firing frequency of neurones. These neurones fire at different frequencies and can be categorised into tonic and phasic rhythms. Tonic regular firing of dopamine neurones occur at a rate of 2 – 5 Hz, and can increase to bursts of action potentials having a frequency of 15 – 100 Hz (Hyland *et al.*, 2002). These fast temporal processes can be mimicked by electrical stimulation using a bipolar stimulation electrode, and stimulations at specific frequencies. Additionally a technique to measure these signals with a high temporal resolution (*i.e.* subsecond) is required, for example fast electrochemical techniques (see below).

Constant potential amperometry, high-speed chronoamperometry, fast cyclic voltammetry (FCV) and differential pulse voltammetry (DPV) are the most common electrochemical techniques used to detect monoamine neurotransmitters (*i.e.*, serotonin, dopamine, noradrenaline, adenosine, and histamine). Each method has its advantages and disadvantages; however the technique of FCV is superior when chemical selectivity and high spatial as well as high temporal resolution are required. FCV is a potential sweep technique in which the background subtracted voltammogram gives additional information about the electrolysed species. The current response is measured over a range of potentials in which FCV is a better technique to discern additional current contributions from other electroactive species (*e.g.* 3,4-Dihydroxyphenylacetic acid or DOPAC, pH, and serotonin). FCV is a relatively fast technique with single scans typically recorded every 100 ms (*i.e.* 10 Hz). For more background information and limitation of the technique see the review by Wightman and colleagues (Robinson *et al.*, 2008). In particular, electrochemical detection using the technique of FCV in combination with a carbon fiber microelectrode has been

successfully demonstrated to characterise dopamine release and uptake *in vivo* and *in vitro* (Stamford *et al.*, 1988a; Robinson *et al.*, 2008).
In vitro brain slice preparations are often used to study processes involved in synaptic plasticity and to evaluate the role of native receptor subtypes in neurotransmission (Shankaranarayana Rao and Raju, 2004). Receptors capable of modulating dopamine release in the striatum are not exclusively expressed on dopaminergic terminals within the striatum. Therefore, *in vitro* approaches using more intact tissue preparations, like brain slices, allow recordings from semi-intact neural circuits and neighbouring influences with the advantage of mechanical stability and control over the extracellular environment. This study has focused on a specific area of the brain, the dorsal striatum, in isolated corticostriatal slices, and must not be seen as a single unit but as part of many inter brain pathway connections. Furthermore, this study focused on the dorsal striatal area. As mentioned before, there are important differences between the dorsal striatum and nucleus accumbens (and within the nucleus accumbens), also with respect to dopamine release probability as shown using FCV (Stamford *et al.*, 1988a; Zhang *et al.*, 2009).

In addition to the radiolabelled superfusion studies which were mentioned before, there are some reports which investigated *ex vivo* dopamine release following behavioural sensitisation using FCV. For example, Muscat and colleagues studied the effect of quinpirole- and amphetamine induced behavioural sensitisation on *ex vivo* dopamine release after 3 days of withdrawal, as measured by fast cyclic voltammetry (Muscat *et al.*, 1993). They showed that single pulse evoked dopamine release in rat nucleus accumbal slices was not different after behavioural sensitisation, but that multiple pulse stimulated dopamine release was enhanced in quinpirole- and attenuated in amphetamine sensitised rats. Furthermore, it was shown that the potency of quinpirole to inhibit single pulse stimulated dopamine was significantly increased in quinpirole sensitised, but decreased in amphetamine sensitised rats. Also behavioural sensitisation induced by repeated stress can increase extracellular dopamine concentrations in the nucleus accumbens and striatum, as determined by high-speed chronoamperometry (Doherty and Gratton, 1992).

In contrast to the number of studies assessing the effect of the dopamine D_2 receptor activation or inhibition on dopamine release, considerably less attention has been paid to the role of the dopamine D_1 receptor. Nonetheless, the dopamine D_1 receptor is still very important in dopaminergic neurotransmission. As shown in Chapter 2 of this thesis, dopamine D_1 ligands can alter behavioural sensitisation suggesting an important role for dopamine D_1 receptor mediated neurotransmission in the mechanism underlying behavioural sensitisation. The experiments in this study have examined the contribution of dopamine D_1 receptor activation or inhibition and the effect of nicotine on dopamine release in the dorsal striatum using FCV.

HYPOTHESES AND AIMS

Hypotheses:

Nicotine induced behavioural locomotor sensitisation results from impairment in the dopaminergic system in the dorsal striatum, specifically by the dopamine D_1 receptor.

Aims:

- Study putative changes of basal and electrically stimulated dopamine release in corticostriatal slices.

- Studying the cellular and synaptic mechanisms by assessing the effect of dopamine D_1 receptor ligands on stimulated dopamine release, which might underlie nicotine induced behavioural sensitisation.

3.2 MATERIALS AND METHODS

The described technique of fast cyclic voltammetry (FCV) was established, and experiments were performed at the Department of Chemistry, National University of Ireland, Maynooth (NUIM), Ireland.

3.2.1. Drugs

The following ligands and chemicals were used: D(+)-glucose, dopamine, (-)-quinpirole.HCl (L-171555), GBR-12909.2HCl, (±)-SKF-38393.HCl, and R-(+)-SCH-23390.HCl were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Calcium chloride ($CaCl_2$) was obtained from Baker (Deventer, the Netherlands). (-)-Nicotine ditartrate dihydrate from Acros Organics (Geel, Belgium).
3 mM K^+ Krebs-Ringer Buffer (KRB, Gibco-Invitrogen) contained (in mM): 121 NaCl, 2 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11 D-(+)-glucose, 1.2 KH_2PO_4, with modifications as used during whole experiment: $CaCl_2$ (2.4 mM) and D(+)-glucose (22 mM). The buffers were freshly prepared on each test day, kept at 37 °C and gassed with 5 % CO_2/95 % O_2 (BOC gasses, Dublin, Ireland) for at least 30 min before use.

3.2.2. Animals and tissue preparation

Naïve adult male Wistar Unilever Harlan (WUH) rats, 100-150 g (Harlan, United Kingdom) were used. All experimental procedures were carried out according to the regulations of the Animal Research Ethics Committee of NUIM and national legislation. The rats were killed by decapitation, without anaesthetics (see methodological considerations below), using a guillotine. Brains were removed and dissected (according to Paxinos and Watson, 1998) as quickly as possible. Directly after preparation, the tissue was placed in ice-cold Krebs-Ringer Buffer gassed with 95 % O_2/5 % CO_2 before commencing the slicing procedure.
For striatal recordings, the brain was blocked sagittally to split both hemispheres. The cut was made along the midline between coordinates +1.7 – +0.2 mm

relative to Bregma (Paxinos and Watson, 1998), using a razor blade. Hemispheres were glued, midline side up, to the plate of the vibratome (Campden Instruments, Loughborough, UK). The tissue block was sliced in 350 µm thick slices till 2-3 slices around +1.7 – +0.2 mm relative to Bregma (Paxinos and Watson, 1998) were obtained. The slices were kept in Krebs-Ringer Buffer at room temperature and gassed with 95 % O_2/5 % CO_2 for 0.5 h before starting the experiment.

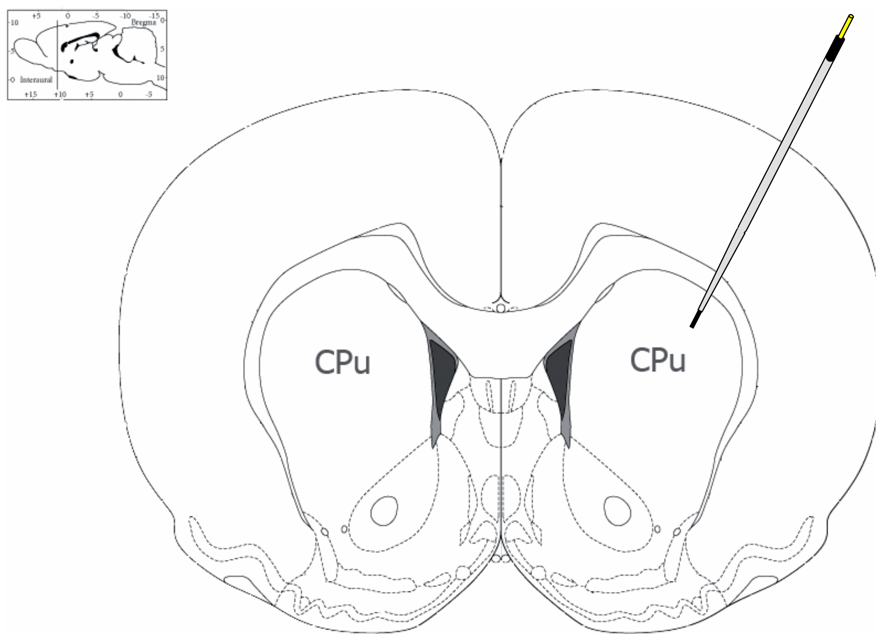


Figure 3-1 Schematic of coronal brain slice including CPu

The vertical line in the sagittal schematic (inset) indicates the coordinate where the coronal schematic was taken (between coordinates +1.7 – +0.2 mm relative to Bregma (Paxinos and Watson, 1998). CPu indicates the caudate putamen in a coronal section. The electrode indicates the position of recording. Diagrams were modified from Paxinos and Watson (1998).

Methodological considerations: Tissue preparation

Although some studies use anaesthetics before decapitation of the experimental animals, in house experience and previous published studies (*e.g.* Hiramatsu *et al.*, 1994; Mantz *et al.*, 1994) showed that the use of anaesthetics can affect the

results of neurochemical experiments. Therefore all experiments in this study were performed using decapitation without the use of anaesthetics.

3.2.3. Fast Cyclic Voltammetry (FCV)

Extracellular recordings were made in slices using a custom-made interface-type chamber. Slices were perfused with Krebs-Ringer buffer (KRB) at 29-30 °C and gassed with 95 % O_2/5 % CO_2. A three-electrode setup was used consisting of a commercially available carbon fiber glass electrode (Ø 5 µm x length 30-60 µm, KationScientific, USA) for current recordings, an auxillary electrode (an Ag/AgCl wire in a 100 uL-pipet tip with KRB), and a reference electrode (Ag/AgCl wire). All electrodes were connected to a headstage (Millar voltammeter probe, 2x amplification). Fast cyclic voltammetry was performed using a Millar-potentiostat (PD systems, West Moseley, UK, kindly donated by Dr. Julian Millar). A triangular waveform from 0 to -1.0 to +1.4 to -1.0 and back to 0 V vs. Ag/AgCl was used at a scan rate of 480 V/s. The sampling rate was 4 Hz and data was digitalised using an A/D-converter (Powerlab 4/20, ADInstruments, Oxford, UK). A standard PC with Chart software (v5.2, AD Instruments) was used for data acquisition and analysis. The faradaic current of the electrode was monitored using a digital oscilloscope (Nicolet 310).

Dopamine release was electrically evoked by a monophasic stimulation pulse (100 µs width, 10 V amplitude) which was generated by a NeuroLog stimulator (NeuroLog NL-800, Digitimer, Hertfordshire, UK). The NeuroLog was connected to a Stimulus Isolation Unit (radio frequency transformer isolation unit, SIU5, Grass Technologies) to minimise artefacts. The electrical pulse(s) were applied using bipolar tungsten electrodes (100 µm apart; 0.1 MΩ, Molecular Probes Inc, USA). The stimulation pulse had a calculated current of 0.1 µA, according to Ohm's law (*i.e.* $I = V/R$; where I is current (A), V is potential (V) and R is resistance (Ω)). Both stimulating and recording electrodes were placed in the dorsal striatum, approximately 100 µm apart. The shape of the waveform was recorded by the A/D-converter and analysed using Scope software (v3.7.3, ADInstruments).

Methodological considerations: Electrodes

Despite the good quality of home-made carbon fiber microelectrodes, their manufacturing is a very labour intensive process and can provide poor reproducibility. Therefore this study used commercially available carbon fiber microelectrodes (Ø 5 µm, 50 µm exposed, glass sealed; Kation Scientific, USA). The electrodes were manufactured according to the protocol described by Budai and Molnár (2001) using spark etching (Armstrong-James and Millar, 1979; Armstrong-James *et al.*, 1980; Millar, 1992). Electrodes with a diameter of 5 µm were chosen as the capacitance (the ability of the electrode to hold an electrical charge) of smaller electrodes was found to be much larger, resulting in a lower signal to noise ratio (Wipf *et al.*, 1989), and to minimise tissue damage by the electrode. Additionally, the use of smaller electrodes would result in less tissue damage.

3.2.4. Experimental protocol

The carbon-fiber microelectrode was placed into the CPu where transient dopamine release was monitored, and its position was adjusted to a site where single pulse electrical stimulated dopamine release was achieved. Single pulse electrical stimulation (0.1 ms, 10V) at 5 min intervals elicited reliable dopamine release detected in the range 0.05 to 0.15 µM. Peak release was reached within 1 s and returned to baseline between 1 and 2 s. Stable release was maintained for 25 min before experiments with drug perfusion were initiated.

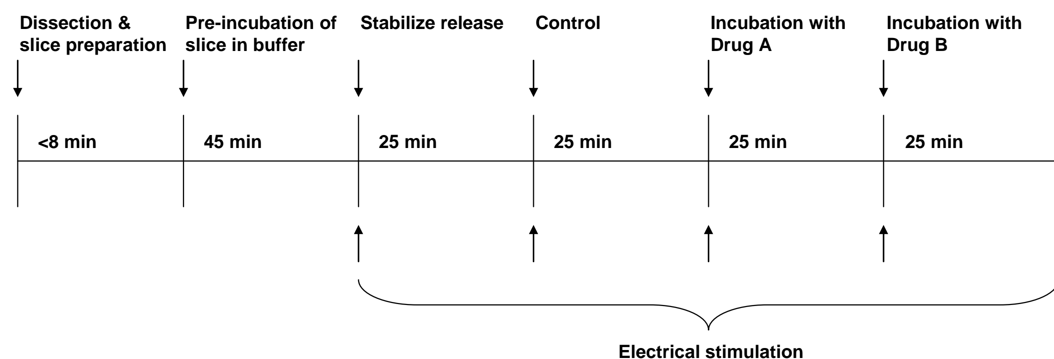


Figure 3-2 Experimental scheme of a typical release experiment

Experimental scheme showing the actions and their respective time frames.

After preparation, the brain slice was pre-incubated at 32°C in the slice bath. Experiments were started after achieving a stable single pulse signal for at least 25 min. At the end of the control period (*i.e.* incubation without test compound) different types of electrical stimulations were examined with 5 min recovery periods between each stimulation. Then the incubation was repeated in the presence of a test compound (*i.e.* Drug A or Drug B).

3.2.5. Data analyses

Chart software (ADInstruments) was used to evaluate the voltage-time potential traces obtained by averaging data in a 10 mV window centred on the peak oxidation potential for dopamine. Data were analysed using Chart software to detect peak amplitudes. Amplitudes (voltages, V) were converted to currents (amperage, A) to correct for gain settings.

Graphs and statistical analyses were performed using PRISM graphing software (GraphPad, USA). The main effects of treatments were analysed using a one-way analysis of variance (ANOVA): [F (df_b, df_w) = x; P = x]. When a significant difference was observed, the ANOVA was followed by a *post hoc* Bonferroni's multiple comparison test analysis where appropriate. Or using an unpaired two-tailed t-test (all data with $P < 0.05$ were considered as significant) where indicated. The correlation (r^2) value was calculated from the Pearson correlation coefficient using PRISM software.

3.3 RESULTS

Prior to studing the interactions between nicotine and changes in dopamine functionality, a system for fast cyclic voltammetric measurements was successfully established (as previously described by *e.g.* Millar *et al.*, 1985; Millar and Barnett, 1988; Palij *et al.*, 1990; Kruk and O'Connor, 1995; O'Neill *et al.*, 2007).

To investigate the quality of the commercially available carbon fiber microelectrodes (KationScientific, USA), a comparison of faradaic currents was made between custom-made and commercial carbon fiber microelectrodes. The measured faradaic current showed a clear representation of the applied triangular waveform by first a negative potential followed by a positive and negative potential, respectively. It was found that the commercial electrodes had comparable faradaic currents to the home made electrodes (Figure 3-3). Furthermore, the sensitivity of the commercial electrodes was determined by measuring different concentrations of dopamine in a physiologically relevant range, perfused through the experimental slice-bath. The signal-to-noise ratios and sensitivity of the commercial electrodes to the dopamine standard solutions (Figure 3-4) were comparable to the home-made electrodes.

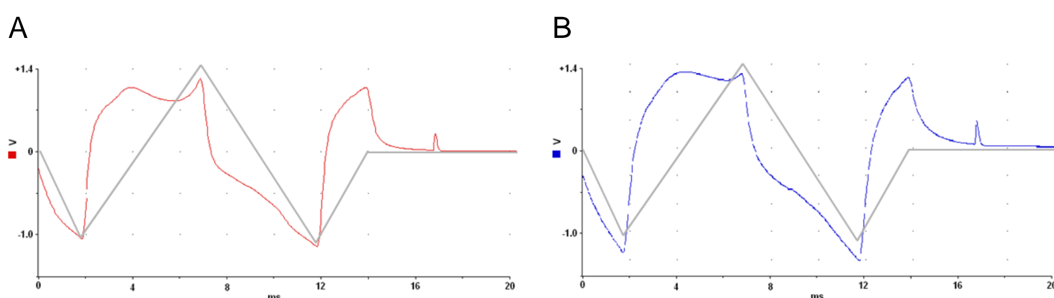


Figure 3-3 Faradaic current commercial vs custom-made electrode

Representative faradaic currents of a carbon fiber microelectrode (Ø 5 µm) in a perfusing Krebs-Ringer Buffer salt solution, using a triangular waveform (superimposed linear grey-line) as described in the methods section. A.) A commercial available carbon fiber microelectrode (KationScientific). B.) A custom-made carbon fiber microelectrode (kindly donated by Dr. Carmel O'Neill). y-axis, potential (V); x-axis, time (min).

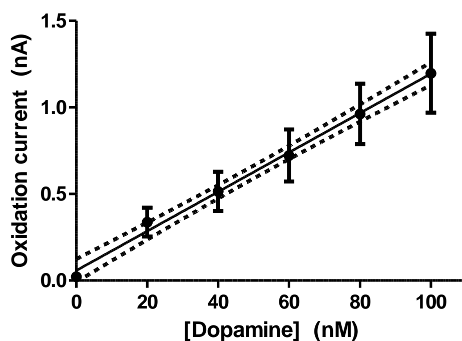


Figure 3-4 Calibration curve of dopamine

Increasing concentrations of dopamine were applied to the perfusion bath using a complete wash-out between each concentration. Mean ± s.e.m. of n = 4 experiments on individual days using different electrodes. Dotted lines represent 95 % confidence interval. r^2 = 0.9952, y= 0.0114x + 0.0579.

In order to validate the FCV technique in a biological system, rat cortico-striatal brain slices were prepared as described in the methods section. After inserting the microelectrode into the slice, the presence of dopamine transients and stimulated release confirmed that the electrode was placed in a microenvironment with functional dopaminergic terminals that release dopamine. To validate the system, the effect of single pulse and multiple pulse (4 pulses at 1 Hz, 4p@1Hz) electrical stimulation on dopamine release in rat striatal slices were examined. It was shown that 4p@1Hz induced twice as much dopamine release compared to a single pulse stimulation (Figure 3-5). The experiment was performed in the presence of GBR-12909, a dopamine transporter inhibitor, which explains the relative long decay time (indicated by a relative large half width) and is in consensus with previous literature (*e.g.* Palij *et al.*, 1990).

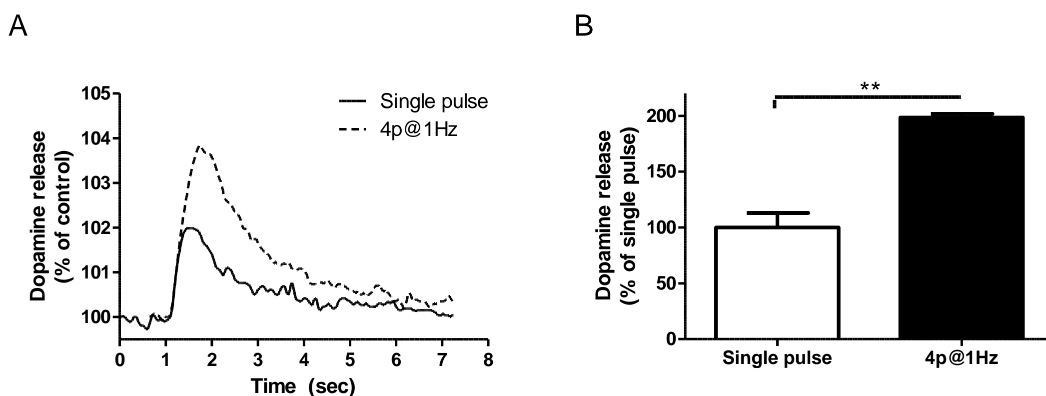


Figure 3-5 Effect of single pulse and 4 pulses at 1 Hz stimulation on the release of striatal dopamine release

A.) Representative example of dopamine overflow after single pulse and 4p@1Hz electrical stimulation. B.) Effect of single pulse and 4 pulses at 1 Hz on dopamine release. Unpaired two-tailed t-test showed a significant increase of 4p@1Hz in transient amplitude compared to single pulse (** $P < 0.01$). $n = 2$ slices tested individual days from separate rats, in the presence of GBR-12909 (1 µM).

Pharmacological validation of the FCV setup was achieved by examination of the effect of quinpirole on single- and multiple pulse stimulation in rat striatal slices. A test concentration of quinpirole (1 μM) was incubated for 60 min, reaching its maximal effect by 15 - 20 min. Therefore further concentrations were incubated for 25 min before stimulation. Quinpirole inhibited dopamine release at single pulse and 4p@1Hz electrical stimulation in a concentration dependent manner (Figure 3-6). However, quinpirole had a lower potency at 4p@1Hz stimulation compared to single pulse stimulation (pEC_{50} 6.7 ± 0.1 vs. 7.5 ± 0.1, respectively).

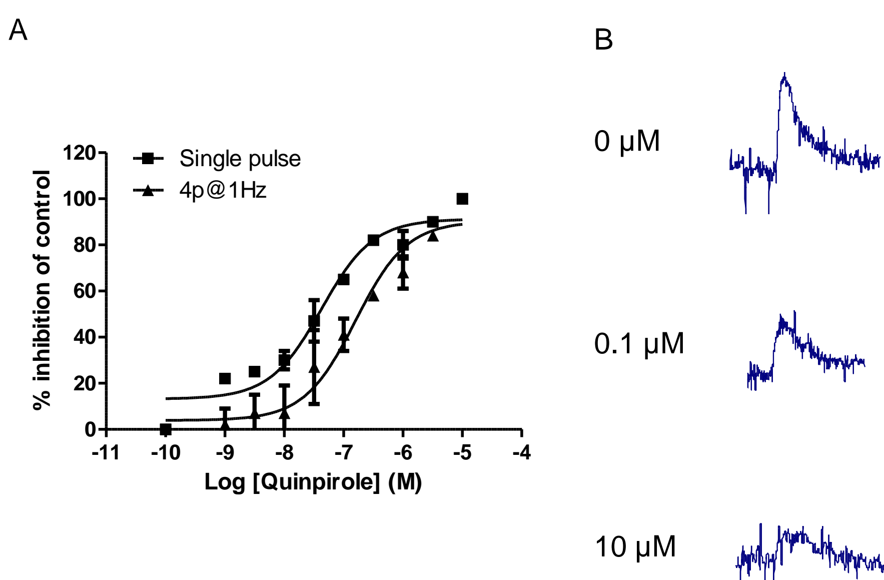


Figure 3-6 Effect of quinpirole on electrically stimulated dopamine release
A.) Quinpirole was tested at a concentration range from $1E^{-05}$ to $1E^{-10}$ M. Single pulse (■) and 4p@1Hz (▲). pEC_{50} and E_{max} (at 10 μM) values of quinpirole were; single pulse (7.4 ± 0.2 and 80%, respectively) and 4p@1Hz (6.8 ± 0.1 and 55 %, respectively). B.) Representative measured current of sample-and-hold trace at +600 mV potential (*i.e.* dopamine oxidation potential). Effect of 0, 0.1 and 10 μM quinpirole on single pulse induced dopamine release. *n* = 2 experiments at two individual days from two separate rats, in the presence of GBR-12909 (1 μM).

The effect of nicotine on dopamine release was investigated. After equilibration of the slice in the superfusion bath for 1 hr, the carbon fiber electrode was positioned into the dorsal striatum and dopamine release was evoked by single pulse electrical stimulation. Single pulse stimulation was repeated every 5 min and after a 20 min stable release, nicotine (500 nM) was added to the bath. Nicotine decreased single pulse induced dopamine release by 32 ± 7 %. The effect of nicotine (500 nM) on dopamine release was rapid and its maximal effect (E_{max}) was achieved by 10 min (Figure 3-7).

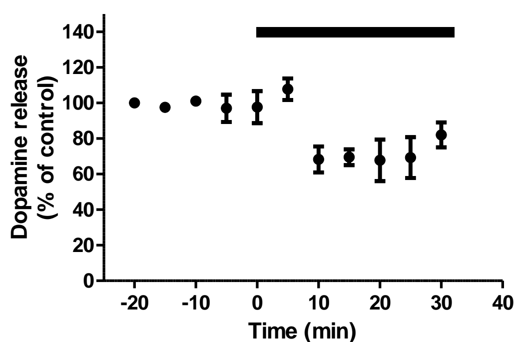


Figure 3-7 Effect of nicotine on single pulse induced dopamine release in time

Single pulse stimulation was repeated every 5 min and after a 20 min stable release, nicotine (500 nM) was added to the bath (horizontal bar). Results of 4 individual experiments performed on four different days ($n = 2$-4).

Neurotransmission is highly dependant on the firing frequency of neurones. Using FCV it was shown that dopamine release produced by low frequency (5p@10Hz) or high frequency (5p@100Hz) electrical stimulation was not significantly different compared to single pulse stimulation in the absence of a dopamine reuptake inhibitor (Figure 3-8). Addition of nicotine (500 nM) to the slice bath for 25 min did significantly decrease dopamine release at single pulse and at low frequency stimulation. Interestingly, the presence of nicotine increased dopamine release at high frequency stimulation (Figure 3-8).

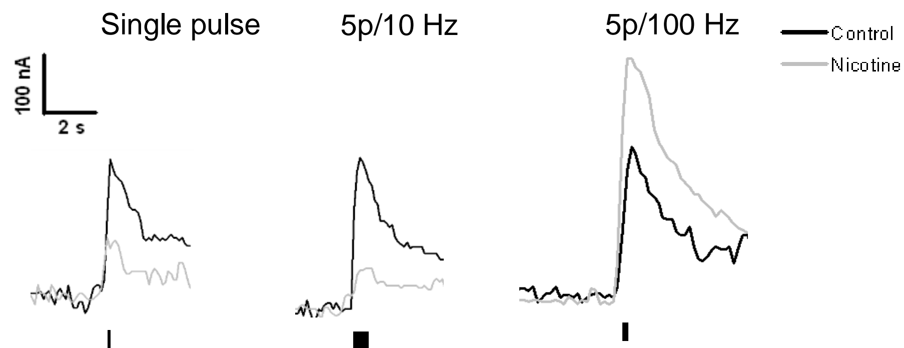


Figure 3-8 Representative sample and hold measurements of the dopamine signal following addition of nicotine

The effect of different electrical stimulation frequencies (single pulse, 5 pulses at 10 Hz and at 100 Hz) on dopamine release in corticostriatal slices (350 µm) in the absence (black lines) or presence (grey lines) of nicotine (500 nM). Insert: Vertical bar indicates current (A) and horizontal bar represents time (s).

These experiments were repeated for quantification and statistical assessment of this biphasic effect of nicotine (Figure 3-9). There was no significant effect found between the different stimulation parameters in the absence of nicotine (control group [F (2,8) = 2.044; P = 0.1918]), but did show a significant difference in the presence of nicotine compare to its respective control group (Nicotine group [F (2,9) = 52.58; P < 0.001]). Further *post hoc* Bonferroni's analysis showed that addition of nicotine significantly decreased single pulse stimulated dopamine release (100 % vs. 64 ± 8%, P < 0.01), and also significantly decreased low frequency multiple pulse, 5p@10Hz (128 ± 12 % vs. 48 ± 14 %, P < 0.01). However, interestingly, addition of nicotine significantly increased dopamine release at high frequency multiple pulse stimulation, 5p@100Hz (116 ± 13 % vs. 215 ± 15 %, P < 0.01).

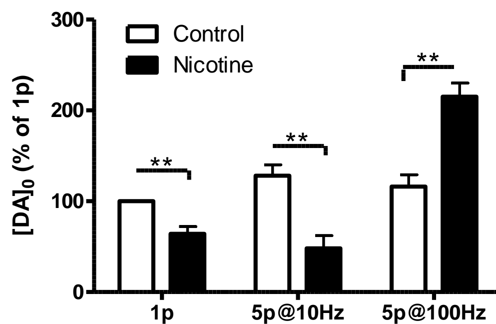


Figure 3-9 Effect of nicotine on electrically stimulated dopamine release at different frequencies

The effect of nicotine (500 nM) on electrically evoked dopamine in dorsal striatal slices with single pulse or multiple pulses (5p@10Hz or 5p@100Hz). $[DA]_0$, extracellular dopamine concentration. $[DA]_0$, extracellular dopamine concentration. Unpaired two-tailed t-test, ** P < 0.01. Data of 4 individual experiments ($n = 4$).

As previously shown in Chapter 2, nicotine induced behavioural sensitisation can be attenuated by dopamine D_1 ligands. Therefore the effect of dopamine D_1 ligands on electrically stimulated dopamine release, at different frequencies, was

investigated. Firstly, the effect of the dopamine D_1 receptor agonist SKF-38393 on electrically stimulated dopamine release was examined.

Rat corticostriatal slices were incubated with SKF-38393 (1 μM) for 25 min and dopamine release was induced by a single pulse and multiple pulse (5p@10Hz or 5p@100Hz) stimulation, followed by incubation of SKF-38393 in the presence of nicotine (500 nM) for another 25 min. Statistical analysis using a one-way ANOVA showed no significant treatment effect of SKF-38393 in the absence or presence of nicotine on single pulse stimulated dopamine release ([F (2, 8) = 0.8763; P = 0.4528], Figure 3-10). Following 5p@10Hz stimulation there was a significant treatment effect observed, [F (2, 8) = 30.84; P = 0.0002], and *post-hoc* Bonferroni's analysis showed a significant decrease of dopamine release following SKF-38393 in the presence of nicotine (P < 0.01, Figure 3-10). The same effect was observed at 5p@100Hz: Following 5p@100Hz stimulation there was a significant treatment effect observed, [F (2, 7) = 18.39; P = 0.0016], and *post-hoc* analysis showed a significant decrease of dopamine release following SKF-38393 in the presence of nicotine (P < 0.01, Figure 3-10). Compared to nicotine alone, SKF-38393 fully antagonised the effect of nicotine on single pulse release but not at multiple pulse (5p@10Hz and 5p@100Hz) stimulated release (Figure 3-9 vs Figure 3-10).

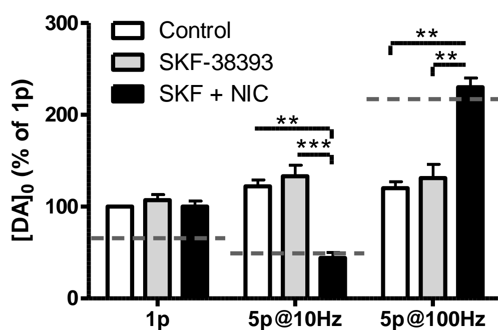


Figure 3-10 Effect of a D_1 agonist and combination with nicotine on electrically stimulated dopamine release

The effect of the dopamine D_1 receptor agonist SKF-38393 on dopamine release and pre-incubation of SKF-38393 (1 μM) on the effect of nicotine. Dotted-line represents nicotine control level (Figure 3-9). *Post hoc* Bonferroni's, ** P < 0.01, *** P < 0.001. Data from 4 individual experiments (n = 4).

The effect of the prototypical dopamine D_1 receptor antagonist SCH-23390 on electrically stimulated dopamine was examined.

Rat corticostriatal slices were incubated with SCH-23390 (1 µM) for 25 min and dopamine release was induced by a single pulse and multiple pulse (5p@10Hz or 5p@100Hz) stimulation, followed by incubation of SCH-23390 in the presence of nicotine (500 nM) for another 25 min. Statistical analysis using a one-way ANOVA showed no significant treatment effect of SCH-23390 in the absence or presence of nicotine on single pulse stimulated dopamine release ([F (2, 9) = 3.610; P = 0.0706], Figure 3-11A). Following 5p@10Hz stimulation there was a significant treatment effect observed, [F (2, 8) = 19.55; P = 0.0008], and *post-hoc* Bonferroni's analysis showed that SCH-23390 significant reversed the effect of nicotine on decrease of dopamine release (P < 0.05, Figure 3-11A). The same effect was observed at 5p@100Hz: Following 5p@100Hz stimulation there was a significant treatment effect observed, [F (2, 3) = 8.370; P = 0.0109], and *post-hoc* analysis showed a significant decrease of dopamine release following SCH-23390 in the presence of nicotine (P < 0.05, Figure 3-11A). Compared to nicotine alone, SCH-23390 fully antagonised the effect of nicotine on single pulse release but did not alter the effect of nicotine at multiple pulse (5p@10Hz and 5p@100Hz) stimulated release (Figure 3-9 vs Figure 3-11A).

Furthermore, the effect of SCH-23390 following pre-incubation with nicotine was assessed. SCH-23390 blocked the inhibitory effect of nicotine on single pulse induced dopamine release, but not the multiple pulse induced release (Figure 3-11B vs Figure 3-9). No significant effect of treatments was observed compared to their respective controls (unpaired two-tailed t-test, P > 0.05). These results are comparable to those obtained after pre-incubation of SCH-23390 followed by nicotine, although the reversal effect of SCH-23390 on single pulse stimulated dopamine release was not as efficacious compared to pre-incubation of SCH-23390.

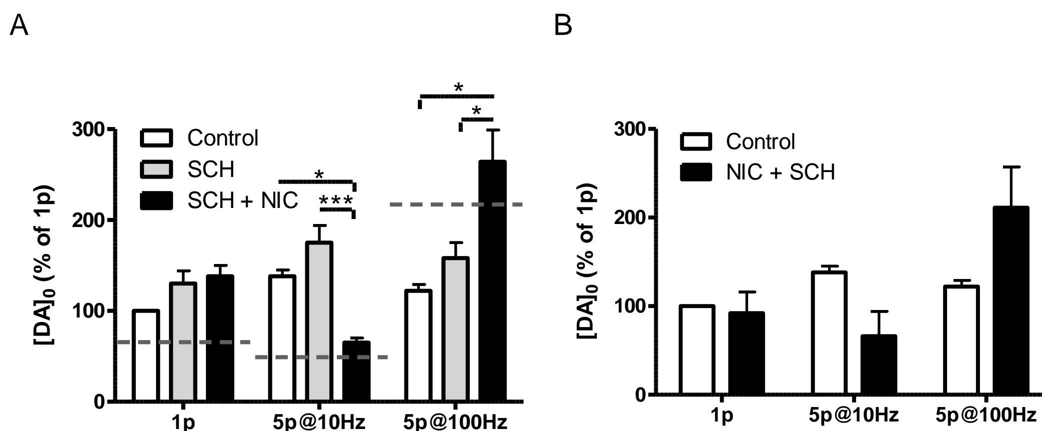


Figure 3-11 Effect of a D_1 antagonist and nicotine on electrical stimulated dopamine release

A. The effect of the dopamine D_1 receptor antagonist SCH-23390 (1 μM) alone on dopamine release and the effect of nicotine in the presence of SCH-23990. Dotted-line represents nicotine control level (Figure 3-9). *Post hoc* Bonferroni's, * $P < 0.05$; *** $P < 0.001$. Data from 4 individual experiments ($n = 4$). B. The effect of SCH-23390 (1 μM) after nicotine pre-incubation. Data from 3 individual experiments, unpaired two-tailed t-test showed $P > 0.05$ for all groups ($n = 3$).

3.4 DISCUSSION

This study showed that nicotine affects stimulated dopamine release in slices of rat caudate putamen *ex vivo* and that its effect is dependent on the frequency of the electrical stimulation. Furthermore, this study showed for the first time that dopamine D_1 ligands can affect the stimulation frequency dependent effect of nicotine on dopamine release.

The present study investigated the effect of nicotine and dopamine D_1 ligands on dopaminergic neurotransmission. Studying the role of dopamine transmission can be achieved with various methodological approaches. In functional terms, the brain is highly spatially and temporally organised. Its function may be studied using many different techniques. Basically four main approaches can be distinguished, microdialysis, electrophysiology, imaging (*e.g.* magnetic resonance imaging (MRI) or positron emission tomography (PET)) and voltammetry. Each method has its advantages and disadvantages, which makes them complementary to each other. For example, electrophysiology offers a very high temporal resolution but provides only an indirect estimate of dopamine transmission and is often not sufficient to identify dopaminergic neurones (Margolis *et al.*, 2006). Moreover, the limitations of these techniques are mainly based on spatial and temporal sampling constraints. Microdialysis and *in vitro* superfusion techniques are known for their superior selectivity in combination with chromatography and suitable detectors. However, they have very poor spatial resolution and relatively long sampling times of *ca.* 10-20 min. The technique of fast cyclic voltammetry (FCV) offers high spatial and high temporal resolution, which is superior to the other techniques. Moreover, FCV is a less-invasive technique (compared to *e.g.* superfusion of microdialysis) which makes it suitable for *in situ* and long term analysis (Bull *et al.*, 1990), and has been extensively used to study neurochemistry *in vivo* and *in vitro.* A good introduction to FCV and other electrochemical methods, including their disadvantages and limitations, is given in the book ‘Electrochemical Methods for Neuroscience’ (Michael and Borland, 2007).

The selectivity of the FCV technique for dopamine is extensively discussed in previous literature (see introduction Chapter 3). For example, the selectivity of the technique for dopamine was examined by oxidation – reduction peak characterisation, corresponding dopamine standards, abolishment of signal by reserpine, desipramine selectively blocked noradrenaline but not serotonin signal, nialamide (a monoamine oxidase inhibitor, MAOi) had no effect, and so metabolites did not interfere with the signal (Stamford *et al.*, 1988b; Stamford, 1990).

FCV is also a versatile method and determinations of various neurotransmitters in brain slices using fast-cyclic-voltammetry are described, for example: dopamine (Palij *et al.*, 1990; Bull *et al.*, 1990; Kelly and Wightman, 1987), noradrenaline (Palij and Stamford, 1992; 1993; 1994), serotonin (O'Connor and Kruk, 1991; Threlfell *et al.*, 2004, 2010), adenosine and adenosine-5′-triphosphate (Swamy and Venton, 2007; Cechova and Venton, 2008; Xu and Venton, 2010) histamine (Cechova and Venton, 2008), tyramine and octopamine (Cooper and Venton, 2009), and nitric oxide (Ledo *et al.*, 2005; Cechova and Venton, 2008). The cyclic voltammogram produced by FCV contains extensive electrochemical information which can be used to identify the individual compounds. By simultaneous measurement of both the oxidation and reduction potentials of the analytes it is possible to distinguish between chemically related and distinguished compounds. Although a great advantage of FCV is to chemically identify compounds by their oxidation and reduction potentials, the FCV system as designed by Dr. Julian Millar uses a sample and hold device at either the oxidation or reduction peak but not simultaneously. This study used a Miller-potentiostat and it was therefore not possible to identify compounds using their voltammogram alone.

For the pharmacological validation of the FCV system, the prototypical dopamine D_2/D_3 agonist quinpirole was used. A dopamine D_2-like agonist was chosen as this receptor is a known autoreceptor which regulates the release of dopamine (Palij *et al.*, 1990; Moquin and Michael, 2009). The inhibitory effect of quinpirole on dopamine release and pEC_{50} values found in this study, are in agreement with previous literature (O'Neill *et al.*, 2007). The shift in potency of quinpirole seen at a multiple pulse stimulation (4p@1Hz) compared to a single pulse stimulation,

could be explained by competition of quinpirole and synaptic available, released endogenous dopamine. This is suggested as 4p@1Hz induced twice as much dopamine release compared to single pulse stimulation.

Neurotransmission (including the release of neurotransmitters) is highly, but not exclusively, dependent on the firing rate of the neurones. The physiological firing frequency range of dopaminergic neurones in the brain can be divided into tonic (5-10 Hz) and phasic burst-like (20-100 Hz) firing frequencies (Hyland *et al.*, 2002). This physiological tonic and burst firing can be simulated by electrical field stimulation. A bipolar stimulating electrode is positioned in the slice, near the recording electrode, and can generate single or multiple electrical pulses at a desired frequency. When focussed on multiple pulse stimulation, these results showed that a 4p@1Hz gives a larger peak of dopamine release compared to 5p@10Hz or 5p@100Hz (Figure 3-5 and Figure 3-9). This is likely due to accumulation of dopamine release at 4p@1Hz, where the accumulation is less at higher stimulation frequencies.

Previous studies on the effect of bath applied nicotine on dopamine release have yielded conflicting results. This may be a consequence of the different techniques used and the fact that results relate to both *in vivo* and *ex vivo* experiments. For example, *ex vivo* superfusion studies using [^{3}H]-dopamine and K^+-stimulation showed that nicotine increased extracellular dopamine levels. However, studies using high-speed dopamine measurement techniques described a bidirectional effect of nicotine on dopamine release (Rice and Cragg, 2004; Zhang and Sulzer, 2004). When stimulating electrically at a low frequency (representing tonic release), nicotine decreased dopamine release, which is in contrast to what is observed with superfusion studies. When stimulating at high frequency (representing phasic release), nicotine increased dopamine release. This latter observation is comparable with the findings using [^{3}H]-dopamine and K^+-stimulation suggesting a common phasic dopamine release. Furthermore, preferential effects of nicotine on dopamine release within the striatum (*i.e.* between dorsal and ventral) have been reported (*e.g.* Imperato *et al.*, 1986; Rice and Cragg, 2004; Zhang and Sulzer, 2004).

A long-held view of psychostimulant addiction (for example by nicotine, cocaine, or amphetamine) is that the reinforcing properties are mediated by an increase in dopamine levels, especially within the striatum (*e.g.* Di Chiara and Imperato, 1988). Paradoxically, it has been reported in human, animal and cell based studies, that nicotine administration desensitises nAChRs on dopaminergic neurones (Pidoplichko *et al.*, 1997; Zhou *et al.*, 2001) and suppresses striatal dopamine release evoked by single action potentials (Zhou *et al.*, 2001). This phenomenon was further studied using fast cyclic voltammetry in brain slices and determined that nicotine has a preferential, bidirectional effect on dopamine release, *i.e.* nicotine decreases stimulated dopamine release at a single pulse or low frequency stimulation, whereas nicotine increased dopamine release at high frequency electrical stimulation (Rice and Cragg, 2004; Zhang and Sulzer, 2004). The present studies confirmed these findings of the effect of nicotine on electrically stimulated dopamine release.

The present study additionally investigated for the first time the pharmacological "modification" of nicotine induced "frequency-dependent-signalling" on dopamine release. The study examined the effect of the dopamine D_1 agonist SKF-38393 and the D_1 antagonist SCH-23390 on nicotine's biphasic modulation of dopamine release. SKF-38393 or SCH-23390 alone were ineffective in altering electrically stimulated dopamine release, which can be explained by the fact that the dopamine D_1 receptor is localised post-synaptically on GABAergic and cholinergic interneurones and therefore may not directly affect dopamine release. This finding is also in agreement with previous literature (*e.g.* Palij *et al.*, 1990; O'Neill *et al.*, 2007). However, it has been reported that SKF-38393 increased dopamine release in vivo rat striatum (Walters and Howard, 1990). For the first time, this study showed that SKF-38393 and SCH-23390 selectively affect nicotine induced frequency dependent dopamine release. It was found that pre-incubation with either D_1 ligand significantly reversed or attenuated nicotine's effect on single pulse, but not at multiple pulse (high frequency) evoked dopamine release.

When nicotine was pre-incubated before the addition of SCH-23390 to the bath, no significant effect of SCH-23390 was observed (Figure 3-11). This could be

explained by the low number of experiments (*i.e.* $n = 3$) or by (de-) sensitisation of nAChRs by nicotine during its pre-incubation time.

It is not yet known how to explain the bidirectional effect of nicotine on dopamine release. There are a few hypotheses to explain this phenomenon. For example there may be different nAChR subtypes involved in single and multiple-pulse release. *In vivo* single cell recordings in rat VTA indicated that the nAChR subtypes contribute to increased firing rate but not bursting (e.g β2 and α7, Schilstrom *et al.*, 2003; Mameli-Engvall *et al.*, 2006). Suggesting from the results presented in this thesis, it may be that co-activation or localisation of different nAChR subtypes occurs with dopamine D_1 receptors. Another concerted hypothesis for the nicotinic control of burst firing suggests the rapid and preferential desensitisation of nAChRs on dopaminergic neurones (Wooltorton *et al.*, 2003; Piccotto *et al.*, 2008). In addition to all of these hypotheses, it must be considered if single or multiple nAChR subtypes play this essential and complex role in the bidirectional effect of nicotine on dopamine release (Livingstone and Wonnacott, 2009).

This phenomenon of preferential or bidirectional effects on neuronal activity at different stimulation frequencies is also described in electrophysiology measuring firing frequency and is called 'frequency dependent signalling' or FDS. Further research into the quantal neurotransmitter release and frequency relationships is needed to evaluate if this phenomenon also applies to functional neurotransmitter release. In this study only 5p@10Hz and 5p@100Hz were tested representing low and high frequency stimulation respectively. Future experiments should assess various stimulation frequencies within the 2 to 100 Hz range and thereby creating frequency-response curves.

There are a few reports which studied the effect of behavioural sensitisation on *ex vivo* dopamine release, as measured by FCV. It was shown that there is no difference between single pulse stimulated dopamine release in NAc and VTA slices from (+)-amphetamine- or quinpirole sensitised groups (Muscat *et al.*, 1993, 1996), or from caudate and accumbens slices from cocaine sensitised rats (Jones *et al.*, 1996; Lee *et al.*, 1998), when compared to vehicle. Dopamine

release was enhanced after multiple pulse stimulation (20-50p@5-200Hz) in nucleus accumbens and VTA slices from rats sensitised to quinpirole (Muscat *et al.*, 1993, 1996). The potency of bath applied quinpirole to inhibit dopamine release was decreased in slices containing nucleus accumbens and VTA from rats sensitised with quinpirole (Muscat *et al.*, 1993, 1996), and caudate slices from cocaine sensitised rats (Jones *et al.*, 1996). In nucleus accumbens and VTA slices from (+)-amphetamine sensitised rats, the potency of bath applied quinpirole was increased and decreased respectively (Muscat *et al.*, 1993, 1996). However, no release experiments following nicotine sensitisation are known and future experiments should include the examination of frequency dependent release in tissue from chronic nicotine treated animals, to investigate the role of frequency dependent signalling in behavioural sensitisation. Many studies have shown that the length of time from tissue extraction to analysis is important for the effects seen when measuring [^{3}H]-dopamine release *ex vivo* (*e.g.* Peris *et al.*, 1991). This should also be taken in consideration when testing/preparing tissue from (chronic) treated animals.

Conclusion

The present study has shown the effect of only single concentrations of SKF-38393, SCH-23390 and nicotine on frequency dependent dopamine release. However, future experiments should assess various concentrations in order to determine the potency (pEC_{50} values) and efficacy (E_{max} values) of these compounds. The present experiments focused on dopamine release where it investigated the role of nicotine on dopamine signalling. However, the extensive literature available describing behavioural sensitisation also indicates that many different compounds can induce and block behavioural sensitisation. This could suggest that more than one mechanism, besides the dopamine system, is involved in sensitisation.

Chapter 4. Development and in vitro application of a method for measuring (cyclic-) nucleotides using LC-MS/MS

Abstract.

OBJECTIVES: Cyclic-adenosine monophosphate (cAMP) plays an important role in cell signalling and is widely used as a marker for receptor activation and as a molecular target for pharmacological research. This chapter presents the development, validation and potential application of a novel method for the determination of cAMP and ATP (adenosine triphosphate) and other nucleotides in a biological system by combining zwitterionic hydrophilic interaction liquid chromatography (HILIC) and tandem mass spectrometry (MS/MS).

METHODS: The HILIC-MS/MS method was developed for the simultaneous quantitative analysis of cAMP and ATP, and was validated by assessment of linearity (over a range from 0.5-100 nM for cAMP and 50 nM-50 μM for ATP (r^2 >0.999)), resolution, limit of detection (0.5 and 50 nM for cAMP and ATP, respectively) and reproducibility.

RESULTS: The method was validated and applied *in vitro* to determine cAMP accumulation in cell cultures and brain tissue samples. The effect of several dopamine D_2 receptor (partial-) agonists and antagonists on cAMP accumulation was assessed by determination of the cAMP/ATP ratio in cells transfected with the human dopamine D_{2L} receptor. Furthermore, the method was applied *ex vivo* to determine the effect of a dopamine D_1 receptor agonist on cAMP accumulation in brain slices.

CONCLUSIONS: These results demonstrate that the described bioanalytical method was robust, fast, sensitive, and selective. Moreover, it showed utility in determining cAMP/ATP in biological systems and the ability to study the effect of (partial-) agonists and antagonists which makes it a useful tool for drug discovery.

Keywords: cAMP, ATP, HPLC, MS/MS, HILIC

4.1 INTRODUCTION

Cyclic nucleotides (*e.g.* cAMP and cGMP) are intracellular second messengers which play an important role in many physiological processes. cAMP is synthesised from ATP by the enzyme adenylate cyclase (AC), and is metabolised by the enzyme phosphodiesterase (PDE). cAMP is widely used in biochemical, pharmacological and drug research as a marker to screen the activity of compounds for specific receptors. Additionally, cyclic nucleotides are used to study the activity of PDEs, which play an important role in various neuropsychiatric and cardiological diseases.

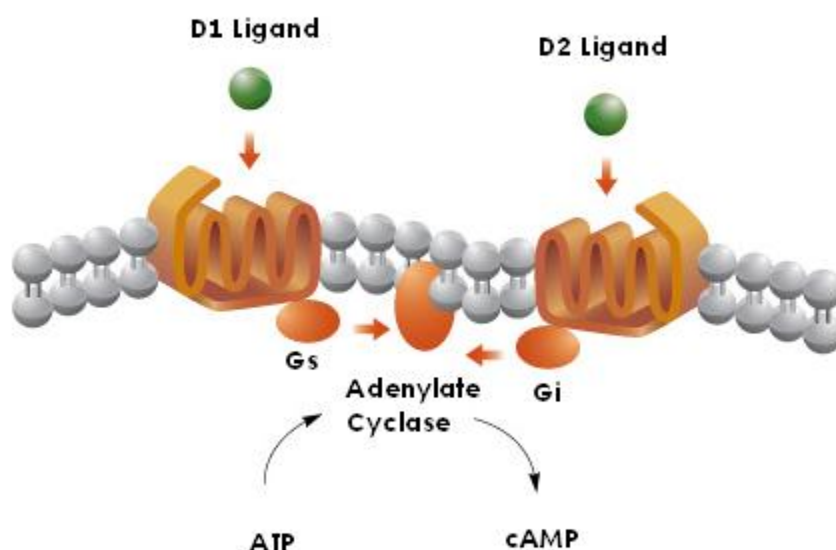


Figure 4-1 Cartoon of dopamine receptor coupled cAMP-pathway

Activation of the dopamine D_1 receptor or D_2 receptor will uncouple Gαs or Gαi from their heterotrimers (together with Gβ and Gγ subunits), respectively. The Gαs subunit can activate the enzyme adenylate cyclase (AC) which catalyzes the formation of cAMP from ATP. Gαi can inhibit AC which attenuates cAMP formation.

Development of methods for the measurement of (cyclic-) nucleotides has received considerable interest in the past few decades, and two major approaches for the determination of cAMP accumulation have been developed.

Firstly, the classical metabolic labelling method employs tritium-labelled adenine as the substrate, followed by anion-chromatographic separation of [^{3}H]-cAMP and [^{3}H]-ATP (Solomon *et al.*, 1974; Feenstra *et al.*, 2003). Secondly, the more recently developed techniques are based on high throughput, including electrochemiluminescence, enzyme complementation, radiometric- and luminescence- proximity, fluorimetry, fluorescence polarisation, reporter genes (*e.g.* luciferase and green fluorescent protein) and time-resolved fluorescence assays (see Williams, 2004 for review). Although very sensitive, the disadvantages of current methods are the requirement of radioactive substrates, high false-positive hits and/or the limitation of measuring only one component at a time and are highly labour intensive (Williams, 2004). Simultaneous analysis of cAMP and ATP, to calculate a cAMP/ATP ratio, is often required to better estimate the efficacy (or intrinsic activity) of partial agonists, especially for inhibitory coupled receptors like the dopamine D_2 receptors. Furthermore, ATP is the basic energy source of the living cells and the additional detection of ATP can be used as an indicator for cell viability to detect possible cytotoxicity (Kangas *et al.*, 1984).

More recently, another analytical method, liquid chromatography – tandem mass spectrometry (LC-MS/MS), has gained interest due to its specificity, reproducibility and sensitivity (Banoub *et al.*, 2005). Because of the extremely polar properties of nucleotides, due to the presence of (multiple-) phosphate groups, most LC methods use anion exchange chromatography or reversed stationary phases in combination with ion-pairing agents. Unfortunately, both of these methods have their drawbacks when used in combination with MS/MS analysis (for review see Niessen, 1998). However, hydrophilic interaction liquid chromatography (HILIC) is able to separate very polar compounds, without the needs of ion-pairing agents, and uses mobile phases which are compatible with mass spectrometry analysis.

The involvement and importance of dopamine as an endogenous neuromodulator is indisputably in many neuropsychiatric disorders (Marsden, 2006), and is therefore the key focus of pharmacological research. In numerous of cases, studies were designed to specifically screen for dopamine receptor

subtype selective ligands. A commonly used method to assess the effect (*i.e.* selectivity, potency and efficacy) of these selective ligands *in vitro* is to test their effect on intracellular cAMP accumulation in cells transfected with specific receptor subtypes.

Although cell based assays have their advantages (*e.g.* cells can be cultured in large amounts for high throughput screening, cells can contain one specific receptor subtype), it is still desirable to assess the effect of the compounds in a more physiological advanced system. One alternative between *in vitro* cell based assays and *in vivo* assays is the use of tissue isolated from the animal. Hereafter referred to as *ex vivo*. This can be tissue from non-treated animals or tissue from animals (chronically) treated *in vivo*.

In the past it was very difficult to examine nucleotides, *e.g.* cAMP, in rat brain slices due to large inter- and intra- assay variation. Hypothetically, like the cell based assay, the variation might be caused by the variation in amount of slices per sample, and that the level of ATP could be used as a marker for the amount of protein. By calculating a cAMP/ATP ratio, a correction could be made for the amount of slices in each sample-well, which will reduce variation.

This project initially started with the hypothesis that the radiometabolic labelling method for the measurement of [^{3}H]-cAMP and [^{3}H]-ATP, as described by Salomon and colleagues (Salomon *et al.*, 1974; Salomon, 1979), could be optimised. The optimisation included miniaturisation from 24-well plate cell cultures and individual solid-phase-extraction (SPE-) columns to a 96-well plate cell culture and online 96-well SPE-filterplates. This optimisation was hypothesised to improve the quality of the assay by reducing variation, and subsequently improve the ability to measure the effect of compounds with reduced efficacy (*i.e.* partial agonists). Furthermore the miniaturisation would increase the robustness, performance and have a higher throughput, which is required for future *ex vivo* studies (later to be discussed in Chapter 5). However, miniaturisation of the radioactive assay to 96-well plates did not succeed due to radiolabel concentrations reached below limit of detection (data not showed and not to be discussed further in this thesis). A second hypothesis was then investigated which include the measurement of the two nucleotides of interest (*i.e.* cAMP and ATP) using mass spectrometry. Preliminary studies investigated flow

injection analysis (FIA) to quantify cAMP and ATP using the mass separation resolving power of the mass spectrometer only. Unfortunately, the limit of detection was approximately 1 µM for cAMP, where the expected levels were around 1 nM of cAMP. Therefore a hyphenated/hybrid technique by combination of liquid chromatography with mass spectrometry was proposed.

The aim of this study was to develop and validate a rapid, sensitive and selective method for the simultaneous analysis of cAMP and ATP in biological samples. Here, for the first time, we present a method and its application using the powerful combination of hydrophilic interaction liquid chromatography and tandem mass spectrometry (HILIC-MS/MS) to measure nucleotides. The analytical method was applied to *in vitro* biogenic samples, and the effect of several dopaminergic D_2 (partial-) agonists and antagonists on intracellular cAMP accumulation was assessed. Furthermore, a ratio of cAMP/ATP was calculated were ATP was used as a biomarker for cell viability and provide indices of cell toxicity and/or cell number which significantly reduced variation.
To investigate the application of the developed method using brain tissue, slices from rat dorsal striatum (caudate putamen) were prepared. The striatum is known to contain all different subtypes of the dopamine receptor (*i.e.* $D_{1\text{-}5}$) (Tarazi and Baldessarini, 1999). The dopamine D_1-like receptors (*i.e.* D_1 and D_5) are positively coupled to AC, and activation will lead to an increase of cAMP levels (Kebabian and Calne, 1979; Seeman and Grigoriadis, 1987). The dopamine D_2-like receptors (*i.e.* D_2, D_3 and D_4) are negatively coupled to AC, and activation will lead to a decrease of cAMP levels (see Chapter 1).

Dopamine receptors are coupled to adenylate cyclase and subsequently their activity can be monitored by the measurement of cAMP accumulation. One of the first examples of making the *in vitro* cell-based experiments possible is the dopamine D_2 receptor, which was firstly cloned and transfected into cells by Bunzow and colleagues (Bunzow *et al.*, 1988). By culturing these cells on a large scale it is possible to assess the effect of D_2 receptor ligands on receptor binding and changes in intracellular pathways such as cAMP, although, the biological relevance of transfected cell systems has been questioned. Therefore native

systems, like *ex vivo* slice preparations, remain pivotal to our understanding of the complex pharmacological systems within the brain.

Many neuropsychiatric disorders are suggested to be the result of changes in dopaminergic function. The basal ganglia, specifically the striatum, are suggested to play an important role in modulation of movement and cognitive processes involving executive function and reward (for review see, Balleine *et al.*, 2007). Especially located in the striatum, the dopamine D_1 receptor appears to play a pivotal effect in neuropsychiatric disorders (see Chapter 1 and Chapter 2). Therefore this chapter also assessed the utility of brain slices to study putative changes of intra-cellular (cyclic-) nucleotides in tissue from *in vivo* disease-models (*e.g.* behavioural sensitisation).

HYPOTHESES AND AIMS

Hypotheses:

- Due to the very polar physical properties of nucleotides, chromatographic retention of cAMP and ATP can be achieved using a zwitterionic (ZIC®-pHILIC) column.
- A high throughput cAMP-method is required for the large amount of samples from future studies; the development of a 96-well format assay in combination with LC-MS/MS will increase throughput and keep simultaneous analysis of cAMP and ATP.

Aims:

- Development and analytical validation of the simultaneous analysis of cAMP and ATP by using a ZIC®-pHILIC column in combination with electrospray ionisation mass spectrometry.
- Achieve the separation of cAMP and ATP from biological matrices (*i.e.* cell lysate and brain tissue lysate), and validate this method conform generally accepted bioanalytic criteria as given by the United States Food and Drug Administration (FDA; FDA, 2001).

- Development and pharmacological validation of a 96-well format cell based assay to measure the effect of compounds on cAMP accumulation.
- Validation of a 96-well filterplate method for the *ex vivo* measurement of cAMP accumulation in brain slices, to increase throughput in such way that samples from multiple treatment groups *in vivo* can be processed simultaneously.
- Pharmacological validation using dopamine D_1 and D_2 receptor (partial-) agonists and antagonists, which can be used for drug discovery, to ensure integrity of the assay.

4.2 MATERIALS AND METHODS

The described analytical method validation and *in vitro* pharmacological experiments were performed at Abbott Healthcare Products, the Netherlands.

4.2.1. Chemicals

Adenosine-cyclic-3',5'-monophosphate (cAMP), adenosine-triphosphate (ATP), 25 % aqueous ammonium hydroxide (NH_4OH), ammonium bicarbonate (NH_4HCO_3), D(+)-glucose, forskolin (colforsin), dopamine, (-)-quinpirole, LY-741626, (±)-SKF-38393, ropinirole, (+)-terguride, haloperidol and (-)-sulpiride were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Lisuride was purchaced from Tocris (Washington, DC, USA). HPLC grade water, LC/MS grade acetonitrile (ACN) and calcium chloride ($CaCl_2$) were obtained from Baker (Deventer, the Netherlands). 1-Methyl-3-isobutylxanthine (IBMX) from Calbiochem (Ontario, Canada). Bifeprunox was synthesised by Solvay Pharmaceuticals, the Netherlands.

4.2.2. Liquid chromatography

High Performance Liquid Chromatography (HPLC) separation was performed using an Agilent 1100 liquid chromatography system equipped with a binary pump operating at 0.5 mL/min, thermostatic auto sampler and column oven (Agilent Technologies, Amstelveen, the Netherlands). The injector was maintained at 4 °C and injection volumes were 10 µL. For the separation of cAMP and ATP a ZIC®-pHILIC (5 µm polymeric beads, 150 x 4.6) PEEK column (SeQuant, AB, Sweden) was used.

Mobile phase A consisted of 10 mM ammonium bicarbonate buffer adjusted to pH 9.4 with ammonium hydroxide in 20 % acetonitrile in HPLC grade water. Mobile phase B consisted of 100 % acetonitrile. A linear gradient profile from 53.7 % B to 0 % B over 1 min followed by a 1 min period of 0 % B and a 3.5 min re-equilibration period was used, after which the column was directly ready for the next injection. Total run time using pre-sampling was less then 6 min. The

column temperature was controlled at 35 °C. The LC system was controlled by Chemstation software (Chemstation for LC version 1.4, B.01.01, Agilent Technologies, Amstelveen, the Netherlands).

4.2.3. Preparation of standards

Stock solutions (10 mM of each analyte) were prepared in the HPLC-grade water. Calibration standards were prepared by serial dilution ranging from 1 - 100 nM for cAMP and 0.1 – 10 µM for ATP. The accuracy (percentage Relative Error, %RE) and precision (percentage Relative Standard Deviation, %RSD) of the method were determined by replicate analysis of five individual calibration curves. This procedure was repeated over five days to get intra- and inter- day variability. Quality control samples (QCs) contained 10 nM of cAMP and 1 µM of ATP, and were measured in between every 12 samples. Dilutions were made in lysis buffer (10 mM NH_4HCO_3, adjusted to pH 9.4 with 25 % NH_4OH solution, in 63 % ACN in HPLC grade water).

4.2.4. Mass spectrometry

In this study, two mass spectrometers were investigated. First, for the development of HILIC in combination with a mass spectrometer and the subsequent *in vitro* pharmacological validation, a linear quadrupole ion trap mass spectrometer (LTQ10000, Thermo Fisher Scientific /Finnigan, San Jose, CA, USA) was used. The HPLC-system, as described above, was coupled to the mass spectrometer using an electrospray ionisation (ESI) source. A post-column split ratio of 1:5 was applied to reduce fouling of the ion source. After the splitter a divert valve directed the column effluent to waste in the period before and after elution of the analyte of interest. During effluent diversion, a second Agilent 1100 isocratic pump delivered a mixture of 50 % (v/v) acetonitrile and water to the mass spectrometer to ensure a stable electro spray and to clean the orifice. The ion spray voltage was set at 2 kV operating in negative electrospray ionisation (ESI). The capillary temperature was set at 300 °C and capillary voltage was set to -16 V and -23 V for cAMP and ATP, respectively. Nitrogen flow for sheet- and auxiliary- gasses were set at 20 and 15 (arbitrary units) respectively. Sweep gas

was not used. The tip of the probe was set off axis to the orifice to ensure system robustness (*i.e.* prevent fouling of the source). Daughter scanning was used as the detection mode, as shown in Table 4-1.
Two segments were used (*i.e.* m/z 328 for cAMP and m/z 506 for ATP) in the negative ESI mode. Before and after the negative ESI segments, segments of positive ESI mode were applied using the same conditions for spray voltage and gas flow to prevent possible charging of ion lenses of the mass spectrometer. Data were acquired with XCalibur v.2.0 SR2 and processed using LCQuan v.2.5 (Thermo Fisher Scientific/Finnigan).

Table 4-1 Mass spectral conditions for LTQ 10000

Parent (m/z)	Isolation width	Collision energy	Activation time (ms)	Scan (m/z)	IT time (ms)	XIC (m/z)
328	1.5	38	30	90 - 350	800	134
506	2.0	25	30	135 - 550	800	408

IT: Injection time; XIC: Extracted ion chromatogram; ms: milli seconds; m/z: mass to charge ratio. Activation type: Collision induced dissociation (CID).

The second mass spectrometer investigated was a triple quadrupole mass spectrometer (Quattro Premier^{TM} XE, Micromass, Waters, Milford, MA, USA). The HPLC-system was directly coupled to the triple quadrupole mass spectrometer by an orthogonal designed electrospray interface (Z-Spray^{TM}-source.) This source design improves protection of the mass spectrometry source from fouling, *e.g.* by non-electroactive molecules. Massa spectrometer parameters were optimised by flow injection analysis of both compounds (*i.e.* cAMP and ATP) using auto-tune.
Two segments were used in the positive ESI mode (*i.e.* m/z 330 for cAMP and m/z 508 for ATP). Data acquisition and analysis were performed with MassLynx v.4.1 software (Waters).

4.2.5. Cell culturing

The human dopamine D_2 long splice variant receptor (hD_{2L}) was cloned and expressed in Chinese Hamster Ovarian (CHO-K1) cells (Dr. D. Grandy, Vollum Institute, Portland, OR, USA). Cells were grown in Dulbecco’s modified Eagle’s

medium (DMEM; Invitrogen, Breda, the Netherlands) supplemented with 2 mM (L)-glutamine and 10 % heat-inactivated foetal bovine serum (FBS; Invitrogen) in 93 % air/7 % CO_2. Cells were cultured twice a week. For the cAMP assay, CHO-hD_{2L} cells were trypsinisated and transferred into 96-well plates (Nunclon™; VWR, Amsterdam, the Netherlands), 48 h before the assay to produce a 100 % confluent monolayer (*ca.* 40000 cells/well).

4.2.6. cAMP accumulation assay (cell based)

In vitro functional adenylate cyclase activation assay

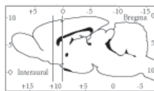
Test compounds were diluted in a 96-well plate using assay buffer (DMEM, containing 1 mM IBMX and 0.3 µM forskolin). Incubations were performed in 96-well plates at 37 °C for 20 min in a total volume of 100 µL and each condition was tested in duplicate. The incubation was initiated by addition of 100 µL assay buffer, in the presence or absence of the test compound, to a 96-well plate with cultured cells. Additionally for antagonists, the assay buffer also contained 1 µM (-)-quinpirole. Prior to nucleotide extraction, adherent cells were rapidly washed with water (HPLC grade) to remove the incubation medium and salts, preventing fouling of the mass spectrometers orifice and ion suppression. The nucleotides were extracted by addition of 100 µL lysis buffer. The plate was cooled at 4 °C for a few min and heat-sealed (ThermoSealer Abgene, Thermo Fisher Scientific, Breda, the Netherlands). The samples were centrifuged (4000 G, 5 min) and stored at 4 °C until analysis.

In this method, the specific lysis-buffer (pH 9.4 controlled with 63 % ACN in HPLC grade water containing 10 mM NH_4HCO_3) is crucial as pre-validation studies showed that other concentrations or reagents will affect subsequent analysis (*e.g.* chromatography and ionisation).

4.2.7. Tissue preparation

Naïve adult male Wistar Unilever Harlan (WUH) rats, 200-250 g (Harlan, the Netherlands) were used and two rats per 96-well filter plate were needed. The rats were decapitation, without anaesthetics, using a guillotine and various brain regions were dissected (according to Paxinos and Watson, 1998) as quickly as

possible. Directly after preparation, the tissue was kept in ice-cold Krebs-Ringer Buffer. 3 mM K^+ Krebs-Ringer Buffer (KRB, Gibco-Invitrogen, Breda, the Netherlands) contained (in mM): 121 NaCl, 2 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11 D-(+)-glucose, 1.2 KH_2PO_4, with modifications as used during whole experiment: $CaCl_2$ (1.2 mM) and D(+)-glucose (22 mM). The buffers were freshly prepared on each test day, kept at 37 °C and gassed with 5 % CO_2/95 % O_2 for at least 0.5 h before use.



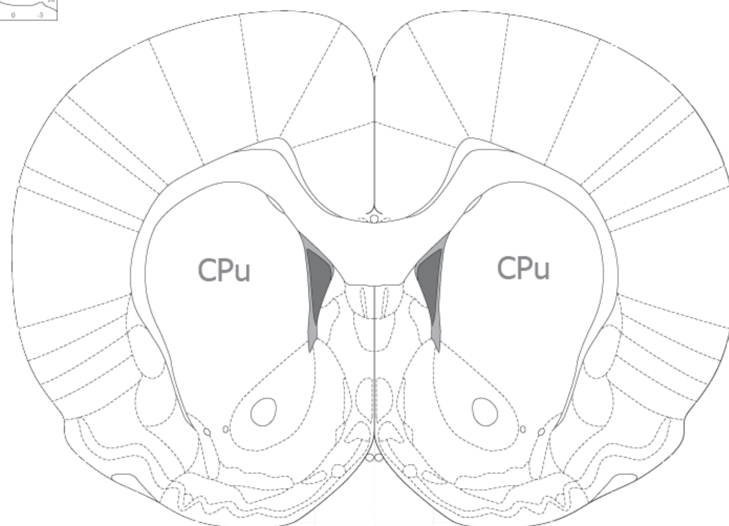


Figure 4-2 Schematic of dissected brain area (CPu)

The pair of vertical lines in the sagittal schematic (top left) indicate the anterior and posterior limits of the taken caudate putamen (CPu), coordinates were +1.7 – +0.2 mm relative to Bregma (Paxinos and Watson, 1998). CPu indicates the region of interest in coronal sections. Diagrams were modified from Paxinos and Watson (1998).

The same brain areas from animals within the same group were pooled (*i.e.* from 4-6 rats) and sliced together. The fresh tissue was sliced in two directions (coronal, 90° apart) using McIlwain tissue chopper (300 µm setting), and subsequently washed with three 5-mL aliquots of ice-cold buffer and allowed to settle. Then the slices were washed 3x times with warm (37 °C) oxygenated KRB followed by one h incubation in 37°C water bath under 5 % CO_2/95 % O_2

atmosphere. The methodology was approved by the animal ethics committee (Abbott Healthcare Products, the Netherlands) and is in accordance with all local laws.

Methodological considerations

Variation within *in vivo* treated groups made it impossible to use only a single rat for an $n = 1$ *ex vivo* experiment. To overcome this problem the dissected brain areas from at least four animals within the same treatment group were pooled. This significantly reduced intra-experiment variation.

An $n = 1$ experiment consisted of all concentrations conduced in quadruplo in 1 individual *ex vivo* experiments using 4 – 8 rats from 1 individual *in vivo* experiment.

4.2.8. cAMP accumulation assay (brain tissue)

Activation and inhibition of adenylate cyclase activity was monitored by measuring levels of cAMP and ATP in 96-well plates by using filterplates (1.2 µm Multiscreen-plates, Millipore). Briefly, the slices were washed with three 5-mL aliquots KRB followed by taken up in 10 ml warm (37 °C) KRB. After pre-wetting the filterplates with 100 µL assay-buffer (KRB with 1 µM Forskolin and 1 mM IBMX), constantly suspended slices (100 µL) were transferred to 96-well filter plates using large orfice pipette tips. The buffer was removed using vacuum-filtration (Millipore) and 100 µL of assay-buffer was added to wash the slices. After removal of the assay-buffer, 100 µL of the diluted test compounds were added. After 30 min the incubation was stopped by vacuum-filtration, slices were washed with 100 µL HPLC-grade water and were lysed by the addition of 100 µL ice-cold lysis-buffer. After 30 min incubation at 4 °C the plate is vacuum-filtered collecting the fraction in a normal 96-well plate. The plate wass heat-sealed (Thermosealer, Abgene) and stored at 4 °C till analysis. Heat sealed plates can be stored at 4 °C for maximal 2 weeks. Sealed plates could not be stored at -80 °C, as the acetonitrile would adversely affect the plate seal causing sample evaporation (*i.e.* freeze-drying).

4.2.9. Data analyses

Chromatographic peak resolution was calculated by $Rs = 2 (Rt_{(a)} – Rt_{(b)})/(W_{(a)} + W_{(b)})$, where Rt is the retention time and W is peak width at peak baseline. Complete peak separation is achieved at Rt > 1.5. Calibration curves of cAMP and ATP were constructed using linear curve fitting based on the areas under the curve of the measured standards. All points were equally weighted and the origin ignored. For each biological sample, the cAMP concentration was corrected for the ATP concentration by dividing cAMP levels by total amount of nucleotides: cAMP/(cAMP+ATP)*100.

Data represents mean ± s.e.m. from at least four independent experiments, conducted in duplicate (*in vitro*) or quadruple (*ex vivo*). Values are expressed as a percentage of respective control. Curves were fitted by non-linear regression using Hill 4 parameter sigmoidal equation. Graphs, pEC_{50} and E_{max} values were calculated using SigmaPlot (v9.01, Systat, Chicago, IL, USA). Antagonist pA_2 values were calculated according to the Cheng-Prusoff equation (Cheng *et al.*, 1973).

4.3 RESULTS

4.3.1 Analytical Method Development

For the development of an analytical method to measure cAMP and ATP in cell based samples, a linear ion trap mass spectrometer (LTQ, Finnigan) was used as this type of mass spectrometer was available at the time. The mass spectrometry was coupled to a HPLC system (HP1100, Agilent) with ZIC-pHILIC column (SeQuant, Sweden) to achieve temporal separation of the analytes, see below.

Optimisation of HPLC conditions

In order to optimise the resolution of cAMP and ATP chromatography, the resolution was measured at different flow rates (Table 4-2). Although 0.2 mL/min gave the best resolution, 0.5 mL/min was chosen as a compromise between the resolution and analysis time. At this flow rate the cAMP and ATP peaks are still baseline separated.

Table 4-2 Flow rate vs chromatographic resolution of cAMP and ATP

F (mL/min)	Rt cAMP (min)	Rt ATP (min)	Rs
0.2	7.11	9.05	5.84
0.3	9.94	6.17	4.54
0.4	3.75	4.79	4.28
0.5	3.00	3.92	3.98
0.6	2.42	3.3	3.77
0.7	2.16	2.97	3.60

F, flowrate; Rt, retention time; Rs, resolution.

Table 4-3 shows the influence of the pH of mobile phase A on the retention time behaviour of cAMP and ATP over a range of pH 8.0 - 10 by adding dropwise ammonium hydroxide (25 %). At all tested flow rates the criteria for complete peak separation (*i.e.* Rs > 1.5) was achieved. Experiments were performed at a flow rate of 0.5 mL/min.

Table 4-3 Influence of pH on the retention times of cAMP and ATP

pH mobile phase A	Rt cAMP (min)	Rt ATP (min)
8.0	2.87	3.87
9.4	3.07	4.02
10.0	3.08	4.03

Rt, retention time.

With current salt-free buffer composition, chromatographic peaks were relatively wide. Therefore the buffer strength of the mobile phase was varied between 1 and 100 mM ammonium bicarbonate (NH_4CO_3: a non-volatile salt which can be used in combination with ESI-MS) and adjusted to pH 9.4. Peak broadening occurred at the 1 mM level and improved with increasing the buffer strength. To avoid ion suppression, which occur at high concentrations of ammonium bicarbonate, a 10 mM concentration was chosen.

The column temperature was varied between 20 and 50 °C which did not affect the retention time of the measured analytes.

Optimisation of mass spectrometric condition

Preliminary experiments using flow injection analysis (FIA) showed that nucleotides (cAMP and ATP) could be identified using mass spectrometry but that quantification using FIA is not sufficient due to high background noise; LOD ~1 nM for cAMP. These preliminary results demonstrate that chromatography is needed to concentrate sample and remove contaminations.

Single ion mode (MS) was found to be not sensitive enough and tandem mass spectrometry was investigated. Experiments performed with an LTQ (negative ESI) showed that MS^3 fragment m/z 107 was approximately 1000 times less sensitive instead of measuring MS^2 fragment m/z 134 (adenine). This result shows again no advantage of LTQ above using a triple quadrupole mass spectrometer.

Under the described conditions the MS-MS fragmentation of cAMP (m/z 328) results in the daughter ion m/z 134 which corresponds to the purinylamine part of the molecule (*i.e.* adenine). For ATP (m/z 506) the loss of a phosphate- and a water group results in the most intense fragment ion at m/z 408 (see Figure 4-3). The fragmentation and tuning of the ion optics for both compounds was optimised using the auto tune capabilities of the instrument software. In the acquisition method cAMP and ATP were measured in separate segments with a maximised injection time to obtain the best possible sensitivity. The lowest calibration standard still contained 15 scans to describe the chromatographic peak. A tandem mass spectrum (MS/MS) with the selected daughter ions and a proposed fragmentation is presented in Figure 4-3. An example extracted ion chromatogram is shown in Figure 4-4.
Using single reaction monitoring (SRM) mode in two separate segments, resulted in better sensitivity then simultaneous segments (multiple reaction monitoring, MRM) because the mass spectrometer does not have to switch between the two mass during the 800 ms interval between ionisation and detection.

Negative ESI was found the most sensitive mode for this assay. Additionally, more selectivity was obtained using the negative ESI mode whilst the majority of biological molecules in cell lysate were ionised in positive ESI mode.

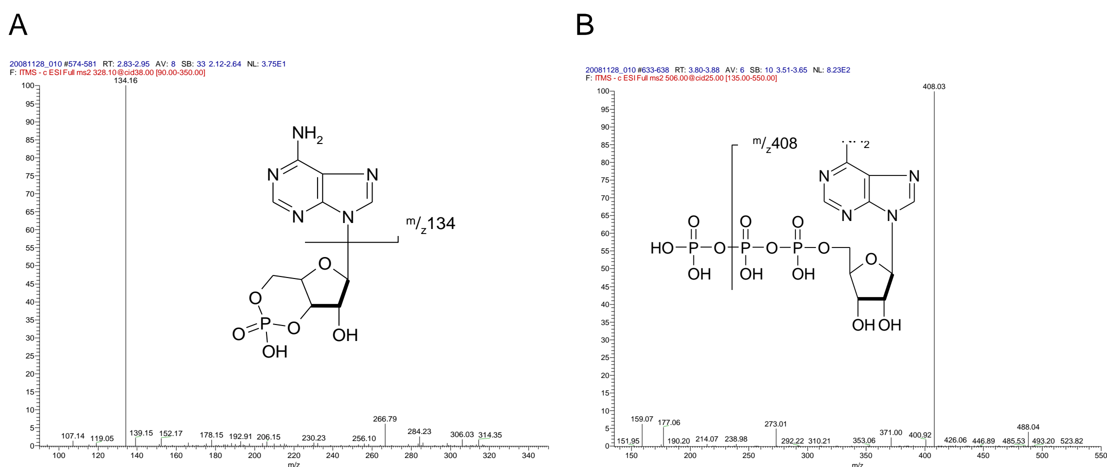


Figure 4-3 Mass spectra of cAMP and ATP with their proposed fragmentation

A. MS/MS spectra of cAMP resulting in the main peak at m/z 134. Insert: Structure of cAMP with proposed fragmentation m/z 328 > 134. B. MS/MS spectra of ATP resulting in the main peak at m/z 408. Insert: Structure of ATP with proposed fragmentation m/z 506 > 408. y-axis, relative abundance (%); x-axis, mass to charge ratio (m/z).

The probe was positioned away from the inlet of the source as far as possible, this prevents non-ionic molecules entering the MS. Further settings like collision and sheet gas flows, source temperature, and ionisation voltage were optimised using the AutoTune function in XCaliber Software (Waters). Another feature to reduce fouling of the source of the mass spectrometer was to split post-column before the inlet flow to the MS source. Since mass spectrometry is a concentration dependent technique, reducing the volume is not of any consequence for quantification. Note that the flow rate can have consequences for the optimum electrospray, depending on used ESI-probe, ESI-type and MS-source. By applying a split of 8:2, the contamination was theoretically reduced by 80%.

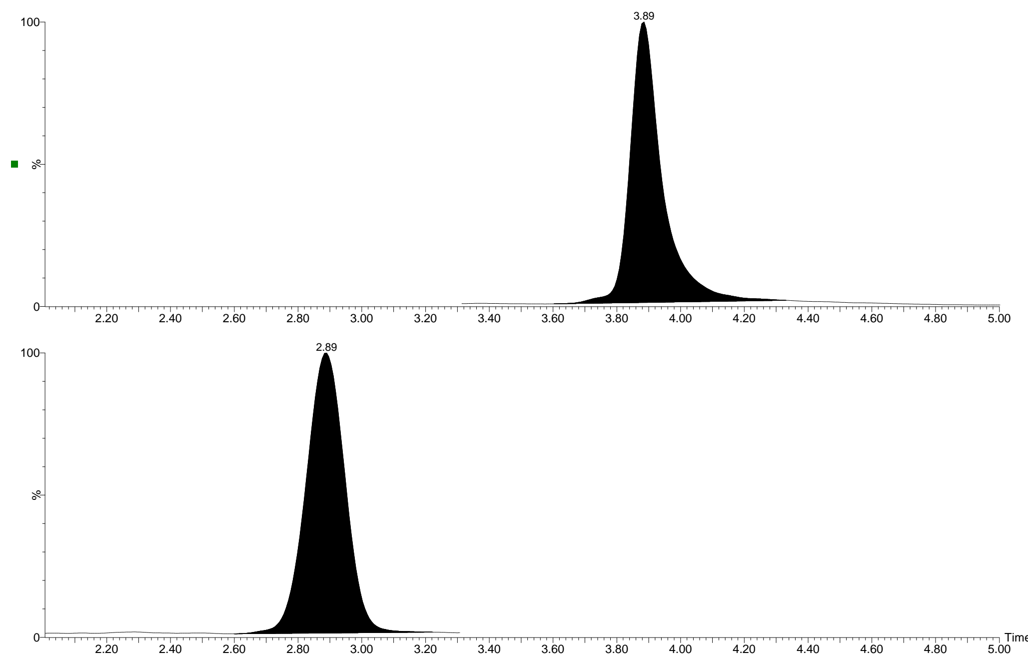


Figure 4-4 Extracted ion mass chromatogram (XIC) of cAMP and ATP

Top: XIC of ATP, m/z 506 > 408. Bottom: XIC of cAMP, m/z 328 > 134. y-axis, relative abundance (%); x-axis, time (min).

Validation of the LC MS MS method

The assay was linear over at least the range of 0.5 – 100 nM for cAMP and 50 nM – 50 µM for ATP. For both components, the use of higher concentrations resulted in a non-linear calibration curve. Regression of the calibration curves (Figure 4-5) yielded the following for cAMP: slope (s.d.), 0.032 (0.002); y-intercept, 0.136 (0.346) nM; r^2 = 1.000, and ATP: slope, 0.149 (0.003); y-intercept, 75.639 (14.719) nM; r^2 = 0.9999.

The addition of matrix (*i.e.* cell lysates) to the calibration curves affected neither the accuracy nor the precision of the concentrations within the working range.

The LOD (3*SD) were at least 0.5 and 200 nM for cAMP and ATP respectively.

The working range (LLOQ; 5*SD, %RSD < 20 %) was at least 1-100 nM for cAMP and 500 – 10000 nM for ATP.

Carryover was determined by a blank injection after a 10 µM cAMP and 1 mM ATP standard and resulted in a carryover of < 0.1 %.

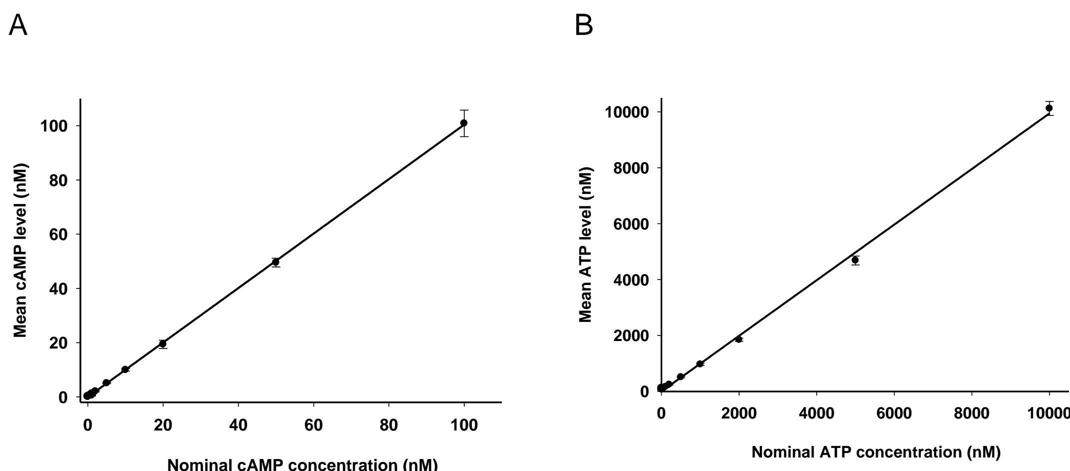


Figure 4-5 Calibration curves of cAMP and ATP

A.) Calibration curve of cAMP in the range of 0.5 – 100 nM. B.) Calibration curve of ATP in the range of 50 – 10000 nM. Graphs represent mean ± s.d. of measurements from 5 individual days, *n* = 5.

The intra-run accuracy and precision ranged from −8 to 9 % RE and 1 to 17 % RSD, respectively, for both of the analytes (Table 4-4). The inter-run accuracy and precision ranged from -20 to 8 % RE and 2 to 12 % RSD, respectively, for both of the analytes (Table 4). All mean concentrations had an accuracy and precision of >85 %, except for LLOQ (>80 %), which is in good agreement with FDA guidelines (FDA, 2001).

The robustness of the analysis was monitored by the measurement of quality controls (QC) containing 10 nM cAMP/1 µM ATP in lysis buffer. The intra-assay variation was assessed by a total of 65 QCs per experiment, *i.e.* a QC in between every twelve biological samples. A typical run contained 65 QCs with RE and RSD < 15 % for both cAMP and ATP.

To investigate storage conditions and stability of standards and samples, a standard solution of cAMP (10 nM) and ATP (1 µM) was prepared and aliquoted in vials. The vials were stored in the refridgerator (4 °C) for a period of 14 days. Every day fresh standard solutions were prepared and the stored standard solution was measured. No decrease in the concentration of cAMP or ATP was

observed. Additionally, the same experiment was performed with CHO-hD$_{2L}$ cell lysate; cAMP and ATP were stable for a period of at least 14 days.

Table 4-4 Standard curve statistics for cAMP and ATP

	Intra-run			Inter-run		
Nominal (nM)	Mean (nM)	RE (%)	RSD (%)	Mean (nM)	RE (%)	RSD (%)
cAMP						
0.5	0.5	3	24	0.7	-41	30
1	1.1	-5	17	1.3	-20	12
2	1.8	9	11	2.1	-3	10
5	5.0	0	3	5.1	-2	4
10	10.5	-5	1	10.0	0	5
20	21.5	-8	6	19.4	3	8
50	50.9	-2	4	49.5	1	3
100	104.7	-5	7	100.8	-1	5
ATP						
50	144	-188	2	119	-138	19
100	187	-87	5	162	-62	16
200	268	-34	3	245	-22	10
500	536	-7	3	514	-3	5
1000	1007	-1	3	968	3	4
2000	1891	5	2	1847	8	3
5000	4706	6	2	4682	6	3
10000	10415	-4	2	10120	-1	2

Statistics indicate nominal concentration (nM), calculated concentration (Mean, nM), percentage relative error (%RE), and percentage relative standard deviation (%RSD). All curves were regressed using a linear with 1/X fit. n = 5 curves analysed for all statistics.

Heat sealed 96-well and 384-well plates could be stored in the fridge for a period up to 14 days without evaporation of the acetonitrile fraction or decrease of cAMP or ATP concentration, which exclude possible adhesion of cAMP or ATP to the well plates.

4.3.2 Pharmacological validation (cell based assay)

To verify adenylate cyclase (AC) activity in CHO-cells transfected with the hD_{2L} receptor, the effect of the AC activator forskolin on cAMP accumulation was assessed. Forskolin produced a concentration dependent increase in cAMP accumulation (Figure 4-6). Forskolin was tested in a concentration up to 100 µM, as the stimulating effects of higher concentrations were considered to be not physiologically relevant, and not necessary for assay development. Because the maximal effect was not achieved yet, no pEC_{50} or E_{max} values could be calculated. Basal cAMP and ATP levels found in 96-well plate cultured CHO-D_{2L} cells (*ca.* 40000 cells/well) were between 0.5 - 5 and 1000 - 3000 nM, respectively. ATP values lower then 1000 nM/well indicate that no cells were cultured or indicate cell toxicity. Cell toxicity is often recognised by a concentration dependent decrease of ATP levels caused by the tested drug. Forskolin (0.3 µM) produced approximately 20 fold increase over basal levels of cAMP and was used in future experiments.

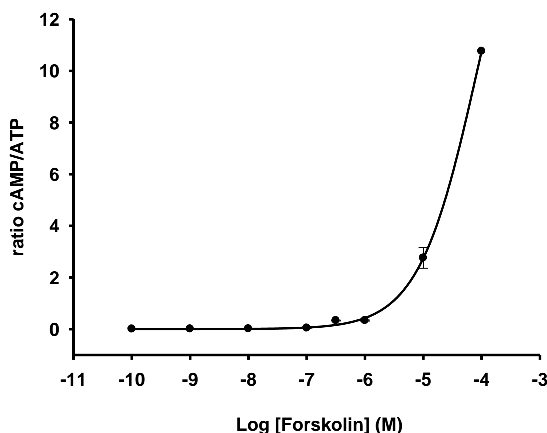


Figure 4-6 Effect of forskolin on cAMP accumulation in CHO-hD_{2L} cells

The AC-activator forskolin stimulated cAMP formation in a dose dependent manner. Data presents mean ratio (cAMP/ATP) ± s.e.m. of two individual experiments performed in duplicate.

Hypothetically, ratio calculations between cAMP and total nucleotide content could make a correction for the relative amount of cells per well/sample, and will reduce variation. As proof of concept, the dopamine D_2 partial agonist terguride

was tested and calculated using only cAMP or the cAMP/ATP ratio. The advantage of the cAMP/ATP ratio over cAMP-only for partial agonist terguride was tested in a concentration range of 1 pM to 1 µM. When the effect was expressed as cAMP levels only, variation was large (Figure 4-7). However, when the effect was expressed as the cAMP/ATP ratio, there was a clear concentration dependent effect of terguride resulting in a pEC_{50} of 10.2 ± 0.1 and intrinsic activity (E_{max}) of 35 ± 3 % (Figure 4-7).

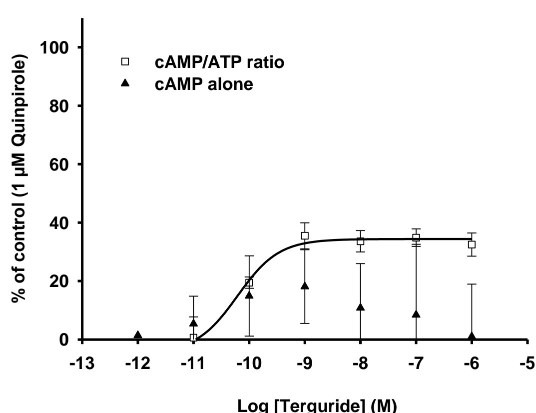


Figure 4-7 The value of cAMP/ATP ratio calculation

The effect of terguride on cAMP accumulation was tested and calculated using cAMP-only (▲) or cAMP/ATP ratio (□). Calculations using cAMP-only resulted in a non-significant effect, with large variations. Calculation using cAMP/ATP ratio revealed a significant, partial effect resulting a pEC_{50} of 10.2 ± 0.1 and E_{max} of 35 ± 3 %, $n = 6$.

Effect of dopamine receptor (partial-) agonists on cAMP accumulation

The quantitative determination of cAMP in cells was further explored to illustrate the utility of this method. As a proof of concept the effect of different D_2-like ligands, including dopamine (endogenous ligand for the dopamine receptor), the prototypical agonist quinpirole and ropinirole (selective D_2-like agonists), on cAMP accumulation in CHO-cells transfected with hD_{2L} receptors was tested. Under the conditions used, the application of 10 µM quinpirole reduced forskolin (0.3 µM) stimulated cAMP levels by more then 70 %. The potencies of the agonists were quinpirole > ropinirole > dopamine (Figure 4-8A, Table 4-5). All

compounds were confirmed as full agonists as they were without any effect in presence of 1 µM quinpirole (data not shown).

Furthermore, the dopamine D_2 receptor partial agonists terguride, bifeprunox and lisuride were tested and showed partial activity on cAMP accumulation under the present conditions. The relative efficacies (E_{max}) of these partial agonists were lisuride > terguride > bifeprunox (Figure 4-8B and Table 4-5). The relative potencies of terguride, bifeprunox and lisuride were essentially equal to each other.

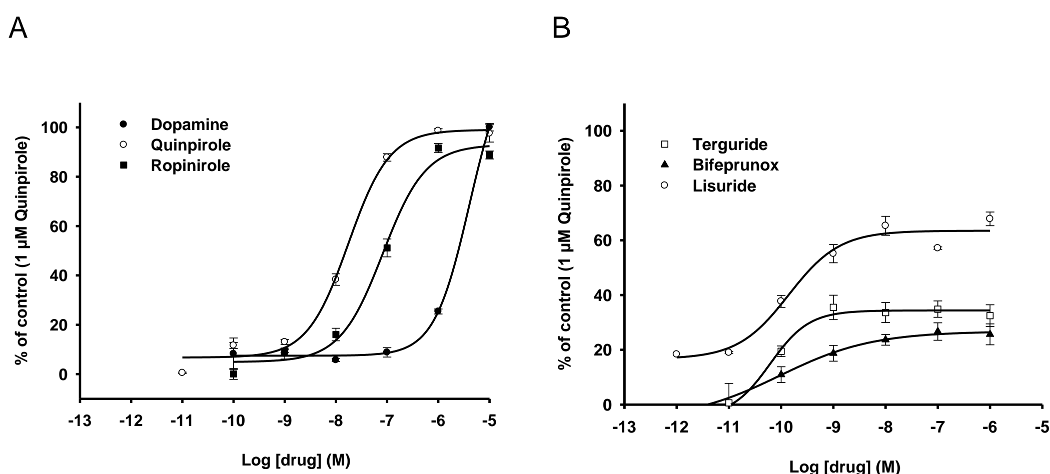


Figure 4-8 Effect of D_2-like (partial-) agonists on cAMP accumulation

A. Dopamine (●), quinpirole (○) and ropinirole (■) showed full inhibition of cAMP accumulation; B. Terguride (□), bifeprunox (▲) and lisuride (○) showed only partial inhibition of cAMP accumulation. See Table 4-5 for pEC_{50} and E_{max} values. Data are expressed as % maximal forskolin stimulated cAMP inhibition by 1 µM quinpirole ± s.e.m.

Table 4-5 Functional data of agonists for hD_{2L} receptor in CHO cells

Compound	pEC_{50}	E_{max} (%)
Quinpirole	7.8 ± 0.1	98 ± 4
Dopamine	5.4 ± 0.6	100 ± 2
Ropinirole	7.1 ± 0.1	89 ± 2
Terguride	10.2 ± 0.1	35 ± 3
Bifeprunox	10.0 ± 0.2	26 ± 4
Lisuride	9.9 ± 0.2	68 ± 2

Functional data represents mean ± s.e.m. (*n* = 4-12 individual experiments).

Effect of dopamine receptor (partial-) antagonists on cAMP accumulation

The effects of the selective D_2-like antagonist LY-741626, and the prototypical antagonists haloperidol and (-)-sulpiride on cAMP accumulation were investigated. LY-741626, haloperidol and (-)-sulpiride (1 pM – 10 µM) did not alter cAMP accumulation and were therefore found as silent antagonists, *i.e.* exhibited neither partial nor inverse agonistic activity at the hD_{2L} receptors in this system (data not presented). In order to test antagonism of these ligands, the ligands were tested in presence of 1 µM quinpirole. All ligands fully inhibited the quinpirole-mediated effect on cAMP accumulation in a dose dependent manner. Relative potency: haloperidol > LY-741626 > (-)-sulpiride (Figure 4-9A, and Table 4-6).

In order to test the ability of the assay to determine antagonism of partial agonists, the partial agonists terguride and bifeprunox were tested in the presence of 1 µM quinpirole and partially reversed the effect of quinpirole; relative efficacy (E_{max}) of bifeprunox > terguride, and the relative potency of terguride > bifeprunox (Figure 4-9B, and Table 4-6). The agonistic and antagonistic effects of the partial agonists were found complementary as expected.

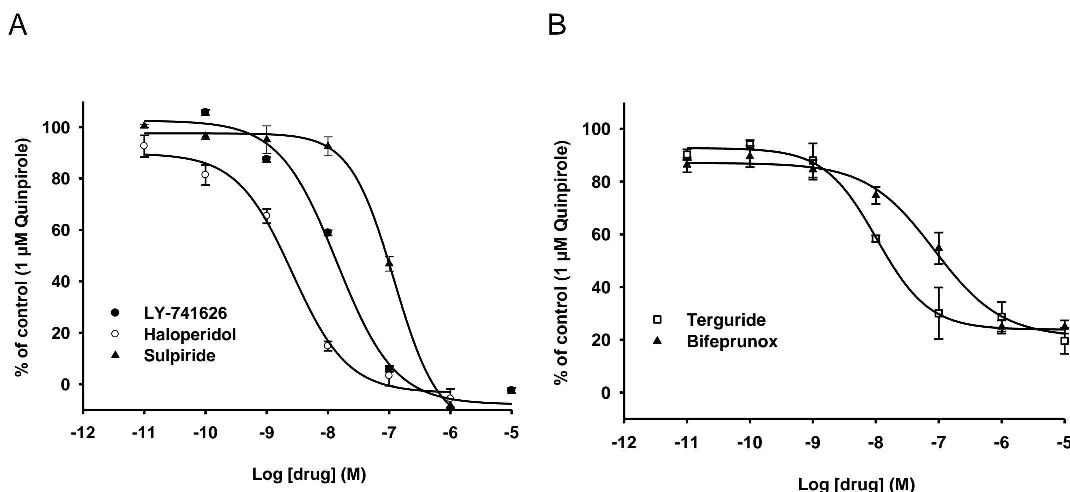


Figure 4-9 Effect of D_2-like partial- and full- antagonists on cAMP accumulation

A. LY-741626 (●), haloperidol (○), and (-)-sulpiride (▲) fully antagonised the effect of quinpirole; B. Terguride (□) and bifeprunox (▲) only partially reversed the effect of quinpirole. All antagonists were tested in presence of 1 µM of quinpirole. See Table 4-6 for pA_2 and E_{max} values. Data are expressed as % maximal cAMP inhibition by 1 µM quinpirole ± s.e.m.

Table 4-6 Functional data of antagonists for hD_{2L} receptor in CHO cells

Compound	pA_2	E_{max} (%)
Haloperidol	9.9 ± 0.1	93 ± 4
(-)-Sulpiride	8.2 ± 0.1	100 ± 1
LY-741626	9.2 ± 0.2	102 ± 1
Terguride	9.3 ± 0.1	66 ± 4
Bifeprunox	8.4 ± 0.2	75 ± 2

Functional data represents mean ± s.e.m. (*n* = 2-5 individual experiments).

These results show that the linear ion trap mass spectrometer (LTQ) is suitable to measure cell lysate samples, although, the analysis of brain tissue samples caused severe contamination of the LTQ and result in loss of sensitivity. Pollution of the source of the mass spectrometer was mainly caused by, but not limited to, salts and biogenic components in the tissue samples which were extracted by the

lysis buffer. At this time, a tandem quadrupole mass spectrometer (Quattro Premier™ XE, Micromass) hyphenated with an orthogonal designed electrospray interface (Z-Spray™-source) was available and investigated. The Z-Spray™-source protects the mass spectrometer from pollution by unwanted biogenic compounds in the tissue samples. The HPLC system as described under "Section A: Analytical Method Development" was used, and no future changes on the HPLC system were made.

The robustness of the analysis was monitored by the measurement of quality controls (QC) containing 10 nM cAMP/1 µM ATP in lysis buffer. A typical run contained 41 QCs per experiment, *i.e.* a QC in between every twelve biological samples, and intra-assay variation were RE and RSD < 10 % for both cAMP and ATP.

4.3.3 Pharmacological validation (brain tissue assay)

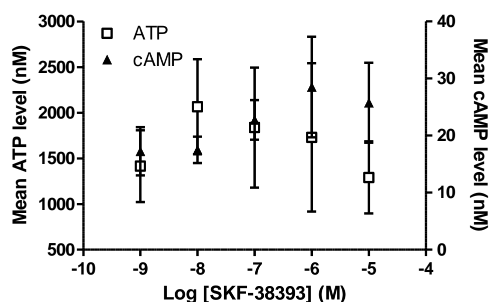
The developed LC-MS/MS method (developed for *in vitro* cell based assays) was applied to *ex vivo* rat striatal slices (350 x 350 µm) using a new developed filter-plate assay. To validate the described LC-MS/MS method using striatal tissue, the effect of the selective D_1-like agonist (SKF-38393) and selective D_2-like agonist (quinpirole) were tested.

Different concentrations of SKF-38393 were incubated in the presence of rat striatal slices for 30 min. A large variation was observed in the measured ATP and cAMP levels (Figure 4-10A). When the effect of SKF-38393 was calculated based on only cAMP levels, a non-sigmoidal increase was obtained due to large variation (Figure 4-10B). However, following correction of the same samples by calculation of a ratio between cAMP and ATP, a concentration dependent effect was observed with very little variation (Figure 4-10C).

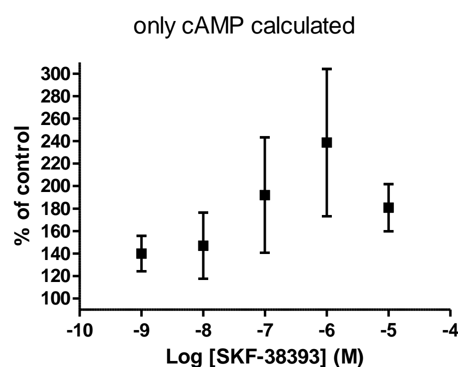
The effect of quinpirole, in presence of 1 µM SKF-38393, on cAMP accumulation was assessed. The inter-run variation (*i.e.* variation between the averages of n = 4 individual experiments) observed with quinpirole was similar to the effect found with SKF-38393, *i.e.* calculations with cAMP-only demonstrated large variations (Figure 4-11A). When calculations were made using a cAMP/ATP ratio, the variation was acceptable and a reliable concentration response curve was fit (Figure 4-11B). Although final pEC_{50} values were the same for both cAMP-only and cAMP/ATP ratio calculations (8.0 and 8.0 ± 0.1, respectively) the reduction in variation is important for the accuracy of the results and calculations of the efficacy.

For validation purposes, also the intra-run data was examined representing the variation within an experiment. When the intra-run data was calculated using cAMP levels only, very large variation was revealed (Figure 4-11C). In addition to the large variation (%RSD of Figure 4-11C and D: > 30 % and < 14 %, respectively), also qualitative pharmacology was affected and in most experiments no significant concentration response curve could be obtained when using only cAMP levels for calculation (Figure 4-11C).

A



B



C

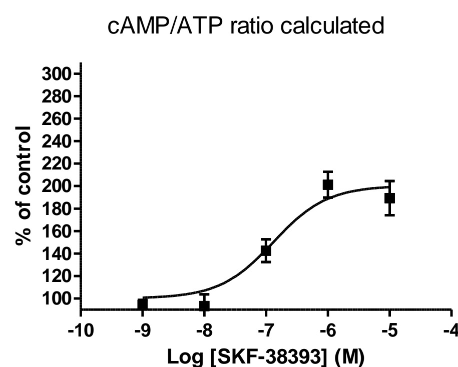


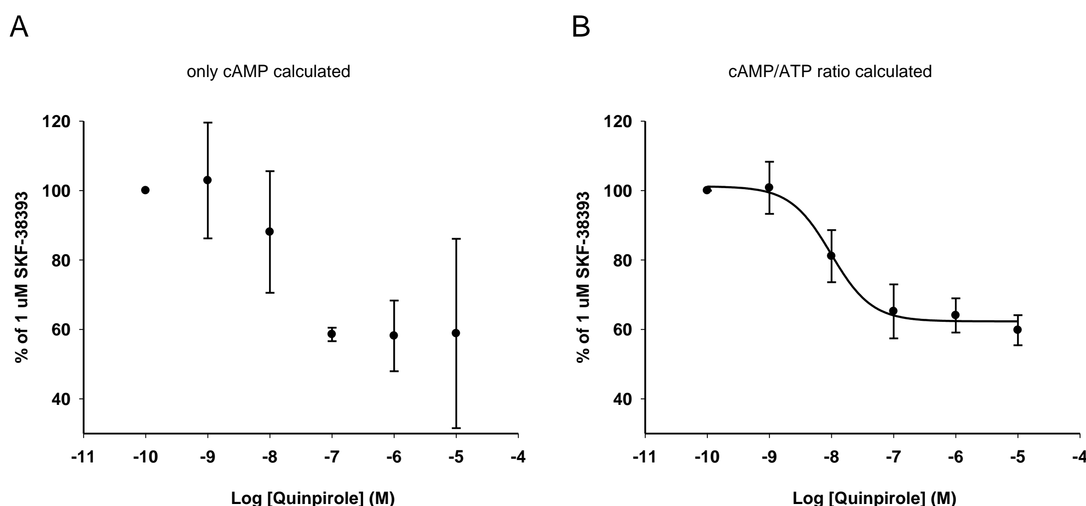
Figure 4-10 Effect of calculations with cAMP levels or cAMP/ATP conversion

SKF-38393 was incubated with rat striatal slices and cAMP and ATP were measured. A. Measured ATP and cAMP levels showing large variation. B. The effect of SKF-38393 calculated with cAMP levels only. C. Effect when calculated using cAMP/ATP conversion. The effect was expressed as mean % ± s.e.m. (tested in quadruple), $n = 4$ individual experiments.

These experiments demonstrate that quinpirole induced a decreased in SKF-38393 (1 µM) stimulated cAMP levels in rat striatal slices when cAMP/ATP ratio was calculated.

Inter-run variation

A B



Intra-run variation

C D

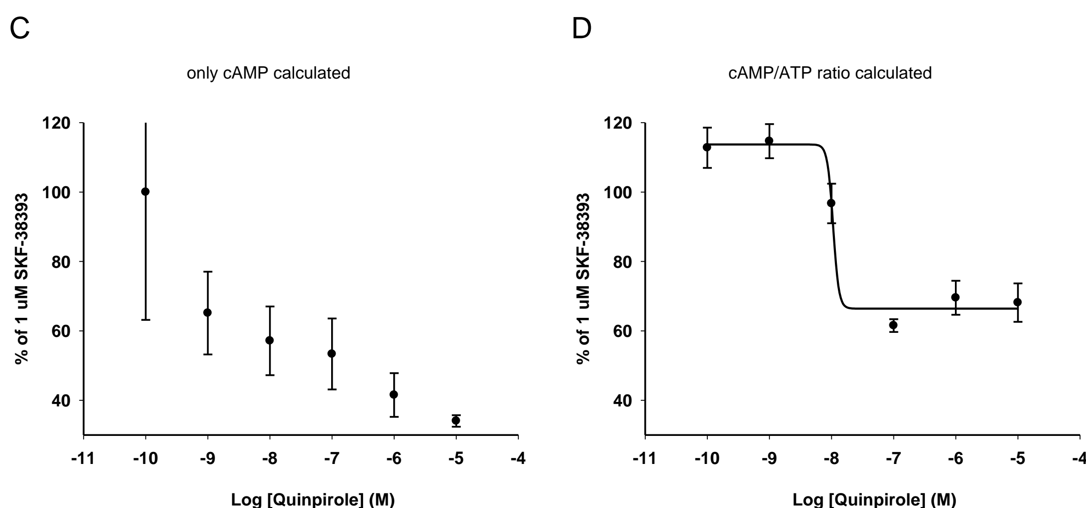


Figure 4-11 Effect of calculation with cAMP levels or using cAMP/ATP ratio

Quinpirole was incubated with rat striatal slices and cAMP and ATP were measured. A.) The effect of quinpirole calculated with cAMP levels only, no pEC_{50} value could be calculated due to large variation. B.) Effect of calculations using cAMP/ATP conversion, pEC_{50} 8.0 ± 0.1. C.) Data from a typical experiment representing intra-run data calculated using cAMP levels only. D.) Data from a typical experiment, calculated using cAMP/ATP ratio. The effect was expressed as mean % of control ± s.e.m. (tested in quadruple), $n = 4$ individual experiments (for A and B).

4.4 DISCUSSION

This chapter describes the successful development, validation and application of a LC-MS/MS method to determine (cyclic-) nucleotides. The potential applicability of the method was evaluated by assessing the utility of measuring cAMP accumulation following *in vitro* (cell based) and *ex vivo* (rat brain slice) experiments with several dopamine receptor ligands.

Due to their extremely polar properties, separation of nucleotides is commonly performed using anion-exchange chromatography, although the mobile phase compositions are highly incompatible with LC-MS/MS analysis. Another approach used is the separation of nucleotides using a reversed phase chromatography, however, because of their polar properties the nucleotides are poorly retained on a reversed stationary phase and therefore often used in combination with an ion-pairing reagent (N,N-dimethylhexylamine, Cai, 2001 and Qian *et al.*, 2004; hexylamine, Coulier *et al.*, 2006, for review see Werner, 1991) or Hypercarb stationary phases combination with diethylamine (Xing *et al.*, 2004). Preliminary experiments using the volatile ion-pairing reagent N,N-dimethylhexylamine (DMHA) showed a main peak of m/z 130 in the mass-spectrum, originating from DMHA, and resulted in poor sensitivity due to substantial background contribution, ion suppression, source pollution and possibly other side effects like space charging in the ion trap mass spectrometer. Possibly this latter phenomenon will not occur in a triplequadrupole mass spectrometer when operating in selective reaction monitoring (SRM) mode. Although Lorenzetti and colleagues (2007) showed that C18 columns can be used without volatile ion pairing reagents, the poor separation of the nucleotides requires good resolving power of the mass spectrometer operating in MRM mode to quantify poorly separated peaks. However, this method did not have the required sensitivity for our samples.

To avoid the use of ion-pairing reagents, a separation was established using a ZIC®-pHILIC column (SeQuant, Sweden). The separation of components was performed by a binary gradient elution using acetonitrile (CH_3OH)/water (H_2O). Substitution of acetonitrile by isopropanol (IPA) was possible but increased

column pressure due to its higher viscosity compared to acetonitrile. The compounds were trapped with acetonitrile and eluted with aqueous buffer, like conventional straight phase chromatography (*i.e.* a polar stationary phase and a non-polar, non-aqueous mobile phase). Several chromatographic conditions were optimised to achieve a reasonable analysis time without compromising chromatographic separation. Addition of 10 mM of ammonium bicarbonate was sufficient for separation of the cAMP and ATP. A tenfold higher buffer concentration resulted in an even better resolution but was not used to prevent ion suppression in the mass spectrometer. A lower (1 mM) buffer concentration resulted in poor peak shape. It was found that the flow rate is an important parameter in the resolving power of this type of column. The Van Deemter equation (van Deemter *et al.*, 1956, *i.e.* $[H = A + (B /u) + C * u]$; were A is the Eddy diffusion, B/u is the longitudinal diffusion and C*u is the equilibration point) for this type of column (4.6 x 150 mm Dp 5 µm) shows an optimum around 200 µL/min. Higher flow rates result in a loss of theoretical plates affecting the resolution. The pH was set to 9.4 and slight variations of *ca.* 0.1 pH did not affect chromatographic behaviour of cAMP and ATP. Changes in temperature within the tested range, *i.e.* 20 – 60 °C, did not affect chromatographic behaviour. A temperature of 35 °C was chosen to keep the column at a conditioned temperature.

Most of the biologically active cyclic nucleotides can behave as zwitterions, due to their purine base and phosphate groups, and could potentially be analysed by positive- as well as negative- electrospray ionisation (ESI). However, under the present conditions, negative ESI was the most sensitive ionisation mode. Good chromatographic resolution of both compounds was achieved in separated optimised segments providing the best sensitivity for each of the analytes. As shown in this study, any mass spectrometer with tandem MS capability and sufficient sensitivity may be used for the analysis (*e.g.* triple quadrupole or ion trap machines). Product ions may differ from different types of instruments and have to be determined before the analysis. The instrument must be tuned for best performance (gasses, temperatures and ion optics). The throughput of the assay was improved by the use of 96-well and 384-well plates. Post-column split and the use of the mass spectrometer's divert valve ensured system robustness by preventing source pollution resulting in decreased sensitivity.

Although some absolute cAMP and ATP values are presented, one should take in account the contribution of matrix effects to the MS signal (*e.g.* sodium-adduct formation). This could have been corrected by using an internal standard.

The described validation study showed intra- and inter- assay variations that are in good agreement with The Guidance for Industry, Bioanalytical Method Validation (issued by the FDA; FDA, 2001). Although this guidance is aimed at the Good Laboratory Practice (GLP) of bioanalytical laboratory, as opposed to the pharmacological non-GLP environment the described method was developed in, the principles highlighted in this guidance do represent important considerations in the development or adaptation of analytical methods. Therefore parameters relevant for the current method, *i.e.* linearity, QCs, accuracy and precision of intra- and inter- assay variations, met the FDA guidelines: *i.e.* The mean value was within ±15 % of the theoretical value and precision did not exceed 15 % of the CV (except for lower limit of detection where it was allowed not to exceed 20 %).

Cyclic nucleotides (*e.g.* cAMP and cGMP) are biologically important as second messengers and are drug targets in their own right. Additionally, cAMP accumulation is often used as a marker for determining receptor activity of candidate compounds and is a widely used tool in biochemical pharmacological research. For this reason, the more recently developed techniques for the measurement of cAMP are based on high throughput analysis (see for review Williams, 2004). However, these methods remain limited by the measurement of only one component at a time and ultimately restrict the quality and increases the variation of the data. The described method was developed to measure cAMP and ATP simultaneously for two major reasons; I.) ATP can be used as an indicator for cytotoxicity, as a large decrease in ATP level is inherent to decreased cell viability. This will prevent false-positive results when assessing the effect of compounds on inhibitory coupled receptors, as the total cAMP/ATP ratio will decrease and not only cAMP levels. II.) By calculating a cAMP/ATP ratio, a significant reduction in variation was seen, which significantly improved the statistical power.

To demonstrate the application of the described method for measuring cAMP in a biological system, the method was validated by determination of cAMP accumulation *in vitro*. First, the effect of forskolin, an adenylate cyclase activator, was examined. Forskolin showed a concentration dependent increase in cAMP accumulation up till the highest concentration tested, *i.e.* 100 µM, but without reaching a plateau. For this reason no pEC_{50} value could be calculated as the E_{max} value is not available. Higher concentrations were not tested as these are not physiologically relevant and might induce cytotoxicity. Furthermore, a selection of dopaminergic D_2 receptor ligands were tested on cells transfected with the human dopamine D_{2L} receptor and cAMP and ATP were measured.
The dopamine D_{2L} receptor, like other G-protein coupled receptors (GPCR), is an important target for psychiatric and neurological disorders, and in particular partial dopamine D_2 agonists show great therapeutic benefit (Lieberman, 2004; Bronzova *et al.*, 2010; Jones *et al.*, 2010). Partial agonists are a challenging class of compounds due to their reduced efficacy, and especially partial agonists for the dopamine D_{2L} receptor because of the relatively small dynamic window. In cAMP functional assays, most biochemical assays have limited resolution to distinguish between the degree of intrinsic activity of full and partial agonists (Williams, 2004). It is noteworthy that this method is able to detect partial agonists (*i.e.* compounds with reduced efficacy compared to full agonists), even for the challenging inhibitory Gαi coupled receptors (*e.g.* dopamine D_{2L} receptor).
The pharmacological results shown were in agreement with the literature: the ranking, potency and efficacy of all tested compounds were in good correlation with quantitative studies using the radiometabolic method (tested in our laboratory, Glennon *et al.*, 2006) and other methods (Jordan *et al.*, 2007). Although the results are comparable with metabolic labelling, the method showed an enlarged induction-factor (or 'dynamic window') of experiments with forskolin and dopamine agonists (*e.g.* quinpirole and dopamine), when compared to the radiolabelling method and several ELISA based kits (data not shown). This property, together with the reduced variation by using the cAMP/ATP ratio, renders this method ideal for the determination of partial agonists.

The power of this method is the simultaneous measurement of cAMP and ATP. Ratio calculations significantly reduce variation and simultaneous ATP

measurement provides an index of cellular viability. This feature increases power easing the determination of partial agonists. Furthermore, this method uses a natural cellular pathway without the use of cellular modification (*e.g.* luciferase) to improve coupling efficiency for signal amplification, which could result in false-positive results of data misinterpretation (*i.e.* partial agonists could be detected as full agonists or even as antagonists). The HILIC-MS/MS method analyses the nucleotides without (radioactive-) pre-labelling, which could be questioned as it labels only a certain pool of intracellular nucleotides and metabolites. In addition, this novel method is simple, robust and straightforward and does not require tedious sample preparation and is therefore applicable for routine analysis.

The present method was also successfully applied in structure-activity-relationship (SAR) studies (*e.g.* Brennan *et al.*, 2010) and in other cell systems. For example, we have validated cAMP accumulation in CHO-cells transfected with the serotonergic 5-HT_{1A} and cannabinoid CB_1 and CB_2 receptors (*e.g.* Lange *et al.*, 2010). Furthermore, it was showed that the described method can be successfully extended for supporting *ex vivo* cAMP accumulation studies using brain slices. Described method was validated using forskolin, dopamine D_1 and D_2 agonists and rat striatal slices.
Studies over the past decade have pointed to a prominent role for cAMP as a second messenger in neural pathways underlying the effects of psychostimulants, and previous stimulant administration increases dopamine D_1 receptor mediated signal transduction (Miserendino and Nestler, 1995; Self and Nestler, 1995; Unterwald *et al.*, 1996). The present results showed that the developed method can be used to study the effects of dopaminergic ligands on the cAMP pathway.

Future perspectives
This LC-MS/MS method is able to measure endogenous cAMP and [^{13}C]-labeled adenine metabolites (*i.e.* [^{13}C]-cAMP, [^{13}C]-ATP) simultaneously, and makes it possible to study intracellular-compartmentalisation-theories. Preliminary studies comparing [^{3}H]-adenine metabolic labelling method (by separation of [^{3}H]-cAMP and [^{3}H]-ATP using Dowex-column, Salomon *et al.*, 1974; Salomon, 1979) with the described HILIC-MS/MS method showed that 1 µM forskolin increased cAMP levels by 4 and 16 times, respectively. The difference in amount of cAMP

accumulation could be explained by intracellular-compartmentalisation. However, further studies are required.
When the chromatography was slightly altered by reducing the slope of the gradient from organic to aqueous mobile phase, another peak appeared at the front of the cAMP peak, with the same unique fragmentation pattern (m/z 328 → 134). Preliminary studies (data not shown) revealed that under the LC conditions described in the method section, 2',3'-cAMP co-elutes at the front of the 3',5'-cAMP peak. Thus, the described LC-MS/MS method is able to identify and quantify 2',3'-cAMP in the same run as 3',5'-cAMP and ATP. Moreover, the measurement of hD_{2L}-CHO-cell lysate showed that < 1 % of the total intracellular cAMP content (the sum of the endogenous 3',5'-cAMP and 2',3'-cAMP levels) was 2',3'-cAMP. Because separation of these isomers requires an additional 2.5 min, the 2',3'-cAMP content was neglected during further analysis of cAMP and ATP. The role of intracellular 2',3'-cAMP is not clear yet, though using described method it is possible to study this subject.

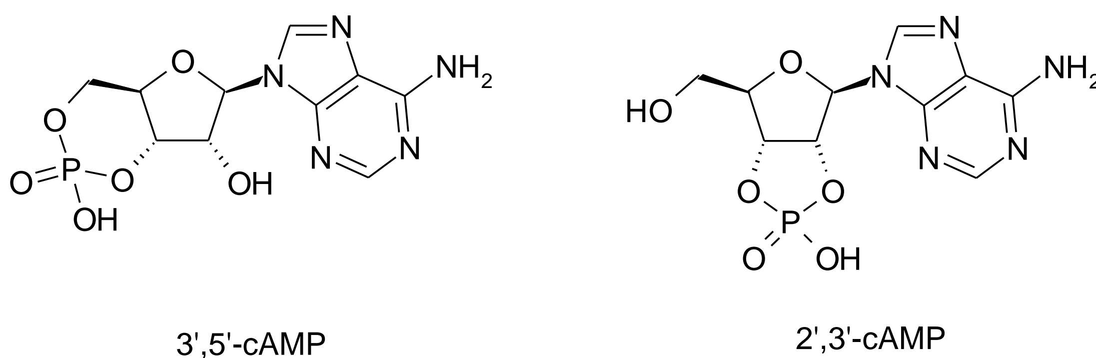


Figure 4-12 Chemical structures of cAMP isomers

Both isoforms of cyclic adenosine monophosphate (cAMP) are endogenously present, although, only 3',5'-cAMP is known to be involved in GPCR signalling. The present study showed that 2',3'-cAMP is also endogenously present but its role is still unknown.

Besides the separation of cAMP (3',5'-cAMP and 2',3'-cAMP) and ATP, the method also addresses the separation of other adenosine and guanosine 5'-mono-, di- and triphosphates *i.e.* cAMP, AMP, cGMP, ADP, ATP, GMP, GDP, in

respective order of elution at a flow rate of 0.4 mL/min. Especially cGMP is of great interest in biomolecular and drug research as cGMP is another second messenger which is exclusively coupled to the D_1-like receptors in the dopamine receptor family. The advantage of yhe described method is the simultaneous analysis of both cAMP and cGMP pathways which could contribute to study the phenomenon of functional selectivity, also called ligand directed signalling (see Gay *et al.*, 2004 for functional selectivity of D_2 receptor ligands and review of other receptors).

If future studies require additional sensitivity, recent advances using nano-electrospray might offer a solution. Applying this technique will not alter the optimised chromatographic method neither requires special mass spectrometric equipment, but using the current method still gain sensitivity. If increased throughput is required, this can be achieved by 'multiplying' or 'parallel LC-MS/MS', an approach where two HPLC systems are coupled to one mass spectrometer (for example see, Korfmacher *et al.*, 1999).

Conclusion

In summary, for the first time, the successful development, validation and application of a novel, robust and sensitive HILIC-MS/MS method for the simultaneous determination of nucleotides in biological samples to study receptor activity (*e.g.* cAMP accumulation) was presented. The simultaneous analysis of cAMP and ATP can be applied to deduce a cAMP/ATP ratio with reduced variation, making this method ideal for the determination of partial agonistic compounds. The method also requires no extensive sample preparation making it suitable for routine analysis and is amendable to 96- and 384- well technology. Additionally, the method can now be used to investigate putative changes in dopaminergic neurotransmission by assessment of cAMP accumulation *ex vivo*, in rat striatal slices of behavioural sensitised animals (see Chapter 5).

Chapter 5. Effect of nicotine induced behavioural sensitisation on dopamine receptor mediated cAMP accumulation in rat striatum

Abstract.

OBJECTIVES: Chronic intermittent administration of psychostimulants (including nicotine) has been shown to induce behavioural sensitisation. Although these drugs have diverse molecular targets in the brain, they share the ability of increasing the release of dopamine. The aim of the present study was to investigate the molecular consequences of nicotine induced behavioural sensitisation in rat dorsal striatum.

METHODS: Behavioural sensitisation in rats was determined by locomotor activity following acute or 5 day chronic intermittent nicotine administration. After the establishment of sensitisation, rats were withdrawn for a 5 day period followed by administration of a nicotine challenge, with or without a pre-challenge with SCH-23390 (a dopamine D_1 antagonist). Either at 45 min or 24 h post-challenge the dorsal striatum was isolated and used to assess the effect of a selection of dopamine D_1 and D_2 receptor ligands on intracellular cAMP accumulation *ex vivo*. The cAMP levels were measured using LC-MS/MS.

RESULTS: The present study showed robust expression of nicotine induced behavioural sensitisation *in vivo* which was significantly blocked by SCH-23390. Nicotine induced sensitisation had no effect on forskolin stimulated cAMP accumulation, however it increased the efficacy of dopamine for the dopamine D_1 receptor, and decreased the potency of D_1 agonists for this receptor. This effect was antagonised by the co-incubation with SCH-23390. Also a time dependent effect was observed between tissues taken at 45 min or 24 h post-challenge.

CONCLUSIONS: These results suggest that the dopamine D_1 receptor signal transduction in the dorsal striatum may play a role in the expression of nicotine induced behavioural sensitisation. Furthermore, a connection between the behavioural effects of sensitisation and the *ex vivo* effects on cAMP accumulation by dopamine D_1 agonists is provided.

Keywords: cAMP, nicotine, sensitisation, *ex vivo*, dopamine receptors

5.1 INTRODUCTION

It is known that a wide variety of drugs including psychostimulants share the ability to induce and express behavioural sensitisation (see Chapter 2). Although these different drugs act on different molecular targets (*e.g.* transporters, receptor systems, ion channels and enzymes) they can directly or indirectly activate common downstream pathways like cyclic adenosine monophosphate (cAMP) and ERK (Nestler, 2001b; Valjent *et al.*, 2006). Previous research has shown that behavioural sensitisation is mediated by long-term neuroadaptations that are secondary to the pharmacological changes elicited by acute drug administration (Wolf, 2003; Niehaus *et al.*, 2010) and studies over the past decade point to a prominent role for dopamine and the second messenger cAMP in the neural pathways underlying the effects of psychostimulants (Wolf *et al.*, 2003). These cyclic nucleotides are powerful signalling molecules, and are hypothesised to play a very important role in behavioural sensitisation. An expanding database indicates that long-term neuroadaptations underlie the symptoms/pathogenesis of neuropsychiatric disorders, more than a consequence of an acute event. Behavioural sensitisation is suggested as a model of synaptic plasticity and several neurochemical substrates have been proposed to account for sensitisation. It is possible that the long-term alterations in dopamine transmission, following sensitisation, may be directly mediated by changes in striatal activity (Wolf *et al.*, 2004; Nestler, 2005).

Previous research has established that dopamine release is involved in different behaviours and it is suggested that dopaminergic neurotransmission plays an important role in behavioural sensitisation (Martin-Iverson *et al.*, 1993; see Chapter 3). In Chapter 2 of this thesis it is shown that the dopamine D_1 receptor plays an important role in the expression of nicotine induced behavioural sensitisation *in vivo*. Both of the dopamine D_1 and D_2 receptor subtypes are localised and co-localised on striatal neurones, and both of these receptor subtypes regulate cAMP accumulation (Stoof and Verheijden, 1986; Kelly and Nahorski, 1987a). Therefore, the enzyme adenylate cyclase is a key component of the dopaminergic signalling pathway and it is hypothesised that increased

adenylate cyclase activity plays a role in behavioural sensitisation. For example, co-administration of cocaine (i.p.) with 7β-deacetyl-7β-[γ-(morpholino)butyryl]-forskolin (7-DMB-forskolin, intracerebroventricular), an adenylate cyclase activator, increased cocaine induced behavioural sensitisation compared to cocaine alone. However, chronic 7-DMB-forskolin (intracerebroventricular) alone for 7 days did not induce behavioural locomotor sensitisation (Schroeder *et al.*, 2004). These data suggests that activation of adenylate cyclase affects the induction of sensitisation but is not able to induce behavioural sensitisation.
Dopamine release can be induced by direct or indirect activation of a variety of receptors located on dopaminergic cell bodies, terminals and neighbouring neurones. The released extracellular dopamine is then able functionally and concomitantly to activate dopamine receptors and intracellular pathways like cAMP. For example, when rat striatal slices were incubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and exposed to different concentrations of the catecholamine-releasing drug amphetamine, this produced both a concentration-dependent release of endogenous dopamine and accumulation of cAMP in the slices (Kelly and Nahorski, 1987b). In addition to cocaine sensitisation it has been shown that acute amphetamine administration did not affect the potency of dopamine induced adenylate cyclase activity, as measured by radiometabolic labelling of striatal membranes which were taken 25 min after the *in vivo* challenge (Barnett et al, 1987). Whereas, chronic amphetamine induced behavioural sensitisation (3 mg/kg s.c., for 6 days followed by 48 h withdrawal) decreased the potency of dopamine stimulated adenylate cyclase activity. Barnett and colleagues used this parameter as an index of dopamine D_1 receptor activation, and suggest its involvement following chronic administration of amphetamine.

As described in Chapter 1, more downstream to adenylate cyclase is the second messenger cAMP. cAMP signalling is at the forefront of intense research interest because its many effects remain controversial and often contradictory. Although much research has been carried out in this area, it is still not clear how nucleotides work and their role in behavioural sensitisation and associated disorders. It has been suggested that adenylate cyclase is implicated in several forms of synaptic plasticity which are also involved in sensitisation and learning

and memory. While acute activation of G(i/o)-coupled receptors, for example dopamine receptors, leads to the inhibition of adenylate cyclase it was shown that chronic activation of these GPCRs produced superactivation or superinhibition, as shown by activation of cannabinoid CB_1 receptors (Rhee *et al.*, 2000) or mu-opioid receptors (Nevo *et al.*, 2000). It is well known that sympathetic nerves, their neurotransmitters and neuropeptides, act upon the target cells via cAMP, which initiates the protein metabolic processes in the cytoplasm, nuclei, chromatin (Svenningsson *et al.*, 2003).

It seems expedient to measure the concentration of intracellular cAMP in the cells of the specific brain area in tissue from behaviourally sensitised animals, as a measure of neuronal changes. The study described in this chapter examined the effect of dopaminergic agonists and antagonists on the accumulation of cAMP in dorsal striatal tissue of the rat *ex vivo*. The cAMP accumulation was assessed in tissue from vehicle, acute nicotine, chronic nicotine and chronic nicotine antagonised with SCH-23390, treated rats. To assess the effect of the post-challenge period after which the tissue was dissected for *ex vivo* studies, tissue from every treatment group was taken at 45 min or at 24 h post-challenge, as indicated in the text.

HYPOTHESES AND AIMS

Hypotheses:

- Dopamine plays an important role in behavioural sensitisation and dopaminergic intracellular pathways are affected by the chronic intermittent dosing regime.

Aims:

- Study putative changes in basal and forskolin-stimulated cAMP levels *ex vivo* following acute- and chronic nicotine administration *in vivo* compared to tissue from vehicle treated groups.
- Investigate the effect of dopamine D_1 and D_2 receptor ligands in tissue of the different treatment groups.
- Investigate the effect of a pre-challenge with SCH-23390 *in vivo*, on *ex vivo* cAMP accumulation in the absence or presence of forskolin or the selected dopamine D_1 and D_2 receptor ligands.

5.2 MATERIALS AND METHODS

The described *in vivo* and *ex vivo* pharmacological experiments, and the LC-MS/MS analyses were performed at Abbott Healthcare Products, the Netherlands.

5.2.1. Chemicals

Adenosine-cyclic-3',5'-monophosphate (cAMP), adenosine-triphosphate (ATP), 25 % aqueous ammonium hydroxide (NH_4OH), ammonium bicarbonate (NH_4HCO_3), D(+)-glucose, forskolin (colforsin), dopamine, (-)-quinpirole, LY-741626, R-(±)-SCH-23390, SKF-82958 and (±)-SKF-38393 were all purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). SB-277011 was synthesised by Syncom (Groningen, the Netherlands). HPLC grade water, LC/MS grade acetonitrile (ACN) and calcium chloride ($CaCl_2$) were obtained from Baker (Deventer, the Netherlands). 3 mM K^+ Krebs-Ringer Buffer (KRB) was purchased from Gibco-Invitrogen (Breda, the Netherlands), and 1-Methyl-3-isobutylxanthine (IBMX) from Calbiochem (Ontario, Canada). Subsequent serial dilutions were performed to obtain the desired test concentration, as stated in the text.

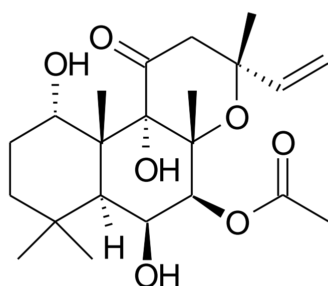


Figure 5-1 Chemical structure of forskolin

Forskolin (colforsin) is an activator of adenylate cyclase, which is biologically active by catalysing the production of the second messenger cAMP. cAMP interacts with proteins such as protein kinase A (PKA) and cyclic nucleotide-gated ion channels (for review see Cooper, 2003).

5.2.2. Animals

For animals used and description of locomotor activity protocol, see Chapter 2.

5.2.3. Experimental design (*in vivo* protocol)

Animals were chronically treated with nicotine and challenged with SCH-23390 as previously described in Chapter 2. The methodology was approved by the animal ethics committee (Abbott Healthcare Products, the Netherlands) and was in accordance with all local laws.

Briefly, one day prior to each experiment (day 0), all animals (n = 8 – 12 rats/group) were habituated to the activity cages for one hr, followed by a saline injection and recording of locomotor activity. On the testing days (days 1 and 5) the rats were habituated for 60 min to the test apparatus, before administration of either saline (s.c., 2 groups) or (-)-nicotine (0.4 mg/kg, s.c., 1 group). After receiving the injections, the rats were directly placed back in the test apparatus and locomotor activity was measured for 45 min, in 5 min time epochs. This first time dose of nicotine was considered the acute dose. On day 2, 3 and 4 all groups received the same treatment as the day before and were directly placed back in their home cage. After a 4 day withdrawal period (day 9), all groups received a saline injection (s.c.) and locomotor activity was measured to assess if the response was context-dependent. On day 10, rats were habituated for 30 min to the test apparatus before administration of a pre-challenge of either saline (s.c., 1 vehicle group and 2 nicotine groups) or SCH-23390 (0.03 mg/kg, i.p., third nicotine group). The rats were directly placed back in the test apparatus and locomotor activity was measured for 30 min. After 30 min of either saline or SCH-23390 administration, the Vehicle - Vehicle group was challenged with saline and all other groups were challenged with 0.4 mg/kg (-)-nicotine (s.c.). Locomotor activity was measured for another 45 min, see Table 2-2 and Figure 5-2 for study design.

A dose of 0.03 mg/kg SCH-23390 (i.p.) was selected as an appropriate dose, as it significantly blocked the effect of chronic nicotine challenge ($P < 0.01$) and at this dose had no significant effect on locomotor activity on its own (see Chapter 2). Locomotor activity was measured 30 min before the pre-challenge with SCH-

23390 followed by another 45 min following the nicotine challenge, as described in Chapter 2.

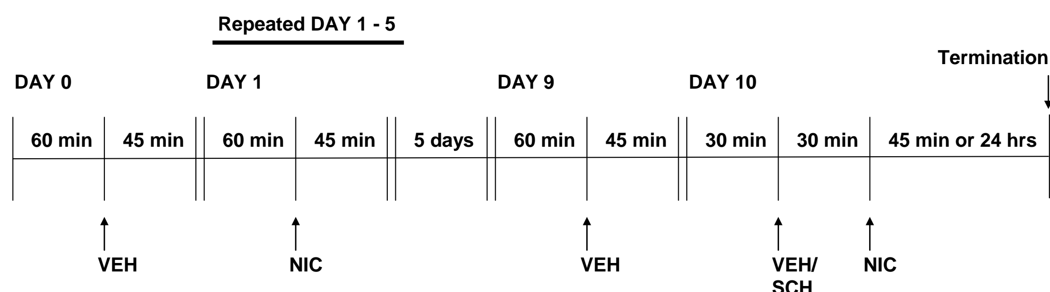


Figure 5-2 Treatment scheme and termination of the *in vivo* experiments
Schematic of the protocol used to determine expression of nicotine induced behavioural sensitisation. The *in vivo* experiments were terminated after either 45 min or 24 h post-challenge and brain tissue was dissected. Locomotor activity was measured as described in Chapter 2. VEH, saline (s.c.); NIC, nicotine (s.c.); SCH, SCH-23390 (i.p.).

5.2.4. Tissue preparation

Brain tissue was prepared as described in the Methods section of Chapter 4, with the addition of an extra post-challenge time point. Briefly, rats were given a challenge injection (saline or nicotine) and locomotor activity was measured for 45 min. This was followed by decapitation at 45 min post-challenge or the following day at 24 h post-challenge. Both striata were dissected as quickly as possible (Figure 5-3) and were kept in ice-cold KRB (for maximal 30 min) until brain-slicing using a McIlwain tissue chopper.

5.2.5. cAMP accumulation assay

See the Methods section of Chapter 4.

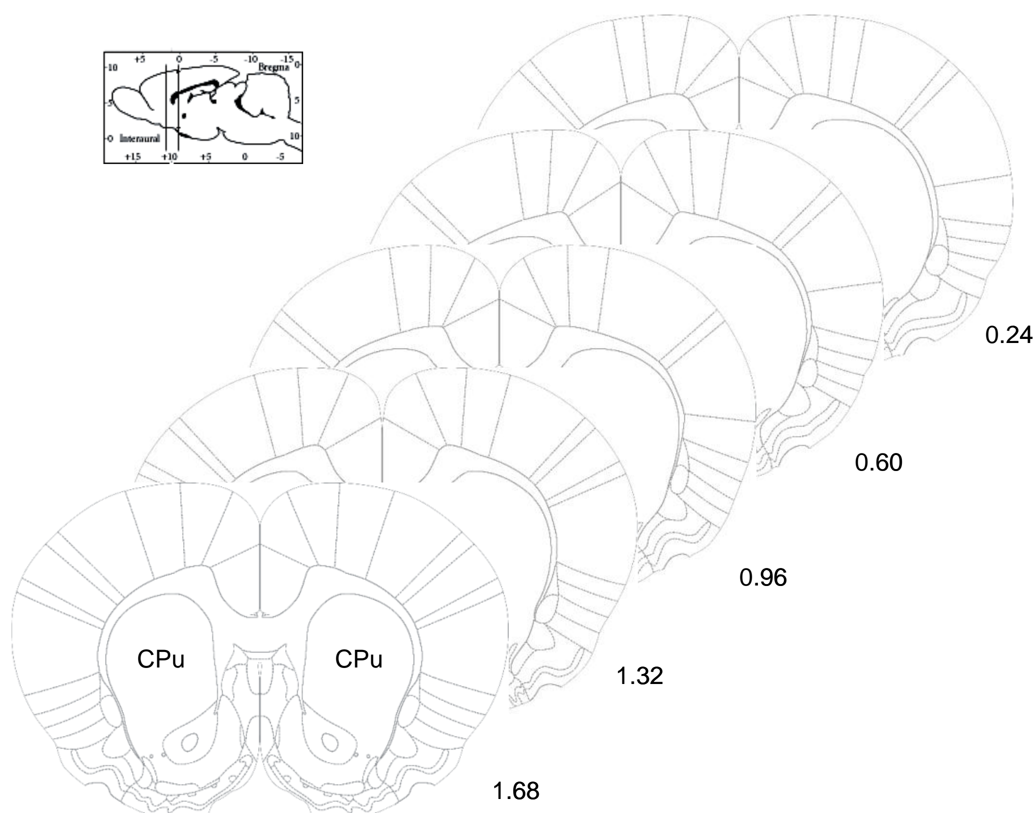


Figure 5-3 Schematic of dissected brain area

The pair of vertical lines in the sagittal schematic (top left) indicate the anterior and posterior limits of the series of coronal schematics, coordinates were +1.7 – +0.2 mm anterior relative to Bregma (Paxinos and Watson, 1998). CPu indicates caudate putamen, the region of interest in the coronal sections, and corresponds to the dorsal striatum. Diagrams were modified from Paxinos and Watson (1998).

5.2.6. Experimental design (*ex vivo* assay)

Striatal tissues (both hemispheres) of 4 animals from the same treatment group were dissected and pooled. All pooled striatal tissues were sliced together resulting in a 'homogenous' suspension which was divided over the 96-well filterplates. Each filterplate contained two controls, *i.e.* basal and 1 μM forskolin, in quadruplo. A single experiment could contain up to twenty 96-well plates and at least 1 plate contained a full concentration-response curve of SKF-38393 as the reference compound (*i.e.* positive control). In order to examine the pharmacological activity of the test compounds, each concentration of the test

compound was tested in quadruplo (4 wells within the same 96-well plate). At the end of each experiment, a mean was calculated from the quadruplo values and outliers (>2x s.d.) were removed. The calculated mean from this individual experiment was considered as n = 1. For example, if stated n = 3 this value is taken from 3 means of individual *ex vivo* experiments after 3 individual *in vivo* experiments.

5.2.7. Sample analyses

Samples were analysed using LC-MS/MS as described in Chapter 4, with the following modifications: The nucleotides were analysed using a different mass-spectrometer, *i.e.* triplequadrupole Premier XE (Waters, Breda, the Netherlands) in the positive electrospray ionisation mode (ESI-MS/MS). A single ion monitoring (SIM) chromatograph was used to only detect the selected mass in the analysis: cAMP m/z 330 → 136, and ATP m/z 508 → 410. Validation of this method was as described in Chapter 4 (data not shown).

5.2.8. Data analyses

For data analyses of the *in vivo* experiments see Methods section Chapter 2. For data analyses of LC-MS/MS measurements see Methods section Chapter 4. Graphs and statistical analyses were performed using PRISM graphing software (GraphPad, USA). For each biological sample, the cAMP concentration was corrected for the ATP concentration by dividing cAMP levels by the total amount of nucleotides: cAMP/(cAMP+ATP)*100.

Data from *ex vivo* experiments represent mean ± s.e.m. of at least three independent experiments (unless stated otherwise), and were conducted in quadruplicate. Values are expressed as a percentage of their respective control. The effect of single concentrations of forskolin tested were analysed using a two-tailed paired t-test. Data with $P < 0.05$ were considered as significant. Concentration response curves were fitted using non-linear regression (*i.e.* Hill three-parameter sigmoidal equation) and mean values were plotted against the logarithm of the drug concentration (in molar). The maximal forskolin induced stimulated conversion was taken as the maximum value and the maximal

inhibition (at 10 μM of the reference compound) as the minimum and these values were fixed during the fitting process. This was followed by a calculation of pEC_{50} ± s.e.m. as presented in graphs and tables. EC_{50} values are defined as the concentration that is able to achieve 50 % of the maximally obtained inhibition by the compound, and pEC_{50} is its respective negative logarithm.

5.3 RESULTS

5.3.1. Tissue preparation following behavioural sensitisation

Initially, the behavioural response of the experimental animals to nicotine administration was examined in detail to determine the optimal parameters for subsequent biochemical studies (see Chapter 2). After the rats were sacrificed by decapitation (without the use of anaesthetics) at time point 45 min or 24 h post-challenge, the dorsal striatum was dissected for *ex vivo* experiments. Figure 5-4A represents a typical locomotor activity measurement, also showing the time of injection (t = 0) and time of decapitation at the end of the experiment (t = 45 min post-challenge). Figure 5-4B shows the mean locomotor activity of a typical sensitisation experiment, illustrating the expression of nicotine induced behavioural sensitisation and its inhibition by a pre-challenge with SCH-23390.

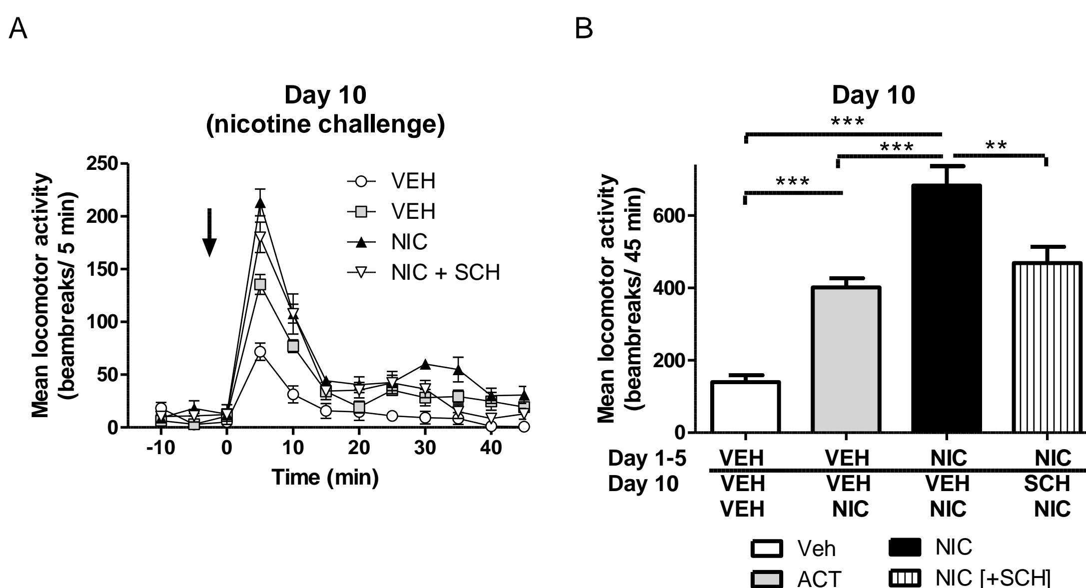


Figure 5-4 Representative *in vivo* experiment for *ex vivo* tissue

Four groups of rats (*n* = 8 animals/group) were treated with either saline (2 groups, vehicle) or nicotine (2 groups) for 5 consecutive days followed by a withdrawal period of 5 days. The animals were pre-challenged with either saline (3 groups) or SCH-23390 (one of the chronic nicotine treated groups), followed by a nicotine challenge. A. Representative experiment showing locomotor activity

in 5 min periods following a saline or nicotine challenge. B. Mean ± s.e.m. beambreaks after the nicotine challenge [F (3, 28) = 30.37; P < 0.0001]. VEH, saline (s.c.); NIC, nicotine (s.c.); SCH, SCH-23390 (i.p.).

The compounds examined in this study were tested at random using tissue from 13 individual *in vivo* experiments (Table 5-1). All *in vivo* experiments showed significant behavioural sensitisation to nicotine.

Table 5-1 Overview of *in vivo* behavioural sensitisation experiments
Expression of locomotor sensitisation following SCH-23390 challenge and/or nicotine challenge. One-way ANOVA followed by *post hoc* Bonferroni's.

Exp (No.)	**ANOVA** (F value)	***post hoc* Bonferroni's** (P value)		
		VEH-NIC	NIC-NIC	NIC- SCH+NIC
1.	[F (3, 44) = 34.15; P < 0.0001]	***	***	**
2.	[F (3, 44) = 23.58; P < 0.0001]	***	***	**
3.	[F (3, 44) = 54.86; P < 0.0001]	***	***	ns$^{\$}$
4.	[F (2, 21) = 56.79; P < 0.0001]	***	***	n/a
5.	[F (2, 20) = 26.28; P < 0.0001]	*	***	n/a
6.	[F (2, 21) = 84.49; P < 0.0001]	***	***	n/a
7.	[F (2, 21) = 56.78; P < 0.0001]	***	***	n/a
8.	[F (3, 27) = 22.01; P < 0.0001]	***	***	ns$^{\$}$
9.	[F (2, 23) = 63.90; P < 0.0001]	***	***	n/a
10.	[F (2, 23) = 33.09; P < 0.0001]	*	***	n/a
11.	[F (2, 23) = 38.85; P < 0.0001]	***	***	n/a
12.	[F (2, 21) = 125.9; P < 0.0001]	***	***	n/a
13.	[F (3, 28) = 30.37; P < 0.0001]	***	***	*

ns, not significant; n/a, not available; $^{\$}$, see discussion for comments. VEH, vehicle; NIC, nicotine; SCH, SCH-23390. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

5.3.2. Effect of forskolin on cAMP accumulation

To examine the effect of *in vivo* nicotine induced behavioural sensitisation on basal and forskolin stimulated cAMP accumulation, the dorsal striatum was dissected following the final nicotine challenge and the slices were incubated with forskolin. As a positive control, basal and 1 µM forskolin stimulated cAMP accumulation were tested in every plate. All cAMP data are presented as a percentage of forskolin, which represents the viability of the physiological system at that time and circumstances. Forskolin did significantly increased cAMP accumulation within all treatment groups (paired t-test, *** $P < 0.001$, Figure 5-5). However, behavioural sensitisation did not affect basal or forskolin stimulated cAMP accumulation, as there was no significant difference observed between the treatment groups, basal [F (3, 19) = 1.408; $P = 0.2712$] and forskolin [F (3, 19) = 0.3484; $P = 0.7907$].

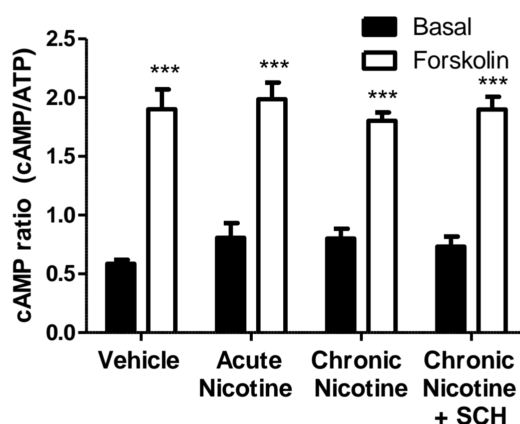


Figure 5-5 Effect of behavioural sensitisation on basal and forskolin (1 µM) stimulated cAMP accumulation in rat striatal slices

Groups (pre-treatment + challenge): Vehicle (saline + saline), acute nicotine (saline + nicotine), chronic nicotine (nicotine + nicotine) or chronic nicotine + SCH (nicotine + SCH-23390/nicotine). Data of $n = 6$ different 96-well plates from a single *ex vivo* experiment representing the effect of basal and forskolin stimulated cAMP accumulation and intra-assay variation. Paired t-test, *** $P < 0.001$.

Forskolin was also tested in a concentration range from 1 nM to 0.1 mM to determine its potency. Forskolin increased cAMP formation in a concentration dependent manner (Figure 5-6A), which is in agreement with results shown above using a single 1 µM concentration. Theoretically no pEC_{50} value can be calculated as the highest concentration forskolin tested (*i.e.* 0.1 mM) did not produce a maximal effect or plateau. Estimated pEC_{50} values, calculated using PRISM software when E_{max} values were given, are showed in Table 5-2. Nonetheless, the results show that there is no change in potency following chronic nicotine treatment compared to vehicle (Figure 5-6A), and that *in vivo* pre-challenge with SCH-23390 before the nicotine challenge also did not affect the potency of forskolin stimulated cAMP accumulation (Figure 5-6B).

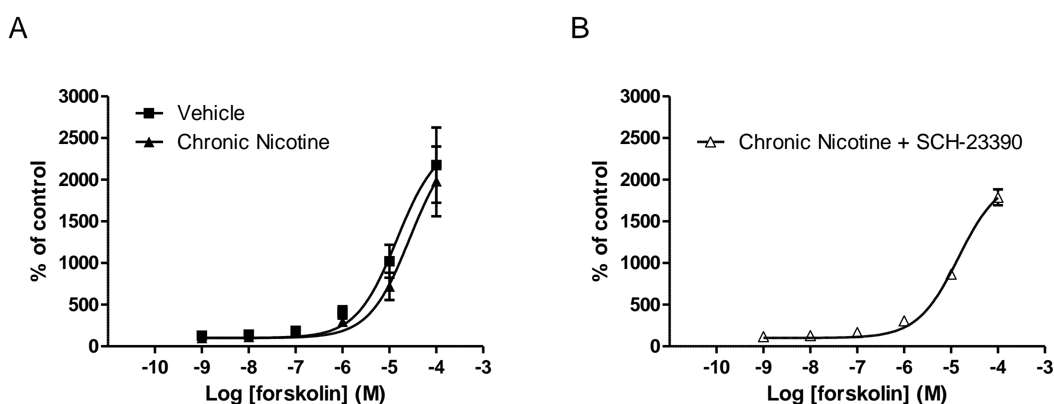


Figure 5-6 Effect of forskolin on cAMP accumulation in rat striatal slices

Effect of forskolin on cAMP accumulation in striatal slices from vehicle and chronic nicotine treated rats (A) and chronic nicotine animals pre-treated with SCH-23390 (B). Tissue was dissected 45 min after the nicotine challenge. $n = 4$ individual experiments, from 4 individual *in vivo* experiments.

Effect of after 24 h post-challenge

The results above were obtained using tissue prepared 45 min post-challenge. To investigate the putative time-response effect of the post-challenge period before dissection of the brain tissue, the striata of animals from the same treatment group were dissected 24 h after the challenge injection. Forskolin stimulated cAMP accumulation in a concentration dependent manner, comparable to the results seen with tissue taken 45 min post-challenge (Figure 5-7). No difference in time-response effect was observed for either treatment group (Table 5-2).

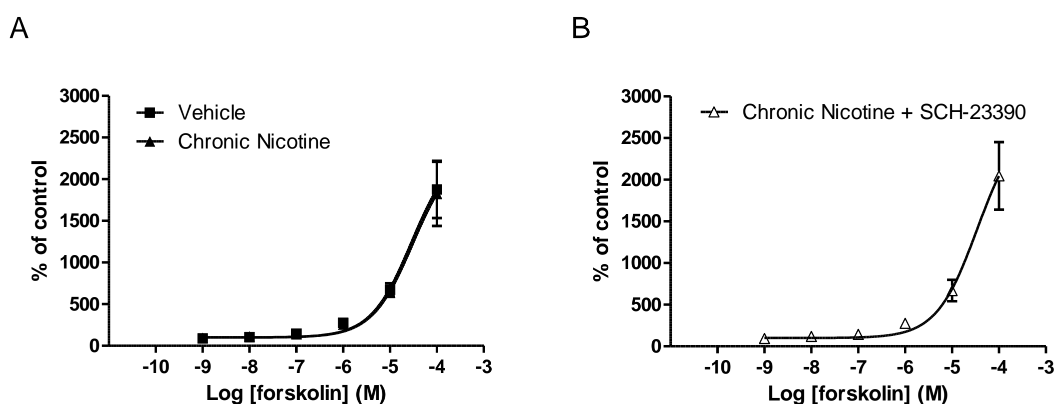


Figure 5-7 Effect of forskolin on cAMP accumulation in rat striatal slices prepared 24 h post-challenge

The effect of forskolin on cAMP accumulation in striatal slices from vehicle and chronic nicotine treated rats (A) and chronic nicotine animals pre-challenged with SCH-23390 (B). Tissue was dissected 24 h after the final nicotine challenge. n = 4-5 individual experiments, from 4 individual *in vivo* experiments which all showed significant behavioural sensitisation.

Table 5-2 Effect of forskolin on cAMP accumulation in rat striatal slices
Values are pEC_{50} mean ± s.e.m. n-number is given between parentheses and E_{max} values (%) between brackets.

	VEH-VEH	VEH-NIC	NIC-NIC	NIC-SCH+NIC
45 min post-challenge				
Forskolin*	4.8 ± 0.2 (4) [2462]	4.5 ± 0.2 (4) [2170]	4.6 ± 0.2 (4) [2488]	4.8 ± 0.1 (4) [2024]
24 h post-challenge				
Forskolin*	4.5 ± 0.2 (5) [2385]	4.5 ± 0.3 (4) [2298]	4.5 ± 0.2 (4) [2352]	4.5 ± 0.2 (3) [2715]

VEH, vehicle; NIC, nicotine; SCH, SCH-23390. * Note: The highest concentration tested (100 µM forskolin) did not produce a maximal effect or plateau; therefore it is theoretically not possible to calculate a pEC_{50} value. However, when the fitting software (PRISM) estimates the maximal value at 0.1 mM, the pEC_{50} values are calculated.

5.3.3. Effect of dopamine on cAMP accumulation

It is hypothesised that dopamine, the endogenous ligand for the dopamine receptor, would have equal affinity for both dopamine D_1-like and dopamine D_2-like receptor subtypes and therefore would have no effect on cAMP accumulation. When the effect of dopamine was tested at a concentration up to 0.1 mM, a small but concentration dependent increase in cAMP production in dorsal striatal slices was observed. Dopamine produced a maximum stimulation of cAMP production of 167 ± 28 % over basal levels in the dorsal striatum of saline administrated animals (Figure 5-8A). Specific antagonism of the dopamine D_2 receptor subtype, by co-incubation of dopamine with LY-741626, increased the potency of dopamine (pEC_{50} dopamine 4.4 ± 0.2 vs. dopamine + LY-741626 co-incubated 5.6 ± 0.4, Figure 5-8C and Table 5-3). In contrast to the results observed with a dopamine D_2 antagonist, co-incubation of dopamine with the dopamine D_1 antagonist SCH-23390 inhibited the effect of dopamine and as a result prevented stimulation of cAMP accumulation ($pEC_{50} < 5.0$, Figure 5-8E). This is in agreement with the *in vivo* behavioural sensitisation observations which are also blocked by SCH-23390.

In addition to the examination of dopaminergic ligands on cAMP accumulation in tissue from naïve animals, the effect of these ligands was investigated in tissue of animals sensitised to nicotine by chronic administration. It was found that behavioural sensitisation did not affect dopamine stimulated cAMP accumulation (Figure 5-8A). However, a large increase in the E_{max} value of dopamine was found in tissue from sensitised animals, when the dopamine D_2 component was blocked by co-incubation with LY-741626 (E_{max} vehicle 162 % vs. chronic nicotine 213 %, Figure 5-8E). Co-incubation of dopamine with SCH-23390 did not increase cAMP accumulation in tissue from sensitised rats ($pEC_{50} < 5.0$, Figure 5-8E).

The effect of dopamine on cAMP accumulation in tissue from sensitised animals which were additionally pre-challenged with SCH-23390 prior to the nicotine challenge was investigated. The E_{max} value of dopamine in tissue from these animals was strongly reduced (Figure 5-8B). The increase in E_{max} value of

dopamine in the presence of LY-741626 following behavioural sensitisation was also reduced by an *in vivo* pre-challenge with SCH-23390 (Figure 5-8D). As expected from the results observed above, no increase was seen by dopamine in the presence of SCH-23390 ($pEC_{50} < 5.0$, Figure 5-8F).

Co-incubation of dopamine with the selective dopamine D_3 antagonist SB-277011 (1 μM) completely blocked the effect of dopamine on cAMP accumulation ($pEC_{50} < 4.0$). This antagonism by SB-277011 was not affected by *in vivo* challenge of nicotine or SCH-23390 (Table 5-3).

This study also investigated the time-response effect when tissue was taken 24 h post-challenge. When striata from animals of the same treatment-group were dissected 24 h after the last nicotine challenge, no effect of dopamine on cAMP accumulation was observed ($pEC_{50} < 5.0$, $n = 3$ for vehicle and chronic nicotine treated groups, see Table 5-3).

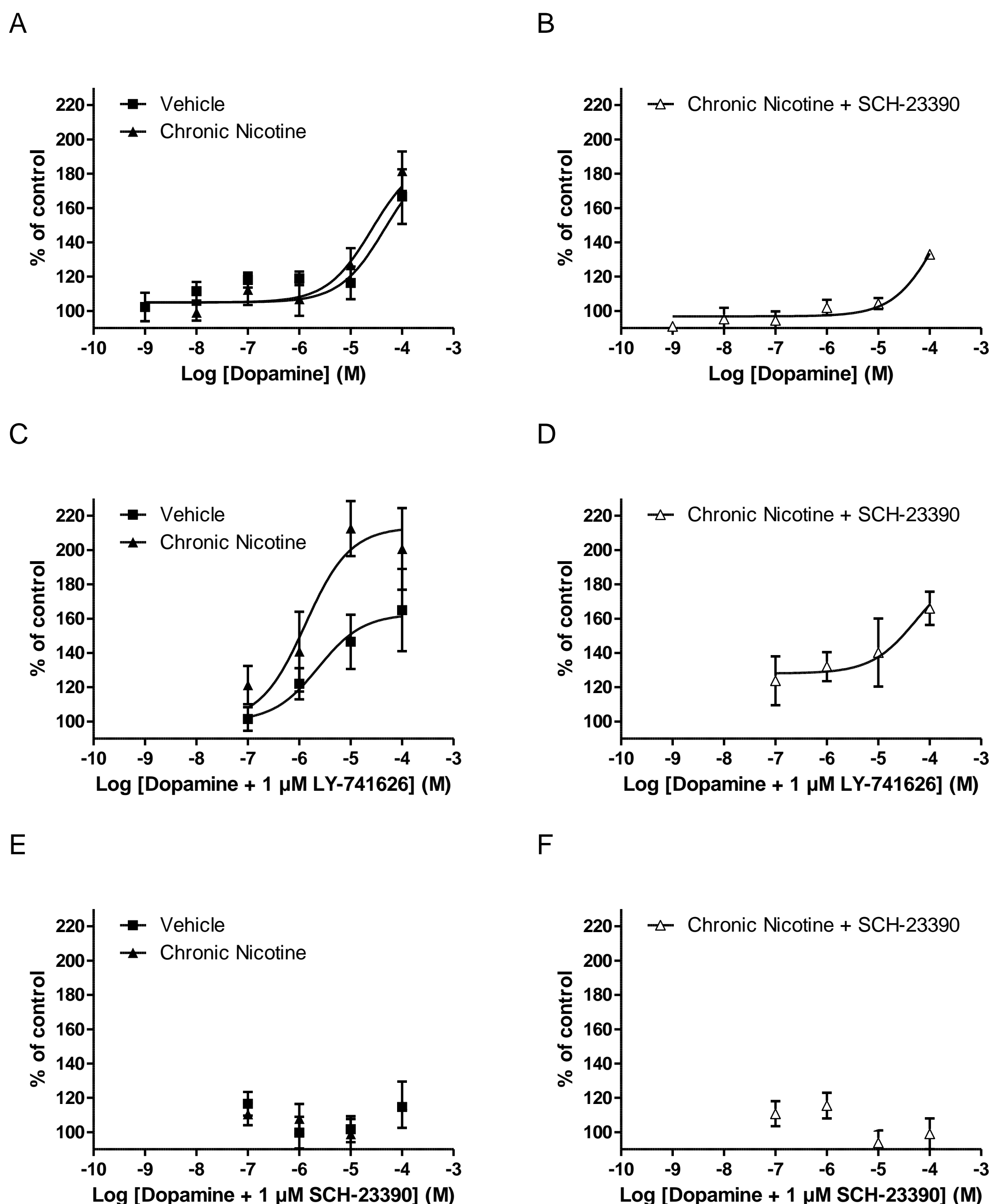


Figure 5-8 Effect of dopamine on cAMP accumulation in rat striatal slices

The effect of dopamine on cAMP accumulation in striatal slices from vehicle and chronic nicotine treated rats (A) and chronic nicotine treated animals pre-challenged with SCH-23390 (B). The effect of dopamine in the presence of 1 μM LY-741626 (C, D). The effect of dopamine in the presence of 1 μM SCH-23390 (E, F). Tissue was taken 45 min post-challenge. *n* = 4-6 individual *ex vivo* experiments from individual *in vivo* experiments.

Table 5-3 Effect of dopamine on cAMP accumulation in rat striatal slices
Values are pEC_{50} mean ± S.D. n-number is given between parentheses and E_{max} values (%) between brackets.

	VEH-VEH	VEH-NIC	NIC-NIC	NIC-SCH+NIC
45 min post-challenge				
Dopamine	4.4 ± 0.2 (6)	3.8 ± 0.1 (4)	4.7 ± 0.2 (6)	3.8 ± 0.1 (4)
Dopamine + LY-741626 (1μM)	5.6 ± 0.4 (4) [162]	5.9 ± 0.3 (4) [172]	5.8 ± 0.3 (4) [213]	4.3 ± 0.4 (4) [166]
Dopamine + SCH-23390 (1μM)	<4.0 (4)	<4.0 (3)	<4.0 (4)	<4.0 (4)
Dopamine + SB-277011 (1μM)	<4.0 (3)	<4.0 (3)	<4.0 (3)	<4.0 (3)
24 h post-challenge				
Dopamine	<5.0 (3)	n/a	<5.0 (3)	n/a
Dopamine + LY-741626 (1μM)	n/a	n/a	n/a	n/a
Dopamine + SCH-23390 (1μM)	n/a	n/a	n/a	n/a
Dopamine + SB-277011 (1μM)	n/a	n/a	n/a	n/a

n/a, not available; VEH, vehicle; NIC, nicotine; SCH, SCH-23390.

5.3.4. Effect of dopamine D_1 ligands on cAMP accumulation

The dopamine D_1 agonists SKF-38393 and SKF-82958 showed a concentration dependent increase in cAMP accumulation using striatal slices from saline treated animals (Figure 5-9A and C, respectively). When SKF-82958 was tested at higher concentrations (*i.e.* 10 µM and 100 µM), the simultaneously measured ATP levels were strongly decreased indicating toxicity.

The effect of the dopamine D_1 agonists was tested in tissue from sensitised animals (tissue taken at 45 min post-challenge). The potency of both dopamine D_1 agonists was reduced after acute and chronic nicotine administration, *i.e.* SKF-38393 also showed a shift in potency of cAMP accumulation in vehicle (pEC_{50} 7.2 ± 0.2) versus acute (pEC_{50} 6.7 ± 0.2) and chronic (pEC_{50} 6.0 ± 0.2) nicotine treated animals. SKF-82958 showed also a shift in potency; Vehicle (pEC_{50} 7.7 ± 0.2), acute (pEC_{50} 7.3 ± 0.4) and chronic nicotine (pEC_{50} 6.2 ± 0.2).

Moreover, the effect of *in vivo* pre-challenge with SCH-23390 on SKF-38393 and SKF-82958 stimulated cAMP accumulation was examined. Interestingly the *in vivo* SCH-23390 pre-challenge increased the potency of SKF-38393, back to the level of vehicle and thus abolished the effect of the sensitisation on cAMP accumulation (Figure 5-9B). The same effect, although to a lesser extent, was observed with SKF-82958 (Figure 5-9D). Furthermore, the maximal effect (E_{max}) of SKF-82958 was decreased.

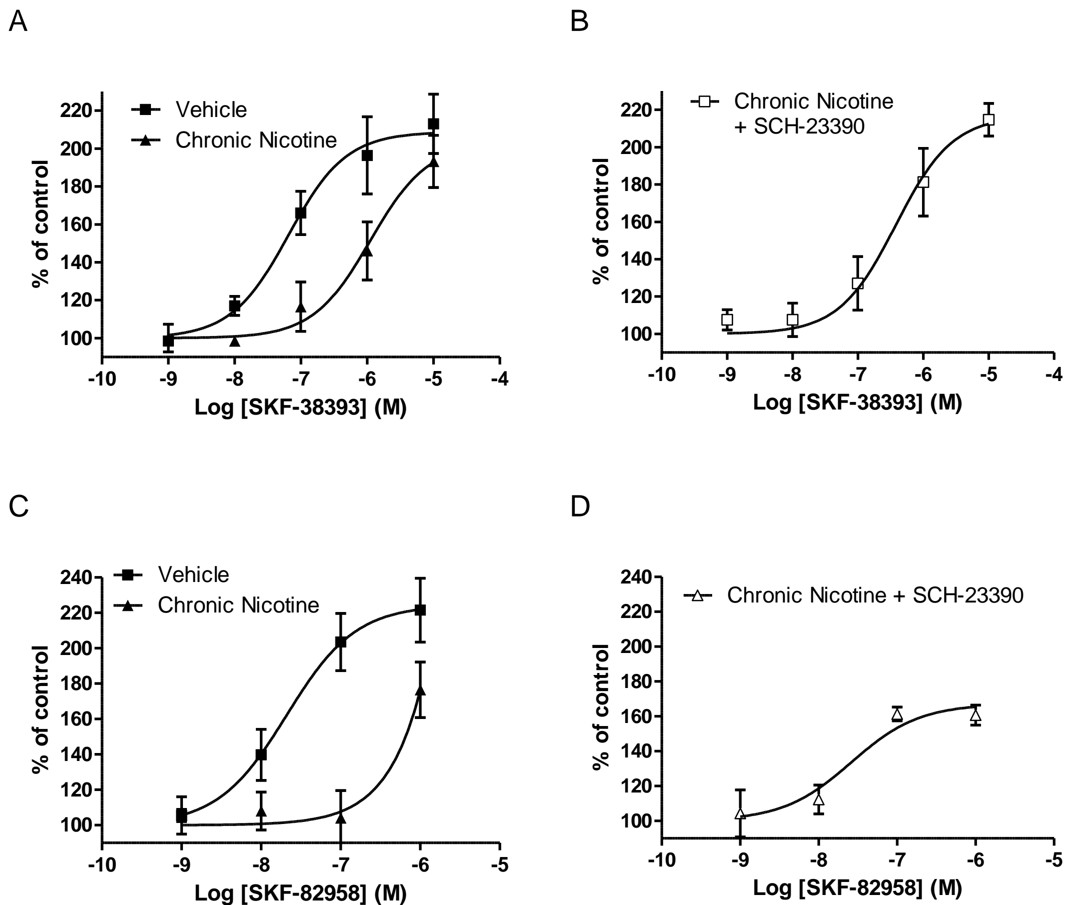


Figure 5-9 Effect of dopamine D_1 receptor agonists on cAMP accumulation

A. Effect of chronic saline and nicotine (A) and chronic nicotine with SCH-23390 pre-treatment (B) on SKF-38393 (A and B) or SKF-82958 (C and D) stimulated cAMP accumulation. Tissue taken 45 min post-challenge. See Table 5-4 for pEC_{50} and E_{max} values, n-numbers and data for acute nicotine treatment.

This study also assessed the effect of the dopamine D_1 antagonist SCH-23390 on cAMP accumulation. Previous research showed that dopamine D_1 antagonists have no intrinsic activity on the dopamine D_1 receptor and as a result have no effect on cAMP accumulation. This was confirmed by this study where the dopamine D_1 antagonist SCH-23390 had no effect on cAMP accumulation in striatal slices (Figure 5-10A). When the dopamine D_1 agonist SKF-38393 (1 µM) was tested in combination with SCH-23390 its effect on cAMP accumulation was

abolished, *i.e.* antagonised by SCH-23390 (Figure 5-10C). The same effects were found for SCH-23390 alone or in presence of SKF-38393 in tissue following chronic nicotine administration (Figure 5-10B and D). It was found that *in vivo* behavioural sensitisation, with or without a SCH-23390 pre-challenge, did not affect the lack of activity of SCH-23390 (Figure 5-10A and B).

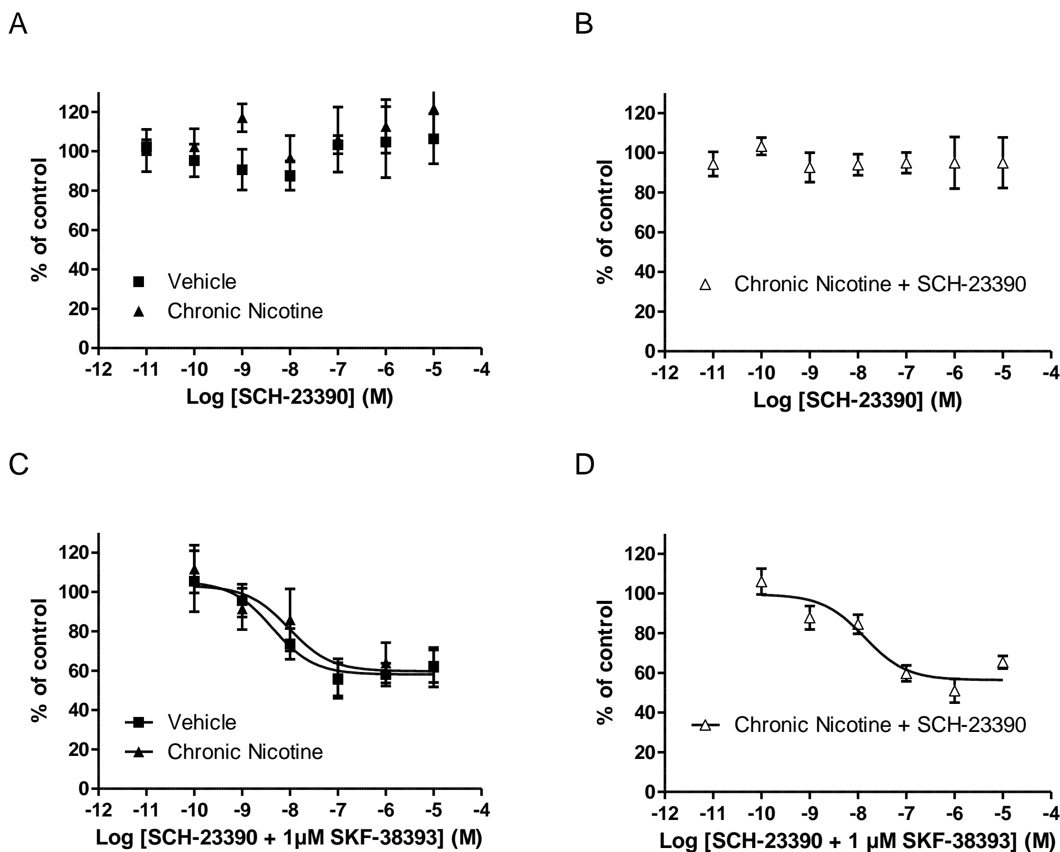


Figure 5-10 Effect of dopamine D_1 receptor antagonist SCH-23390 on cAMP accumulation

The effect of SCH-23390 alone (A) or in the presence of SKF-38393 (C) on cAMP accumulation in striatal slices from vehicle and chronic nicotine treated rats. The effect of SCH-23390 alone (B) or in the presence of SKF-38393 (D) following chronic nicotine with SCH-23390 pre-treatment. Tissue taken 45 min post-challenge. See Table 5-4 for pEC_{50} and E_{max} values, n-numbers and data for acute nicotine treatment.

This study also investigated the effect of dopamine D_1 ligands on cAMP accumulation following a longer post-challenge period, using the striata of vehicle treated rats which were dissected 24 h after the last challenge. A concentration dependent effect of dopamine D_1 agonist SKF-38393 on cAMP accumulation was observed (Figure 5-11A) with a pEC_{50} of 6.9 ± 0.1. This result is comparable to the results obtained using tissue from non-treated animals (see Chapter 4) and tissue taken 45 min post-challenge (7.2 ± 0.2, Table 5-4). In contrast to results seen using tissue taken at 45 min post-challenge, the *in vivo* nicotine induced sensitisation did not alter the potency of SKF-38393 on cAMP accumulation when tissue was taken 24 h post-challenge (Figure 5-11A). Also no difference in potency was observed following a pre-challenge with SCH-23390 (Figure 5-11B).

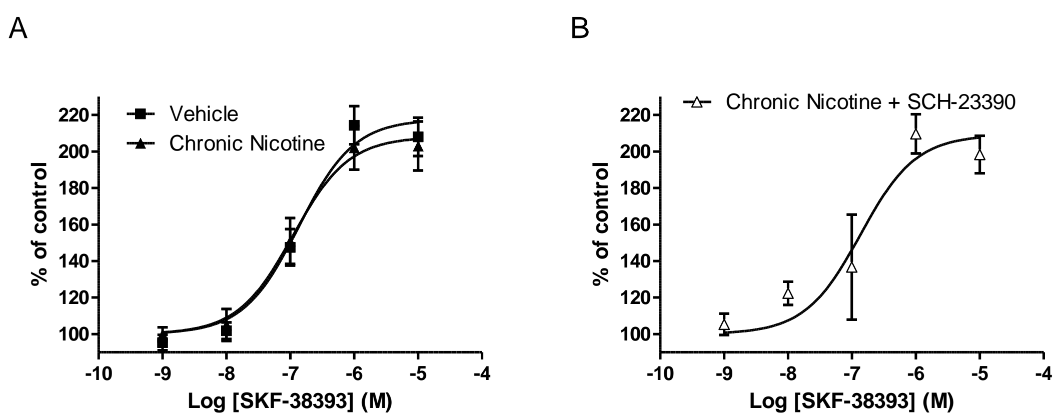


Figure 5-11 Effect of dopamine D_1 agonist SKF-38393 on cAMP accumulation, 24 h post-challenge

A. Effect of SKF-38393 on tissue from vehicle and chronic nicotine treated animals taken 24 h after the final nicotine challenge. B. Effect of SKF-38393 following chronic nicotine and pre-challenged with SCH-23390. See Table 5-4 for pEC_{50} and E_{max} values, n-numbers and data for acute nicotine treatment.

The other dopamine D_1 receptor agonist SKF-82958 showed the same effect when using tissue taken 24 h post-challenge, compared to tissue which was taken 45 min post-challenge. Also, behavioural sensitisation did not affect the potency of SKF-82958 stimulated cAMP accumulation (Figure 5-12A) in contrast to results seen with tissue taken 45 min post-challenge (see Table 5-4). Moreover,

the effect of the partial dopamine D_1 agonist SKF-77434 was examined. Unlike the dopamine D_1 agonists SKF-38393 and SKF-82958, SKF-77434 did not increase cAMP accumulation and no difference was seen between vehicle and chronic nicotine treated rats (pEC_{50} <5.0, Figure 5-12B).

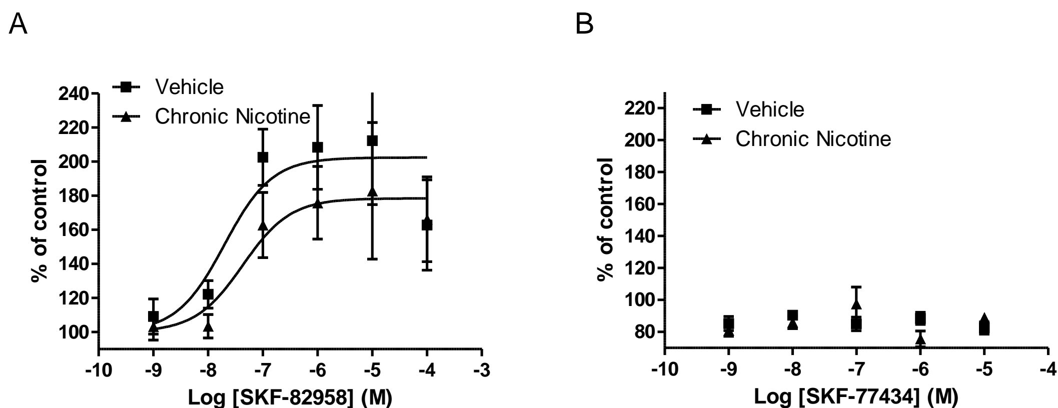


Figure 5-12 Effect of dopamine D_1 receptor agonists on cAMP accumulation, 24 h post-challenge

Effect of SKF-82958 (A) and SKF-77434 (B) on tissue from saline or chronic nicotine treated animals, taken 24 h after challenge. SKF-82958, $n = 7$ and SKF-77434, $n = 4$ from individual *ex vivo* experiments from individual *in vivo* experiments. See Table 5-4 for pEC_{50} and E_{max} values, n-numbers and data for acute nicotine treatment.

The dopamine D_1 receptor antagonist SCH-23390 was tested using tissue taken 24 h post-challenge and did not show any effect on cAMP accumulation (*i.e.* pEC_{50} < 5.0 for both vehicle and nicotine sensitised groups) which is in agreement with previous observations using tissue taken 45 min post-challenge or non-treated animals (see Table 5-4). The effect of SCH-23390 in the presence of SKF-38393 was examined and showed a concentration dependent inhibition with potency comparable to tissue taken 45 min post-challenge. *In vivo* chronic nicotine and chronic nicotine with SCH-23390 pre-challenge did not affect this concentration dependent effect (Figure 5-13, Table 5-4).

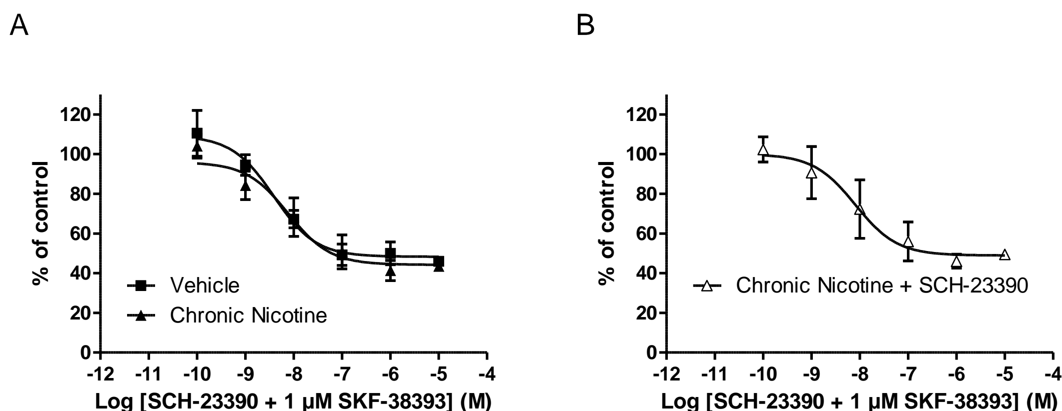


Figure 5-13 Effect of SCH-23390 in presence of SKF-38393 on cAMP accumulation, 24 h post-challenge

A. Effect of SCH-23390 in the presence of SKF-38393 (1 μM) using tissue from vehicle and chronic nicotine treated animals taken 24 h after nicotine challenge. B. Effect of SCH-23390 in the presence of SKF-38393 (1 μM) following chronic nicotine pre-challenged with SCH-23390. See Table 5-4 for pEC_{50} and E_{max} values, n-numbers and data for acute nicotine treatment.

Table 5-4 Effect of D_1 ligands on cAMP accumulation in rat striatal slices

Values are pEC_{50} mean ± S.D. n-number is given between parentheses and E_{max} values (%) between brackets.

	VEH-VEH	VEH-NIC	NIC-NIC	NIC-SCH+Nic
45 min post-challenge				
SKF-82958	7.7 ± 0.2 (4)	7.3 ± 0.4 (3)	6.2 ± 0.2 (4)	7.6 ± 0.3 (3)
SKF-38393	7.2 ± 0.2 (5)	6.7 ± 0.2 (3)	6.0 ± 0.2 (5)	6.4 ± 0.2 (4)
SKF-77434	n/a	n/a	n/a	n/a
SCH-23390	<5.0 (3)	<5.0 (3)	<5.0 (3)	<5.0 (3)
SCH-23390 + SKF-38393 (1µM)	8.2 ± 0.3 (5)	8.4 ± 0.2 (5)	7.9 ± 0.5 (5)	7.9 ± 0.2 (4)
24 h post-challenge				
SKF-82958	7.7 ± 0.4 (7) [202]	7.2 ± 0.5 (2) [165]	7.4 ± 0.5 (7) [179]	n/a
SKF-38393	6.9 ± 0.1 (8) [218]	7.0 ± 0.3 (4) [197]	7.0 ± 0.2 (6) [208]	6.9 ± 0.3 (3) [209]
SKF-77434	<5.0 (4)	n/a	<5.0 (4)	n/a
SCH-23390	<5.0 (3)	n/a	<5.0 (3)	n/a
SCH-23390 + SKF-38393 (1µM)	8.4 ± 0.2 (4)	8.3 ± 0.1 (3)	8.1 ± 0.3 (4)	8.1 ± 0.3 (3)

n/a, not available; VEH, vehicle; NIC, nicotine; SCH, SCH-23390.

5.3.5. Effect of nicotine on cAMP accumulation

The effect of nicotine on cAMP accumulation in the striatum of non-treated animals was investigated. Nicotine was tested in a dose range from 0.01 to 10 μM without any effect on cAMP accumulation and the pEC_{50} values for nicotine in vehicle and chronic nicotine tissue were both < 5.0 (n = 3). Furthermore, the effect of nicotine on SKF-38393 stimulated cAMP accumulation was investigated using tissue from non-treated animals. The presence of 10 μM nicotine did not alter the potency or efficacy of SKF-38393 in rat striatal slices (Figure 5-14).

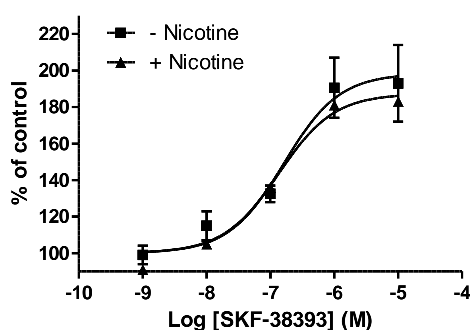


Figure 5-14 Effect of nicotine on the effect of SKF-38393 stimulated cAMP accumulation

The effect of SKF-38393 on cAMP accumulation was tested in the absence (■) or presence (▲) of nicotine (10 μM). Tissue taken from non-treated animals, $n = 2$.

5.3.6. Effect of dopamine D_2 ligands on cAMP accumulation

The dopamine D_2-like full agonist quinpirole showed an inhibition of cAMP accumulation with a pEC_{50} of 9.2 ± 0.5. The potency of quinpirole was decreased following sensitisation using tissue taken 45 min post-challenge (pEC_{50} 8.4 ± 0.5, Figure 5-15A). An *in vivo* pre-challenge with SCH-23390 did not affect the results of the nicotine challenge on cAMP accumulation and quinpirole showed a pEC_{50} of 8.2 ± 0.6 (Figure 5-15B). The selective dopamine D_2 antagonist LY-741626 did not affect cAMP accumulation when tested alone (pEC_{50} < 5.0) and was not affected by either behavioural sensitisation with or without a SCH-23390 pre-challenge (Figure 5-15C and D).

The selective dopamine D_3 antagonist SB-277011 was tested and did not affect cAMP accumulation when tested alone (pEC_{50} < 5.0). Furthermore, its effect was not affected by either behavioural sensitisation with or without a SCH-23390 pre-challenge (Table 5-5).

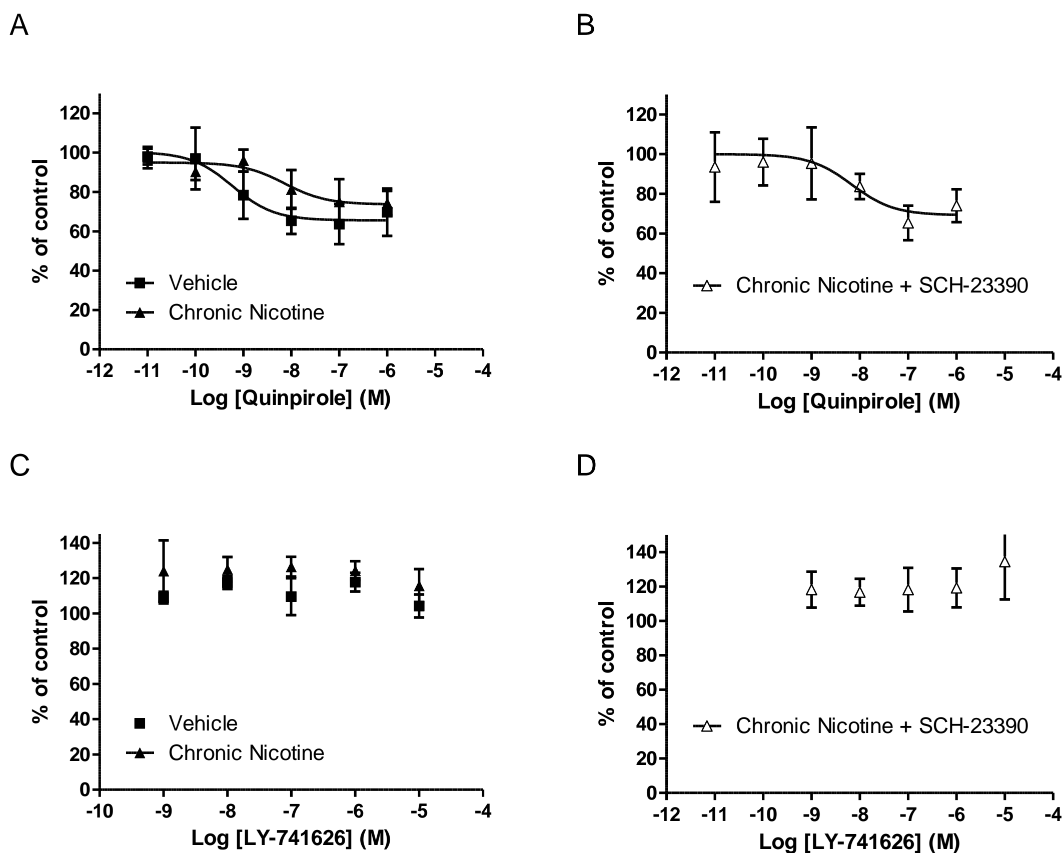


Figure 5-15 Effect of dopamine D_2 receptor ligands on cAMP accumulation

Effect of the dopamine D_2 agonist quinpirole on striatal slices from vehicle and chronic nicotine treated rats (A) and chronic nicotine treated animals pre-challenged with SCH-23390 (B). Effect of the dopamine D_2 antagonist LY-741626 in slices from vehicle and chronic nicotine treated rats (C) and chronic nicotine treated animals pre-challenged with SCH-23390 (D). Tissue was taken 45 min post-challenge. For pEC_{50} values and n-numbers see Table 5-5.

To investigate dopamine D_1/D_2 synergism, the dopamine D_2-like full agonist quinpirole was tested in the presence of the dopamine D_1 receptor agonist SKF-82958. When quinpirole was co-incubated with SKF-82958 (1 μM) in tissue from non-treated animals, the pEC_{50} of quinpirole decreased compared to when quinpirole was tested alone (*i.e.* pEC_{50} 8.1 ± 0.2 and 9.2 ± 0.5, respectively). When quinpirole was co-incubated with SKF-82958 and tissue taken 45 min post-challenge from either vehicle, acute or chronic nicotine treated rats, no difference in potency was seen (Figure 5-16). This is in contrast when quinpirole was tested alone, and suggests that dopamine D_1 receptor activation does have an effect on dopamine D_2 receptor mediated signalling. No significant effect of quinpirole was seen in tissue (taken 24 h post-challenge) from either vehicle or chronic nicotine treated rats (Table 5-5). This might be explained by the high concentration of SKF-82958 used (*i.e.* 10 μM) and therefore the stimulation of cAMP accumulation is too strong to be reversed by 10 μM quinpirole.

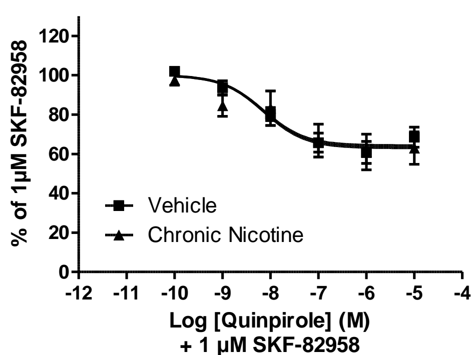


Figure 5-16 Effect of dopamine D_2 receptor agonist in presence of a D_1 agonist on cAMP accumulation

The effect of quinpirole in the presence of SKF-82958 (1 μM) on cAMP accumulation in tissue from vehicle or chronic nicotine treated, behaviourally sensitised animals. Tissue was taken 45 min post-challenge, n = 4. For pEC_{50} values see Table 5-5.

The effect of quinpirole on tissue taken 24 h post-challenge was also investigated. Quinpirole inhibited cAMP accumulation in saline treated animals (VEH) and

resulted in a lower pEC_{50} value compared to tissue taken 45 min post-challenge (*i.e.* pEC_{50} 8.4 ± 0.3 vs. 9.2 ± 0.5). Tissue from sensitised animals resulted in a pEC_{50} of 7.9 ± 0.5 and therefore there was no effect of behavioural sensitisation observed on the effect of quinpirole when tissue was taken 24 h post-challenge. When quinpirole was tested in combination with SKF-38393, it showed a concentration-dependent decrease of cAMP levels in tissue from vehicle (pEC_{50} 8.1 ± 0.2) as well as chronic nicotine treated rats (pEC_{50} 7.8 ± 0.3) when tissue taken 24 h post-challenge. No experiments of quinpirole in combination with SKF-38393 were performed at 45 min post-challenge.

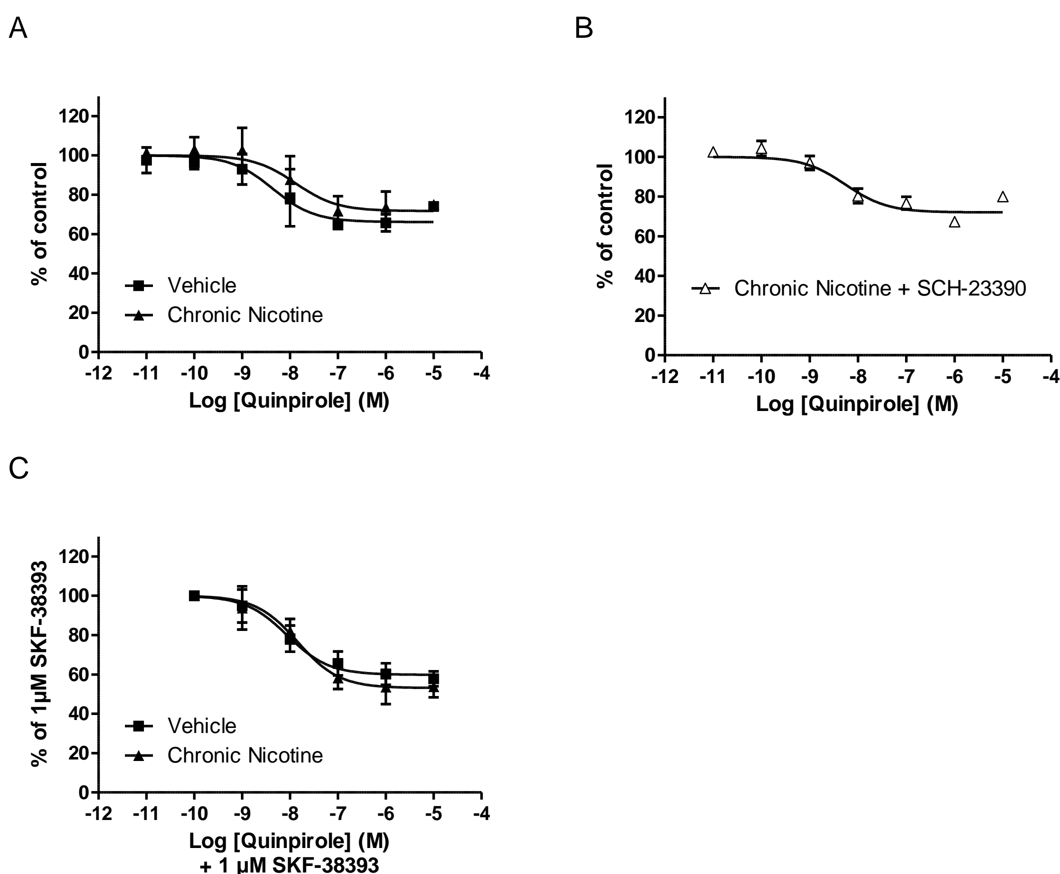


Figure 5-17 Effect of quinpirole alone and in presence of SKF-38393 on cAMP accumulation

The effect of quinpirole on tissue from vehicle or chronic nicotine treated animals (A) or chronic nicotine pre-challenged with SCH-23390 (B). The effect of quinpirole in the presence of 1 µM SKF-38393 (C). Tissue taken 24 h post-challenge.

Table 5-5 Effect of D_2 ligands on cAMP accumulation in rat striatal slices
Values are pEC_{50} mean ± S.D. n-number is given between parentheses.

	VEH-VEH	VEH-NIC	NIC-NIC	NIC-SCH+NIC
45 min post-challenge				
Quinpirole	9.2 ± 0.5 (3)	8.2 ± 0.3 (3)	8.4 ± 0.5 (3)	8.2 ± 0.6 (3)
Quinpirole + SKF-38393 (1μM)	n/a	n/a	n/a	(1)
Quinpirole + SKF-82958 (1μM)	8.1 ± 0.2 (4)	7.9 ± 0.4 (2)	8.1 ± 0.4 (4)	(1)
Quinpirole + SKF82958 (10μM)	n/a	n/a	n/a	n/a
LY-741626	<5.0 (4)	<5.0 (4)	<5.0 (4)	<5.0 (4)
SB-277011	<5.0 (4)	<5.0 (4)	<5.0 (4)	<5.0 (4)
24 h post-challenge				
Quinpirole	8.4 ± 0.3 (3)	8.9 ± 0.3 (3)	7.9 ± 0.5 (3)	8.2 ± 0.2 (3)
Quinpirole + SKF-38393 (1μM)	8.1 ± 0.2 (5)	8.2 ± 0.1 (2)	7.8 ± 0.3 (5)	n/a
Quinpirole + SKF-82958 (1μM)	n/a	n/a	n/a	n/a
Quinpirole + SKF82958 (10μM)	<5.0 (4)*	<5.0 (1) *	<5.0 (4) *	n/a
LY-741626	n/a	n/a	n/a	n/a
SB-277011	n/a	n/a	n/a	n/a

n/a, not available; *, Weak effect, only 10-20%. pEC_{50} *ca.* 7.0. VEH, vehicle; NIC, nicotine; SCH, SCH-23390.

5.4 DISCUSSION

The data presented herein provide further insights into the pharmacological characteristics of striatal cells possessing dopaminergic receptors and demonstrate that the functionality of the dopamine receptor is altered following the expression of behavioural sensitisation. It is shown that the dopamine D_1 receptor plays a role in the expression of nicotine induced behavioural sensitisation. Moreover, this study demonstrated for the first time a direct connection between the involvement of the dopamine D_1 receptor in behavioural sensitisation and the changes in dopamine receptor mediated cellular responses following sensitisation.

Cyclic adenosine monophosphate or cAMP is an important intracellular second messenger and plays a major role in cell signalling. Furthermore, it helps understanding molecular modulations and is therefore a standard assay in the development of new neuropsychiatric drugs. The use of individual techniques has been shown to be insufficient and more multidisciplinary approaches are required. Therefore, the current study combined *in vivo* and *ex vivo* approaches to achieve maximal efficacy in an attempt to identify and pharmacologically characterise behavioural sensitisation. In line with this strategy, the present study used *in vivo* behavioural and *ex vivo* cAMP accumulation assays.

Initial locomotor activity experiments showed that the effect of nicotine returned to basal activity levels after 45 min (Figure 5-4A) and therefore the first time point to terminate the *in vivo* experiment was selected at 45 min after the last nicotine challenge.
As indicated in Table 5-1, two behavioural experiments examining the effect of a pre-challenge with SCH-23390 on sensitisation did not reach significance, although both experiments did show significant sensitisation to nicotine. In experiment number 3, the nicotine sensitised group showed a relative high locomotor response (697 beambreaks/45 min) which should normally read between 550 and 650 beambreaks/45 min. In experiment number 8, the nicotine sensitised group showed a relatively low locomotor response (502

beambreaks/45 min). There were no individual outliers, as defined by the criteria of values higher or lower than the mean ± two times the standard deviation. Moreover, the variation within the groups was small, *i.e.* s.e.m. < 9%. As stated in the methods, tissues from all animals were used regardless of their behavioural response. Tissues from animals within the same treatment group were pooled and test compounds were randomised and tested across tissue from at least three individual *in vivo* experiments (unless stated otherwise).

Forskolin, a diterpene derivative (Figure 5-1), can directly activate the catalytic subunit of adenylate cyclase thereby elevating intracellular cAMP levels. The involvement of adenylate cyclase in the development of behavioural sensitisation has been suggested after repeated co-administration of forskolin and cocaine, which significantly enhance the development of behavioural sensitisation to cocaine (Schroeder *et al.*, 2004). In an attempt to further characterise the role of cAMP signalling in sensitisation, this experiment used forskolin as a tool to investigate the relationship between nicotine induced alterations in behaviour and brain cAMP levels. Forskolin showed a concentration dependent increase in cAMP accumulation (Figure 5-6A) and was active at concentrations as low as 0.01 µM. The highest concentration tested (100 µM) gave a relatively high response, and the E_{max} is expected to be close to this estimated concentration. Therefore an estimated E_{max} was used to calculate pEC_{50} values for forskolin. Forskolin increased cAMP accumulation, however neither acute nor chronic nicotine administration (*i.e.* nicotine induced behavioural sensitisation) affected basal or forskolin stimulated cAMP levels.

The actual percentage of stimulation by forskolin compared to basal can differ greatly between the individual *ex vivo* experiments. Therefore, for comparison of the effect of forskolin between experiments (Figure 5-5), the effect of forskolin was expressed as the cAMP:ATP ratio.

The low potency of dopamine on cAMP accumulation (*i.e.* pEC_{50} 4.4 ± 0.2, Table 5-3) might be explained by the breakdown of dopamine during the incubation. This could be prevented by addition of an anti-oxidant (*e.g.* ascorbic acid/Vitamin C) to the incubation buffer, although the anti-oxidant can also affect the binding kinetics and therefore is not recommended (see Chapter 6). The low potency of

dopamine is in contradiction to the reported high affinity binding of dopamine at the dopamine receptor (see Chapter 6 for discussion). It could be suggested that this effect is due to the non-selectivity of dopamine for dopaminergic receptor subtypes, *i.e.* dopamine will activate both the dopamine D_1-like and D_2-like receptors. As the D_1-like and D_2-like receptors are respectively stimulatory and inhibitory coupled to adenylate cyclase, it could be suggested that the net effect of dopamine on cAMP accumulation could be zero. However, Mottola and colleagues showed that dopamine decreased forskolin stimulated cAMP accumulation when tested on its own (Mottola *et al.*, 2002). In the present study dopamine stimulated cAMP accumulation (Figure 5-8). It should be noted that dopamine produced a maximum stimulation of cAMP production of 167 ± 16 % over basal levels (at 0.1 mM), at which point it had not reached a plateau. An estimated pEC_{50} value was calculated (as described above for forskolin). The low potency observed could also suggest a relative low affinity for the dopamine receptor subtypes.
Dopamine was tested in presence of the selective dopamine D_2 receptor antagonist LY-741626, the selective dopamine D_3 receptor antagonist SB-277011, or the selective dopamine D_1 receptor antagonist SCH-23390. It was proposed that the dopamine D_2 antagonist would prevent dopamine D_2 receptor induced inhibition, and keep the dopamine D_1 receptor available for dopamine stimulation. The present data showed that the dopamine D_1 component of dopamine is revealed when dopamine was tested in combination with the dopamine D_2 antagonist LY-741626 (Figure 5-8C), which is in agreement with previous data (Barnett *et al.*, 1987). When rats were sensitised with nicotine, an increase in potency to stimulated cAMP accumulation was found. This is in contrast with previous data (Barnett *et al.*, 1987) which showed a decrease in dopamine stimulated adenylate cyclase activity after chronic amphetamine administration, were dopamine was used as an index for dopamine D_1 receptor activation. However, the present results of the dopamine induced D_1 receptor activation were observed in the presence of D_2 receptor blockade. A dopamine D_1/D_2 receptor "(de-) activation synergy" could explain the discrepancy between the two studies.

The scientific literature is contradictory, for example, repeated cocaine administration produced no persistent changes in dopamine stimulated adenylate cyclase activity in either caudate putamen or nucleus accumbens *ex vivo* (Mayfield *et al.*, 1992). Although acute administration of a high dose of amphetamine (5 mg/kg) or methylphenidate (50 mg/kg), but not with lower doses, resulted in the desensitisation of dopamine stimulated adenylate cyclase activity, which occurred within 25 min but recovered rapidly within 90 min (Barnett *et al.*, 1986). It might be possible that the decrease of dopamine D_1 agonist (SKF-38393 and SKF-82958) stimulated cAMP accumulation, seen in the present study after 45 min but not after 24 h post-challenge, might be due to desensitisation of the dopamine D_1 receptor. Future studies should investigate tissue taken at additional time points between the 45 min and 24 h post-challenge, in order to fully evaluate the temporality of the effect.

These data suggests that chronic nicotine administration can affect the functionality of the dopamine D_1 receptor. No conclusions can be made about the dopamine D_2 receptor as dopamine does not seem to be the right tool to monitor dopamine D_2 receptor activity by cAMP accumulation.

Behavioural sensitisation experiments in the present study have shown that nicotine sensitisation can be antagonised by the dopamine D_1 antagonist SCH-23390, suggesting a role for the dopamine D_1 receptor in behavioural sensitisation. It is hypothesised that nicotine induced behavioural sensitisation will subsequently affect the dopamine D_1 receptor cAMP pathway. Previous literature showed that activation of the dopamine D_1 receptor increases cAMP levels in rat striatum (Mottola *et al.*, 2002; see also Chapter 4). This study examines the effect of the dopamine D_1 agonists SKF-38393 and SKF-82958 on cAMP accumulation in the striatum following nicotine induced behavioural sensitisation.

The dopamine D_1 agonists SKF-38393 and SKF-82958 increased cAMP formation by stimulation of the dopamine D_1 receptor, as the D_1 receptor is positively coupled to adenylate cyclase through the Gs-protein, and the effect was blocked by a dopamine D_1-like antagonist SCH-23390 (Figure 5-10). In this study, SKF-38393 was found to be a full D_1 agonist, although this compound has

been described as a partial agonist in the literature. For example, using a specific antibody, dopamine and the selective dopamine D_1 receptor agonists SKF-81297 and SKF-82958 behaved as high-efficacy agonists (E_{max} 100 %) in stimulating [^{35}S]-GTPγS (guanosine-5'-O-(3-[^{35}S]-thio)-triphosphate, a non-hydrolysable G-protein activating analogue of GTP) binding to Gs in L-cells over-expressed with the human dopamine receptor subtype 1 (D_1), whereas SKF-38393 displayed partial agonist properties (E_{max} 70 %; Cussac *et al.*, 2004). Other examples include the examination of adenylate cyclase activity in rat striatal membranes, and dopamine D_1 receptor over-expressed in C6-glioma cells, where SKF-38393 displayed an efficacy of only 30–40 % relative to dopamine (O'Boyle *et al.*, 1989; Lewis *et al.*, 1998). The discrepancy between these different assays is probably reflecting a difference in density of dopamine D_1 receptors in the cell systems used, *i.e.* a low receptor density would results in a very poor efficacy whereas a high receptor density would result in full agonistic properties. The present study used intact striatal cells measuring intracellular cAMP accumulation instead of the described indirect methods or *in vitro* cell cultures.

The present study showed that the potency of dopamine D_1 receptor agonist stimulated cAMP accumulation was decreased following nicotine sensitisation. Although this is in contrast to previous reports, this might be explained by the different treatment protocols used. For example, the sensitivity of dopamine and SKF-82958 on adenylate cyclase activity was increased following 14 days of chronic cocaine treatment, but not after acute or 7 days treatment (Unterwald *et al.*, 1996). This study also showed that the maximal effect produced by dopamine or SKF-82958, as well as the pEC_{50} of SKF-82958 were increased. However, Unterwald and colleagues measured the adenylate cyclase activity using [^{3}H]-cAMP membrane binding and tissue was taken 30 min post-challenge, and because of these methodological differences the results from the present study might not be comparable. SCH-23390 was tested in a wide concentration range from $1E^{-11}$ to $1E^{-05}$ M, to assess putative high and low affinity sites as proposed by receptor binding experiments (see Chapter 6).

Considering all the experimental evidence, it is quite conceivable that changes in cAMP-modulation are only seen 45 min after challenge injection, and not after 24 h, as cAMP is a relatively fast acting second messenger as over time (>24 h)

more downstream signalling molecules will get activated. Moreover, changes in dopamine D_1 agonist mediated cAMP accumulation are seen after chronic as well as acute nicotine administration. This study does not show a difference between acute and chronic nicotine administration, and is not sufficient to make conclusions about long term changes.

It was shown that the stimulating effect of dopamine D_1 agonists on *ex vivo* cAMP is affected by behavioural sensitisation and can be reversed by *in vivo* pre-treatment with a dopamine D_1 antagonist; this increases the predictive validity of the hypothesis that the induced cAMP accumulation is related to behavioural sensitisation. Moreover, this suggests that the dopaminergic pathway plays a role in behavioural sensitisation.

The present study observed only an effect of dopamine D_1 agonist stimulated cAMP accumulation when tissue was taken 45 min post-challenge, but not 24 h post-challenge. It might be suggested that there is still nicotine left in the tissue taken 45 min after the challenge injection and no nicotine present 24 h post-challenge. The nicotine still present could then affect the dopamine D_1 stimulated cAMP accumulation, maybe due to the desensitisation of the nAChRs or dopamine receptors.

The shift in potency seen with SKF-38393 after chronic nicotine treatment is in agreement with the given hypothesis (*i.e.* dopaminergic receptors are involved in chronic nicotine induced behavioural sensitisation), the potency shift seen after acute nicotine administration did not agree with the hypothesis. It could be suggested that there is still nicotine present in the tissue after *in vivo* administration. This is supported by the fact that the shift is only seen 45 min and not 24 h after the last injection. However, the extensive washing and stabilisation time of the tissue before testing *ex vivo* contradicts this possibility. Therefore the effect of nicotine on cAMP was investigated, and no significant effect of nicotine was seen (data not shown). To exclude the putative interference of nicotine, if still present in the slices after *in vivo* administration, the effect of SKF-38393 in combination with nicotine (10 µM) was examined. Co-incubation with nicotine did not affect the potency or efficacy of SKF-38393.

The present results showed that nicotine does not have any effect on cAMP accumulation in tissue of naïve animals. Neither does nicotine affect the potency

or efficacy of SKF-38393 stimulated cAMP accumulation. Taken together, these results suggest that the putative presence of nicotine in the tissue after *in vivo* nicotine administration (0.4 mg/kg, s.c., tissue dissected 45 min after the challenge), likely does not interfere with the observed cAMP results, and that this does not explain the effects seen after acute nicotine administration. However, a full concentration curve of nicotine should be performed to achieve a more physiologically relevant concentration, which might act differently on nAChRs and subsequently cAMP levels.

It has been shown that dopamine D_2 receptor antagonists do not block behavioural sensitisation (Vezina and Stewart, 1989; Drew and Glick, 1990) and therefore it was hypothesised that nicotine induced behavioural sensitisation would not affect dopamine D_2-like receptors or cAMP accumulation. The present results show that both acute as well as chronic nicotine administration slightly decrease the potency of quinpirole on cAMP accumulation. Futhermore, the *in vivo* pre-challenge of SCH-23390 did not affect this.
When quinpirole was tested in the presence of SKF-82958 (1 µM), the pEC_{50} value of quinpirole was lower than without the SKF-82958, and in its presence there was no effect of the *in vivo* nicotine treatments on quinpirole. In the presence of 10 µM SKF-82958 there was no effect of quinpirole observed, which could be explained by assuming that the increasing effect of the dopamine D_1 agonist on cAMP accumulation was too strong for quinpirole to compete with. Furthermore, as observed with the dopamine D_1 ligands, there was no effect of the nicotine treatment on quinpirole in tissue taken 24 h post-challenge.

Not investigated in this study, but important to include in further studies is a group chronically treated with nicotine and challenged with saline. It would be interesting to know if chronic nicotine pretreatment does change dopamine stimulated adenylate cylcase activity in the absence of nicotine challenge, or rather lowered the dose threshold for the nicotine induced desensitisation of dopamine stimulated adenylate cyclase.

Schroeder and colleagues showed that activation of adenylate cyclase affects the induction of sensitisation but is not able to induce behavioural sensitisation on its

own (Schroeder *et al.*, 2004). These experiments however, did not show whether adenylate cyclase activation is necessary for behavioural sensitisation. This can be investigated using knock-out animals for specific adenylate cyclase isomers, if available, or by using spectific inhibitors (*e.g.* SQ-22536) in combination with a psychostimulant. Although the 7-DMB-forskolin treatment did not produce a long lasting effect, *i.e.* no effect on the expression of cocaine sensitisation (Schroeder *et al.*, 2004), adenylate cyclase activation and cAMP production still might be necessary for the expression of sensitisation. Future studies should investigate the effect of adenylate cyclase inhibitors (*e.g.* SQ-22536) on the development and expression of behavioural sensitisation.

The present study investigated the role of dopamine D_1 and D_2 receptor subtypes. However, previous literature also suggests a role for the dopamine D_3 receptor in behavioural sensitisation (Le Foll *et al.*, 2003a; 2003b; Harrison and Nobrega, 2009 and references therein). In could be hypothesised that not the selectivity of the dopaminergic ligands for one specific receptor but the synergy (*i.e.* co-activation) of multiple dopamine receptor subtypes is required to achieve optimal efficacy. For example, simultaneous activation of dopamine D_1/D_3 or dopamine D_2/D_3 or dopamine D_1/D_2 receptor subtypes. In the present study it was shown that quinpirole on its own had a pEC_{50} of 9.2 and quinpirole in the presence of SKF-82958 (1 μM) a pEC_{50} of 8.1. This preliminary data suggests an interaction of the two receptor subtype ligands and needs further studying.

Conclusion

The data presented here provides biochemical evidence that dopamine receptors exist on cells in the striatum that possess receptors for neuromodulators known to be important in regulating basal ganglia activity, and inducing or antagonising locomotor activity sensitisation. This information from behavioural and subsequent *ex vivo* biochemical studies shows a direct connection between the behavioural effects of sensitisation and the *ex vivo* effects on cAMP accumulation by dopamine D_1 agonists. Furthermore, the *ex vivo* biochemical assay was able to monitor the *in vivo* reversal of sensitisation by the dopamine D_1 agonist SCH-23390.

Chapter 6. Effect of chronic nicotine induced behavioural sensitisation on ex vivo receptor binding pharmacology

Abstract.

OBJECTIVES: It has been shown that the intermittent administration of psychostimulants can induce behavioural sensitisation and is associated with changes in dopaminergic neurotransmission in the striatum. Psychostimulants (including nicotine) are known to increase dopamine release in the striatum which subsequently can bind to dopamine receptors. The objective of this study was to investigate if nicotine induced behavioural sensitisation affect the dopamine D_1 receptor binding pharmacology in rat striatum *ex vivo*.

METHODS: Rats were administered with saline or nicotine (0.4 mg/kg, s.c.) for 5 consecutive days followed by a 5 day withdrawal period. After the withdrawal period rats were decapitated after 24 h following the final nicotine challenge injection, and dorsal striatum was dissected and frozen on dry-ice. Radioligand binding using [^{3}H]-SCH-23390 was performed to determine the dopamine D_1 receptor occupation in membrane homogenates of dorsal striatum from vehicle and behaviourally sensitised rats. A selection of dopaminergic (D_1-like) ligands was assessed using a newly established 96-well filterplate method.

RESULTS: Behavioural sensitisation was verified by increased intensity of horizontal locomotor activity and it was observed that nicotine significantly induced expression of sensitisation. However, the nicotine induced behavioural sensitisation did not affect the affinity (K_d) of dopamine, apomorphine, SKF-38393, SKF-82958, SCH-23390, or butaclamol for the dopamine D_1 receptor.

CONCLUSIONS: Nicotine induced behavioural sensitisation does not affect the affinity of the tested dopaminergic ligands for the dopamine D_1 receptor. However, functional studies have now shown that behavioural sensitisation only has an effect on tissue taken 45 min post-challenge and further binding studies should include this additional time point. Also, the presence of high and low affinity dopaminergic binding sites is argued.

Keywords: Receptor binding, behavioural sensitisation, nicotine, [^{3}H]-SCH23390

6.1 INTRODUCTION

It is hypothesised that behavioural sensitisation is caused by an excess of dopamine release in the midbrain. This synaptically available, released dopamine is than able to bind to dopaminergic receptors. Receptor binding is the interaction of a ligand binding to form a complex with a biological protein. The ligand can be endogenous or exogenous and upon binding to a receptor, it can alter the structural conformation of the receptor. Subsequently, the functional state of the receptor can be changed which can trigger a physiological response like activation of intracellular signalling cascades and subsequent release of neurotransmitters.

In humans, psychostimulant dependence (for example by cocaine, methamphetamine, and also alcohol or opioids) is associated with lower availability of the dopamine D_2 receptor in the striatum, which is suggested to be a result of increased dopamine release (Volkow *et al.*, 2001; Zijlstra *et al.*, 2008). Moreover, a significant correlation between the duration of (poly) drug use and striatal dopamine D_2 receptor availability has been shown (Zijlstra *et al.*, 2008). In nicotine dependent smokers, significantly less available dopamine D_2/D_3 receptors within the putamen and nucleus accumbens were observed (Fehr *et al.*, 2008). In contrast, the there was no difference in dopamine D_2 receptor binding in the caudate or nucleus accumbens of sensitised rhesus monkeys, two weeks after repeated cocaine injections (Farfel *et al.*, 1992). This suggests a specific role for the dopamine D_1 receptor and the same study showed that the densities of the dopamine D_1 receptor binding sites in the caudate, but not nucleus accumbens, of the sensitised monkeys were significantly decreased two weeks after repeated cocaine injections (Farfel *et al.*, 1992).
In rats, behavioural sensitisation to psychostimulants is correlated with supersensitivity to dopamine agonists (Robinson and Becker, 1986; Robinson and Berridge, 2000), however, it is still unclear if the sensitisation by dopamine agonists is primarily dependent on dopamine D_1 receptor activation (Vezina, 1996) or D_2 receptor activation (Ujike *et al.*, 1990). In rats exhibiting behavioural sensitisation following daily cocaine administration (10 mg/kg, i.p.), the

occupation of dopamine D_1 and D_2 receptors by dopamine (as measured by EEDQ) was increased by approximately 100 % (Burger and Martin-Iverson, 1994). Despite the increased occupation of dopamine D_1 and D_2 receptors following daily cocaine administration, this occupancy of striatal dopamine receptors does not appear to be related to behavioural sensitisation as this increase was observed following either intermittent or continuous cocaine treatment regimes (see for review Martin-Iverson and Burger, 1995; and Chapter 2 for the discussion of different treatment regimes and behavioural sensitisation). This is in agreement with work published by Claye and colleagues who showed that the affinity (K_d) of the radioligands and the density (B_{max}) of striatal dopamine D_1 ([^{3}H]-SCH-23390) and D_2 ([^{3}H]-spiperone) binding sites remained unchanged when measured after 3 days of withdrawal following 14 days of chronic intermittent cocaine administration (Claye *et al.*, 1995). And in agreement with work published by Mayfield and colleagues who observed no differences in dopamine D_1 receptor density and affinities for either [^{3}H]-SCH-23390 or [^{3}H]-dopamine, in the nucleus accumbens or dorsal striatum, following cocaine induced behavioural sensitisation (Mayfield *et al.*, 1992).

In addition to the classical receptor binding hypothesis it is reported that certain receptors, including the dopamine receptors, have two different affinity binding sites *in vitro*, *i.e.* a high and a low affinity binding site. The high-affinity state is considered to be the physiologically functional state (George *et al.*, 1985) and, for example, the high affinity binding site of the dopamine D_2 receptor (D_2^{High}) is suggested to be involved in diseases like schizophrenia and psychosis (Seeman *et al.*, 2005, 2006). The high affinity binding site is also suggested to play a role in behavioural sensitisation, for example following amphetamine induced sensitisation the proportion of dopamine D_2^{High} is increased, as measured by dopamine in competition with [^{3}H]-domperidone (Seeman, 2009) or [^{3}H]-PHNO [(+)4-propyl-9-hydroxynaphthoxazine] (Seeman *et al.*, 2007). A 2-4 fold elevation in the proportion of dopamine D_2^{High} receptors was observed following behavioural sensitisation (Seeman *et al.*, 2002; see for review Seeman *et al.*, 2006). Although the dopamine D_2 receptor availability may be lower in psychostimulant abusers, the proportion of the D_2^{High} versus D_2^{Low} receptors is likely to be elevated, in accord with the clinical observation that such individuals

are dopamine supersensitive (Volkow *et al.*, 2001). Furthermore, it has been shown that the proportion of dopamine D_1^{High} receptor affinity state by dopamine in competition with [^{3}H]-SCH-23390 is increased following methamphetamine sensitisation (Shuto *et al.*, 2008).

In principle, the high affinity state of the dopamine receptor can be labelled by low concentrations of various radioactive dopamine ligands (Richfield *et al.*, 1989). However, it should be noted that literature describing the determination of high and low affinity sites of dopaminergic receptors is not always consistent. For example, the dopamine D_1^{High} receptor affinity state is not always observed in striatal membranes (*e.g.* [^{3}H]-SCH-23390, Leonard *et al.*, 2006 and Schulz *et al.*, 1985), although other groups show that [^{3}H]-SCH-23390 in the presence of 120 mM NaCl and 4 mM $MgCl_2$ can detect the two binding sites in the striatum (Shuto *et al.*, 2008; Sumiyoshi *et al.*, 2005) and might be dependent on the presence of other metals (*e.g.* sodium, lithium and magnesium; Gottberg *et al.*, 1989).

The aim of this study was to investigate the effect of nicotine induced behavioural sensitisation on the binding affinities of dopamine D_1 receptor ligands for the dopamine D_1 receptor. For this purpose, saturation curves and competition experiments were performed using membrane preparations of rat dorsal striatum. Furthermore, the proportion of the high and low affinity binding sites was investigated.

HYPOTHESES AND AIMS

Hypotheses:

Dopamine D_1 receptors are not up-regulated following behavioural sensitisation and behavioural sensitisation will not affect the affinity (K_d) of dopamine D_1 receptor ligands.

Aims:

- Investigate if the dopamine D_1 receptors are up- or down-regulated following behavioural sensitisation by measuring the density of dopamine D_1 receptors by determining the B_{max}.
- Investigate putative changes in the affinity (K_d) of dopaminergic (D_1–like) receptor ligands using a competition experiment with [^{3}H]-SCH-23390 (D_1-like binding).
- Identify and quantitate the high and low affinity receptor states in the striatal membrane preparations and determine if behavioural sensitisation has an effect on their proportions.

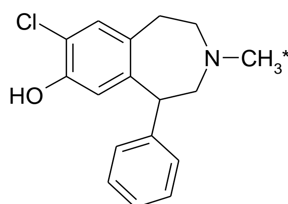
6.2 MATERIALS AND METHODS

The described receptor binding experiments were performed at Abbott Healthcare Products, the Netherlands.

6.2.1. Chemicals

The following drugs were ordered from Sigma (Zwijndrecht, the Netherlands): (+)-butaclamol, (R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-benzazepine-7-ol ((R)-(+)-SCH-23390), (-)-sulpiride, 2,3,4,5,-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine ((±)-SKF-38393), dopamine, 3-allyl-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF-82958), apopmorphine, and salts, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$). All buffers were made using purified water (MilliQ, Waters, Milford, MA, USA).

[^{3}H]-SCH-23390 (N-methyl-^{3}H, specific activity of 2.7047 TBq/mmol) was purchased from Perkin Elmer (Waltham, Massachusetts, USA), see Figure 6-1.



[^{3}H]-SCH-23390

Figure 6-1 Chemical structure of radiolabelled [^{3}H]-SCH-23390

Chemical structure of the selective dopamine D_1 receptor antagonist [^{3}H]-SCH-23390. *, indicates the position of the tritiated isotope ([^{3}H]) according to the manufacturer (Perkin Elmer).

6.2.2. Animals

For animals used and description of locomotor activity protocol, see Chapter 2.

6.2.3. Experimental design (*in vivo* protocol)

Animals were chronically treated and challenged with nicotine as previously described in Chapter 2. The methodology was approved by the animal ethics committee (Abbott Healthcare Products, the Netherlands) and was in accordance with all local laws.

Briefly, one day prior to each experiment (day 0), all animals (n = 8 – 12 rats/group) were habituated to the activity cages for one h, followed by a saline injection and recording of locomotor activity. On the testing days (days 1 and 5) the rats were habituated for 60 min to the test apparatus, before administration of either saline (s.c., 2 groups) or (-)-nicotine (0.4 mg/kg, s.c., 1 group). After receiving the injections, the rats were directly placed back in the test apparatus and locomotor activity was measured for 45 min, in 5 min time epochs. This first time dose of nicotine was considered the acute dose. On day 2, 3 and 4 all groups received the same treatment as the day before and were directly placed back in their home cage. After a 4 day withdrawal period (day 9), all groups received a saline injection (s.c.) and locomotor activity was measured to assess if the response was context-dependent. On day 10, rats were habituated for 30 min to the test apparatus before administration of a pre-challenge of saline (s.c., all groups). The rats were directly placed back in the test apparatus and locomotor activity was measured for 30 min. After 30 min of the pre-challenge, the Vehicle - Vehicle group was challenged with saline and all other groups were challenged with 0.4 mg/kg (-)-nicotine (s.c.). Locomotor activity was measured for another 45 min, see Table 2-1 and Figure 2-1 for study design.

6.2.4. Tissue preparation

The male Wistar rats were decapitated at 24 h after the last challenge, without anaesthetics and the brains were rapidly removed. The brain areas of interest were dissected according to Paxinos and Watson (1998) and directly frozen on dry-ice. The samples were stored at -80 °C until membrane preparation.

Frozen rat striatal samples were weight and homogenised in ice-cold Tris buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA) using GentleMax tissue homogeniser (protocol RNA.01.01, for 30 s.; Miltenyi Biotec, Germany). The membranes were precipitated by centrifugation at 10,000 x g for 10 min at 4 °C and washed by re-

homogenisation using the GentleMax. This was followed by centrifugation at 50,000 x g for 10 min at 4 °C. The final pellet was re-homogenised (GentleMax, 15 s.) in incubation buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, containing 120 mM NaCl, 5 mM KCl, with or without 1.5 mM $CaCl_2$ and 4 mM $MgCl_2$, as stated in the text, and Table 6-1) to obtain the required membrane concentration. The tissue was kept at room temperature for 30 min before starting the incubation.

Methodological considerations

Variation between animals within the same *in vivo* treatment group made it impossible to use tissue from only a single rat for an $n = 1$ *ex vivo* experiment. To overcome this problem the dissected tissue from at least four animals within the same treatment group were pooled and homogenised together. This protocol significantly reduced the intra-experiment variation.

6.2.5. Radiolabel

The actual radioligand concentration was determined in order to calculate the exact radioligand concentration. A stock concentration of the radiolabel was obtained by dilution of 5 µL [^{3}H]-SCH-23390 in 1600 µL incubation buffer. Of this stock, 10 µL was transferred to a volume of 10 mL scintillation liquid (UltimaGold XR, Perkin Elmer) in duplicate and radioactivity disintegrations per minute (DPM) were determined using a liquid scintillation analyser (Perkin Elmer). To correct for the degradation (half-life) of the radiolabel, the current activity was calculated by:

$$\text{Activity (KBq)} = \text{Rad Conc Current (MBq/mL)} * 0.1 * \text{stock volume (mL)}$$

The actual stock concentration was calculated by:

$$\text{Conc (nM)} = \text{activity (KBq)} / \text{volume (mL)} * \text{specific activity (GBq/mmol)}$$

If required, the stock solution was diluted by the following dilution-factor:

$$\text{Conc (nM)} = \text{Conc (nM)} / 10 * K_d \text{ (nM)}$$

This stock is 10 times concentrated as the label will be 10 times diluted at the start of the incubation (as described in the next section).

6.2.6. Receptor binding

Incubations were performed in a total incubation volume of 200 µL using MultiScreen®$_{HTS}$ 96-well filterplates (1.0 µm Glass Fiber Type B Filter, cat.no. MSFBN6B50, Millipore, Billerica, MA, USA). Compounds were diluted in incubation buffer (Table 6-1) and were tested in triplicate. To each well was added 20 µL test compound, 20 µL tritiated label, and the incubation was started upon addition of 160 µL tissue homogenate. The plates were shaken using a well-plate shaker (TeleShake, Variomag, Thermo, MA, USA) and incubated for 2 h in a well-plate incubator at 25 °C. The incubations were terminated by rapid filtration through the MultiScreen®$_{HTS}$ filterplates using a vacuum unit (Millipore), followed by washing the filterplates rapidly three times with 100 µL ice-cold 50 mM Tris-HCl/1 mM EDTA buffer. After air drying the filterplates, each well received 35 µL scintillation cocktail (MicroScint-O™, Perkin-Elmer) and the filterplates were sealed. After 24 h equilibration, radioactivity was determined using a beta scintillation counter (MicroBeta, Perkin-Elmer).

Saturation experiments

Tissue saturation experiments were constructed by the incubation of different concentrations of tissue homogenate with 0.8 nM [^{3}H]-SCH-23390 in the absence or presence of 10 µM (+)-butaclamol (a non-selective dopamine antagonist) to determine total and non-specific binding, respectively.

To determine the K_d and B_{max} of [^{3}H]-SCH-23390 in rat striatum, saturation experiments were performed by the incubation of striatal homogenate (5 mg/mL) with different concentrations of [^{3}H]-SCH-23390 (final concentrations ranging from 0.05 – 8 nM). In these experiments, the non-specific binding was defined using 1 µM (+)-butaclamol.

Competition experiments

Competition experiments were performed by incubation of different concentrations of test compound with [^{3}H]-SCH-23390 (0.8 nM) and 5 mg/mL striatal membranes. Non-specific binding was defined by the binding in the presence of 1 µM (+)-butaclamol.

Table 6-1 Buffer compositions
All buffers were adjusted to pH 7.4.

Salt	Conc. (mM)	Normal	w/o Mg^{2+}, Ca^{2+}	Only TRIS
TRIS.HCl	50	+	+	+
EDTA	1	+	+	+
NaCl	120	+	+	
KCl	5	+	+	
$CaCl_2$	1.5	+		
$MgCl_2$	4	+		

6.2.7. Data analyses

For the saturation experiments, raw data (expressed in counts per minute, cpm) represent radioligand binding at the membrane preparation and the filterplate. Incubations with only radioligand and membranes represent the total binding, and radioligand and membranes in the presence of 10 µM (+)-butaclamol represent non-specific binding. The specific binding was defined as total binding minus binding in the presence of 10 µM (+)-butaclamol (*i.e.* non-specific binding). Binding isotherms were analysed by non-linear regression, using PRISM graphing software (Graphpad software, San Diego, CA, USA) to yield IC_{50} values. The Scatchard plots, *i.e.* Bound/Free versus Bound plot (Scatchard, 1949), were calculated as followed:

Bound [B] (dpm) = Total (dpm) – non-specific binding
Free, [F[(dpm) = Total (dpm) – specific activity (dpm = cpm*3)

Competition receptor binding

Raw data (cpm) representing total radioligand binding (*i.e.* the sum of specific and non-specific binding) are plotted as a function of the logarithm of the molar concentration of the competitor (*i.e.* test or reference compound). Non-linear regression of the normalised raw data (*i.e.* percent radioligand binding compared to that observed in the absence of test or reference compound) was performed with PRISM graphing software using the built-in three parameter logistic model describing ligand competition binding to radioligand-labelled sites:

$$y = \text{bottom} + [(\text{top-bottom})/(1 + 10x\ pIC_{50})]$$

were bottom equals the residual radioligand binding measured in the presence of 10 μM reference compound (*i.e.* non-specific binding) and top equals the total radioligand binding observed in the absence of competitor. The pIC_{50} (*i.e.* the negative logarithm of the ligand concentration that inhibits the specific radioligand binding by 50 %) is estimated from the data and used to obtain the inhibition constant (K_i) by applying the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$Ki = IC_{50}/(1 + [\text{ligand}]/K_d)$$

where [ligand] equals the radioligand concentration (nM) and dissociation constant (K_d) equals the affinity constant of the radioligand for the target receptor (nM).

The actual concentration of the radiolabel at the time of the experiment was calculated by:

$$nM = dpm / 0.02 * 60 * 10 * \text{specific activity (GBq/mmol)}$$

The activity (in dpm) was measured in triplo at the day of the experiment, using a liquid scintillation counter.

6.3 RESULTS

6.3.1. Saturation experiments

The following saturation experiments were performed to determine the optimum tissue concentration for the binding experiments. The experiments were performed at equilibrium (*i.e.* 2 h incubation at 25 °C) with 8 different concentrations of rat dorsal striatal membranes. The various concentrations of rat striatal membranes (0-10 mg/mL) were incubated with 0.8 nM [^{3}H]-SCH-23390. The maximum specific binding (B_{tot} – $B_{non\text{-}specific}$ = $B_{specific}$) observed under the described conditions was 49 % (Figure 6-2). A wet tissue concentration of 5 mg/mL was used for all future experiments as this concentration gave a robust response, maximal specific binding and the results are in agreement with previous literature (*e.g.* Schulz *et al.*, 1985; Leonard *et al.*, 2006).

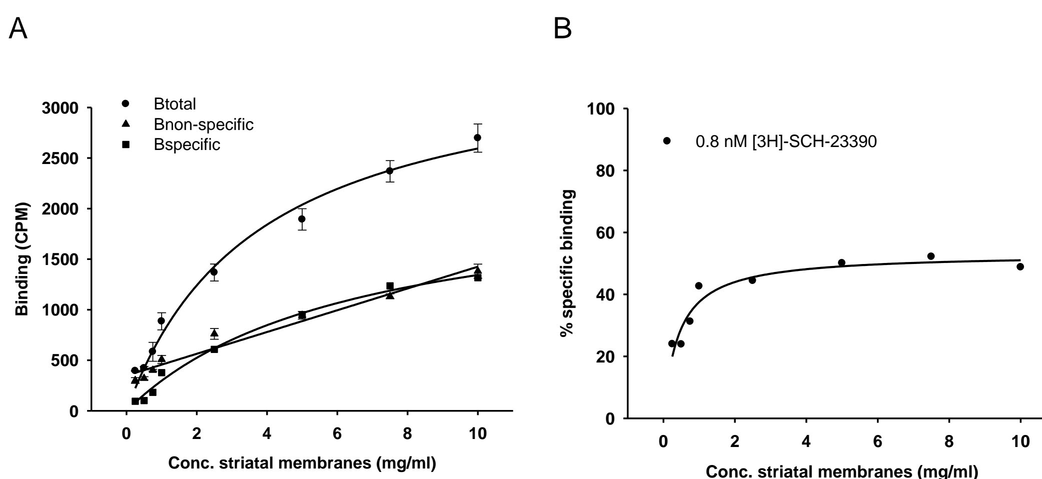


Figure 6-2 Saturation experiment of striatal membranes ([^{3}H]-SCH-23390)

A. The binding isotherm of [^{3}H]-SCH-23390 at different concentrations of dorsal striatal membranes. B. Specific binding (%) of 0.8 nM [^{3}H]-SCH-23390 at different concentrations of striatal membranes (0 - 10 mg/mL). Tissue taken from non-treated animals, $n = 1$.

In order to determine the binding constants of $[^3H]$-SCH-23390, saturation experiments were performed using 12 different concentrations of $[^3H]$-SCH-23390 to determine the K_d and B_{max} values. Rat striatal membranes (5 mg/mL) were incubated with various concentrations of the radioligand ranging from 0 to 8 nM for 2 h at 25 °C. A typical binding isotherm was observed with a parabolic curve equation for total and specific binding and a linear curve equation for non-specific binding (Figure 6-3A). The B_{max} value was determined at 8 nM (highest concentration tested) and the K_d value for $[^3H]$-SCH-23390 was determined at 1.0 ± 0.3 nM.

A maximum of 73 % specific binding was found at a concentration of 0.4 nM $[^3H]$-SCH-23390 (Figure 6-3B). The specific binding of $[^3H]$-SCH-23390 was < 50 % at concentrations lower then 1 nM. The radioligand concentrations for competition experiments were chosen 0.8 nM at which it achieved *ca.* 70 % specific binding.

The calculated Scatchard plot, *i.e.* Bound/Free versus Bound plot (Scatchard, 1949), of the saturation curve resulted in two lines indicating the presence of two different binding sites (Figure 6-3C). However, due to the large variation between the different concentrations tested, no K_d values could be calculated.

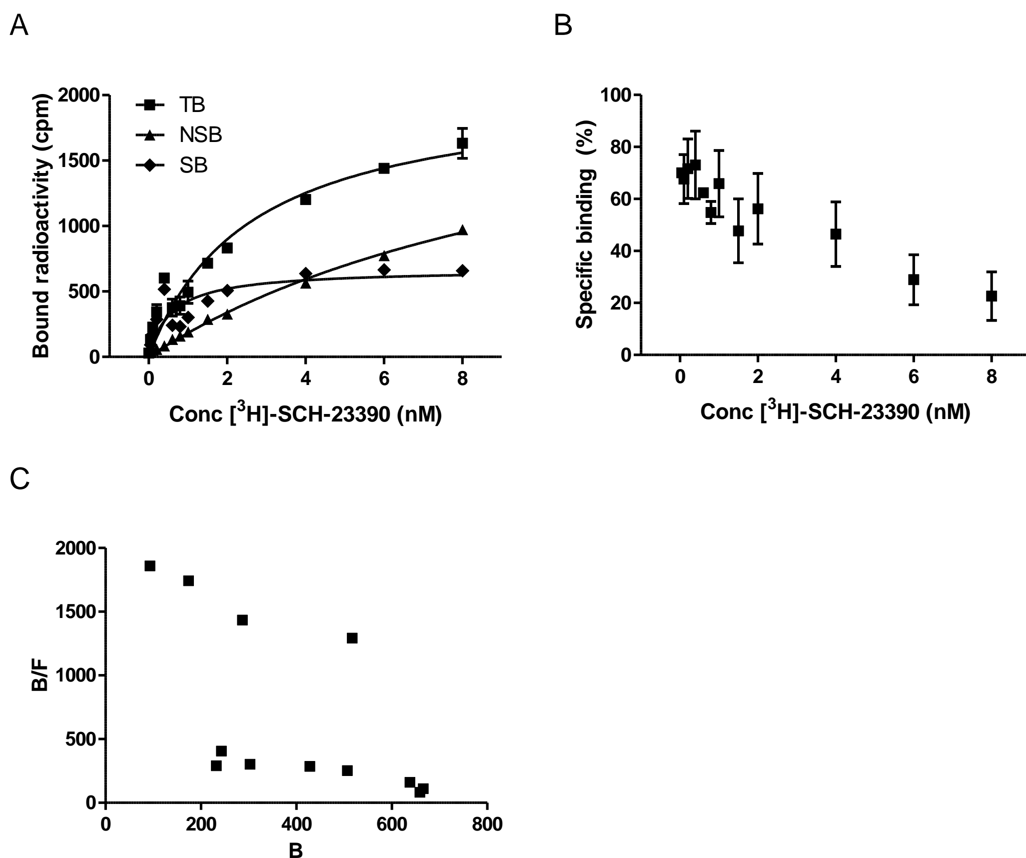


Figure 6-3 Saturation experiment of [^{3}H]-SCH-23390 (striatal membranes)

A. Representative binding isotherm of different concentrations [^{3}H]-SCH-23390. B. Specific binding (%) in striatal membranes, $n = 3$ (5 mg/mL). C. Scatchard plot. B, bound; F, free (dpm). Non-specific binding was determined using 10 µM (+)-butaclamol. Tissue was taken from non-treated animals. Data represents mean ± s.e.m., of three individual experiments performed in triplicate.

6.3.2. Biphasic high and low affinity site

The effect of the alkali cations sodium (Na^+) and potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) on the high and low affinity agonist state of [^{3}H]-SCH-23390 binding site were investigated. This was performed by competition experiments using dopamine as the endogenous substrate to antagonise the receptor binding. Three different incubation buffers were prepared and investigated, containing TRIS/EDTA ("Only TRIS"), TRIS/EDTA + NaCl + KCl ("w/o Mg^{2+}, Ca^{2+}"), and TRIS/EDTA/NaCl/KCl + $MgCl_2$ + $CaCl_2$ ("normal").

The competition experiments with the endogenous agonist dopamine did not reveal the presence of a high affinity (K_h) binding site.

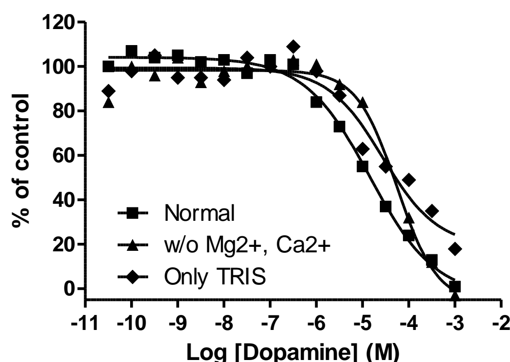


Figure 6-4 Influence of salt concentration on receptor affinity states

The inhibition of specific [^{3}H]-SCH-23390 binding to dorsal striatal membrane preparations by dopamine (at 16 concentrations) using three different incubation buffers. The results shown are from a representative experiment.

6.3.3. Competition experiments

To investigate putative changes in binding affinity of the dopamine D_1 receptor following chronic nicotine treatment, the affinities of a selection of dopaminergic ligands was tested in competition experiments against [^{3}H]-SCH-23390.

The non-selective dopamine receptor agonists dopamine and apomorphine, the selective dopamine D_1 receptor agonists SKF-38393 and SKF-82958, the selective dopamine D_1 receptor antagonist SCH-23390 and the non-selective dopamine receptor antagonist butaclamol, all concentration dependently antagonised the [^{3}H]-SCH-23390 binding (Figure 6-5A to F, respectively). There was no effect of chronic nicotine treatment on the potency or efficacy of these ligands for dopamine D_1 receptor binding.

Competition experiments were performed using buffer without Mg^{2+} or Ca^{2+} (see methods), and in each case a two-site model was considered, however, a single binding site equation showed a significantly better fit according to the F-test (P < 0.05, PRISM software).

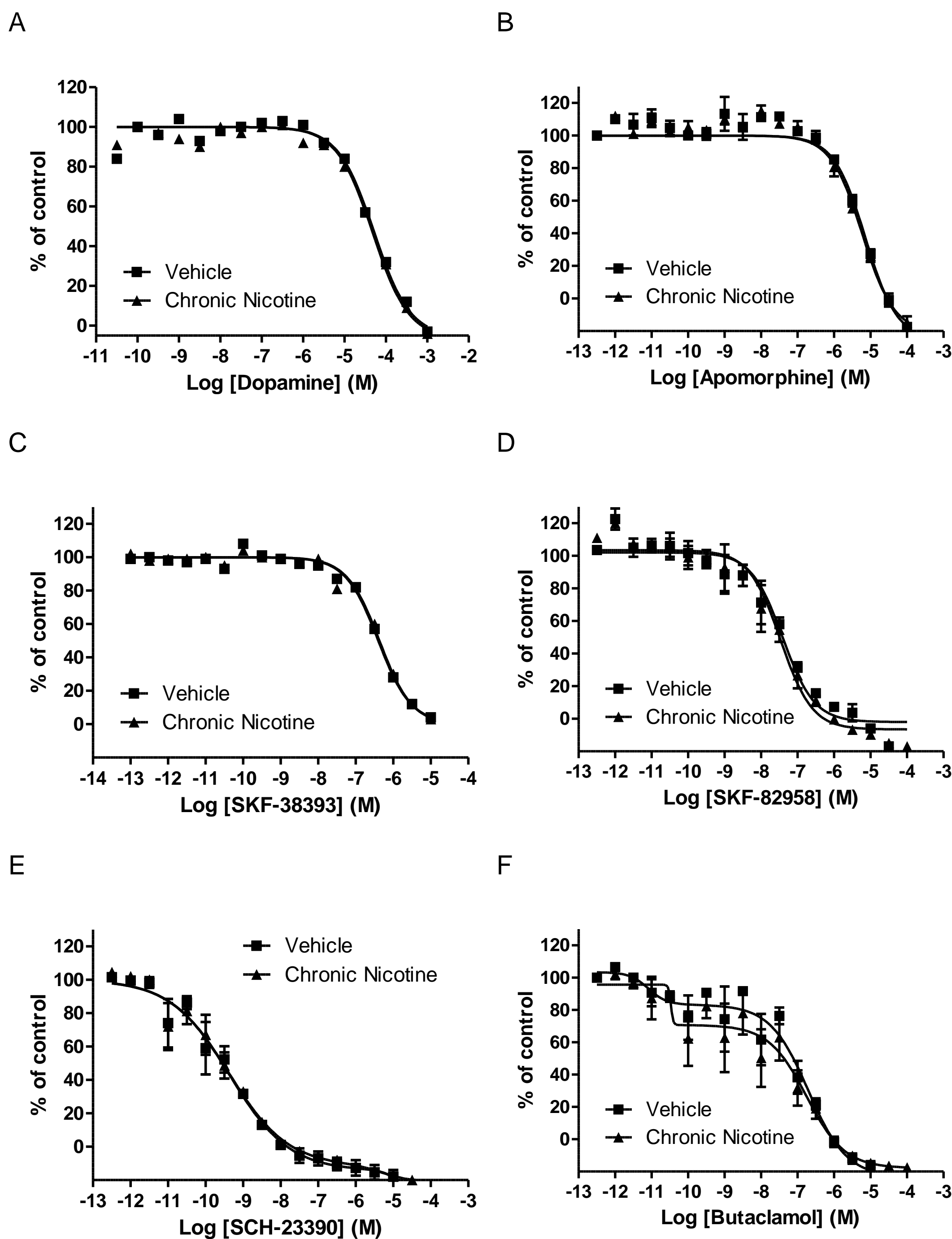


Figure 6-5 Effect of dopaminergic ligands on dopamine D_1 receptor binding
Membrane preparations from vehicle treated or nicotine sensitised rats were incubated with [^{3}H]-SCH-23390 and increasing concentrations of dopamine (A), apomorphine (B), SKF-38393 (C), SKF-82958 (D), SCH-23390 (E) or butaclamol (F). Without Mg^{2+} or Ca^{2+} (see methods). For pIC_{50} values see Table 6-2. Data represent mean ± s.e.m. of $n = 1$ - 4 independently performed experiments.

Table 6-2 Affinity of dopaminergic ligands for dopamine D_1 receptor

[^{3}H]-SCH-23390 receptor binding in rat dorsal striatal membranes. Values are pIC_{50} mean ± s.e.m. n-number is given between parentheses.

	VEH-VEH	VEH-NIC	NIC-NIC
24 h post-challenge			
SKF-38393	6.4 (1)	n/a	6.4 (1)
SKF-82958	7.3 (3)	n/a	7.3 (3)
SCH-23390	9.4 (3)	n/a	9.3 (3)
Apomorphine	5.3 (3)	n/a	5.4 (3)
Dopamine	4.3 (1)	n/a	4.4 (1)
Butaclamol	7.0 (3)	n/a	6.9 (4)

n/a, not available

6.4 DISCUSSION

The present study showed that, under the given conditions, nicotine induced behavioural sensitisation did not affect the affinity of dopamine, apomorphine, SKF-38393, SKF-82958, SCH-23390, or butaclamol for the dopamine D_1 receptor (Figure 6-5).

Prior to the pharmacological study, a MultiScreen®$_{HTS}$ 96-well filterplate-based receptor binding technique was established, using vacuum-filtration instead of the classical harvesting method. The advantages of this method are the high reproducibility, small variation, less chemical waste production, high throughput, and suitablility for tissue homogenates. Prior to the described rat brain tissue experiments, the filterplate based method was first developed using Chinese hamster ovarian (CHO) cells transfected with the human dopamine D_{2L} receptor (data not shown). However, the *in vitro* cell based receptor binding using transfected cells is a very artificial system making translation and predictive validity to animal models very difficult. Therefore more advanced and physiologically relevant experimental systems, such as brain slices (*ex vivo*) are required. For example, dopamine D_1 and D_2 receptor binding in striatal brain slices has been positively correlated to the effect of amphetamine seen in PET and SPECT studies (Gifford *et al.*, 2000). After the validation *in vitro*, this assay was successfully applied to *ex vivo* rat brain tissue. The whole assay is performed in a single 96-well filterplate and is using only a small volume (*i.e.* 200 µL format), which requires less tissue and reagents although still showing high specific binding (*i.e.* > 70 % in this study).

The present study used a dose of 0.03 mg/kg, i.p. of SCH-23390 to antagonise the nicotine induced behavioural sensitisation. This was found both behaviourally the most effective dose (Chapter 2) as well as on receptor occupancy where the dopamine D_1 receptor occupancy following systemic administration of SCH-23390 was evident as an inverted U-shaped dose-dependent effect, with the greatest occupancy observed at the intermediate dose of 0.3 mg/kg i.p (Neisewander *et al.*, 1998).

In the present study the tissue used was taken only at 24 h post-challenge. As discussed in previous chapters, the time of dissection of the tissue following the last challenging dose of nicotine *in vivo* might be crucial to observe effects *ex vivo*. It has been shown that the time frame of dissection can be critical for functional assays (Chapter 5; Kleven *et al.*, 1990) and herein is also suggested to be crucial for binding assays. Further studies should include tissue taken at 45 min post-challenge as tissue taken at this time point, but not at 24 h post-challenge, showed changes in cAMP accumulation (Chapter 5). The present study only investigated tissue from vehicle and nicotine behavioural sensitisised animals. Further studies should also include tissue from acute nicotine treated animals, although no effect is expected as both vehicle and chronic nicotine treatments did not affect dopamine D_1 receptor binding (Table 6-2).

In this study SKF-38393 and SKF-82958 were considered as selective dopamine D_1 receptor agonists and SCH-23390 as a selective dopamine D_1 receptor antagonist (O'Boyle and Waddington, 1984). [^{3}H]-SCH-23390, a selective antagonist of dopamine D_1-like receptors, is the prototypical ligand to determine dopamine D_1 receptor binding parameters (Faedda *et al.*, 1989), however also has affinity for the serotonin 5-HT_2 receptor (see General Discussion).

The absence of high and low affinity binding sites in competition with dopamine might be an experimental artefact due to fast degradation of dopamine by oxidation. This degradation could be prevented by the presence of an anti-oxidant (*e.g.* ascorbic acid or vitamine C, cystein and formic acid, etc.). In the present study no ascorbic acid was used as this was reported to compete with dopamine for dopaminergic receptors (Tolbert *et al.*, 1979; 1992; Wiener et al, 1989). Additionally, it has been shown that treatment of membranes with ascorbic acid could distinguish and visualise the high and low affinity binding sites of dopamine, but not SKF-38393, when competing with [^{125}I]-SCH-23982 (Kimura and Sidhu, 1994). The increase and decrease of high and low affinity binding sites, respectively, is suggested to be the result of the anti-oxidant properties of ascorbic acid preventing the oxidation of dopamine in the *in vitro* system. This was also supported by the finding that ascorbic acid induced inhibition of

dopamine D_1 antagonist binding which was prevented by addition of EDTA through an unknown mechanism (Kimura and Sidhu, 1994).

A review of the literature suggests that the high and low binding sites can only be determined in specific brain areas (or under certain incubation conditions) suggesting a specific role for these binding sites. For example, [^{3}H]-SCH-23390 can distinguish between the two affinity binding sites in the hippocampus and amygdale but only in the absence of $MgCl_2$. Whereas Mg^{2+} does not have any effect in striatum, where only 1 affinity site can be detected (Leonard *et al.*, 2006). Furthermore, it has been shown that [^{3}H]-SCH-23390 can have preferential effects between different brain areas and even within brain structures like striatum (*i.e.* different effects between dorsal and ventral striatum, Savasta *et al.*, 1986). Therefore further studies should include different brain areas and using more advanced techniques look at the distribution of binding sites within the dorsal striatum.

The high affinity state of the dopamine D_2 receptor is postulated to be functionally responsible for its signal transduction. At present, only a few experiments have been carried out to selectively measure dopamine D_2^{High} affinity states in humans and were limited by using [^{11}C]-(+)-PHNO, as no other useful *in vivo* method exists to selectively measure dopamine D_2^{High} affinity states in humans (Willeit *et al.*, 2006). However, following a dual-radiotracer experiment with [^{11}C]-(+)-PHNO and [^{3}H]-raclopride, which label the high affinity state and both affinity states respectively, there was no proof of a distinct high affinity state measured *in vivo* (McCormick *et al.*, 2008). This cast some doubt on the *in vivo* applicability of the dopamine D_2 two-state model, as described by *in vitro* binding experiments.
Numerous reports have been published concerning high and low affinity binding sites, although their occurance in dopaminergic receptors is still susceptible. For example, the homogenate as used by Seeman and colleagues was not washed, centrifuged or pre-incubated (Seeman *et al.*, 1984; Seeman *et al.*, 2007), which can be argued as a crude preparation where non-specific binding can explain the two binding sites.

Another receptor binding parameter which might be important in behavioural sensitisation is the ratio of dopamine D_1 receptor versus D_2 receptor signalling. It is suggested that changes in the ratio of $D_1R:D_2R$ could contribute to psychostimulant induced behavioural plasticity (Thompson *et al.*, 2010).
To study the effect of behavioural sensitisation on dopamine D_2 receptor binding, a receptor binding protocol using [^{3}H]-domperidone was tested. This specific dopamine D_2 receptor radiolabel was chosen as it is reported to distinguish between the dopamine D_2 receptor high and low affinity binding states (Seeman *et al.*, 2003). Although the assay was performed exactly according to the protocol as described by Seeman and colleagues (*e.g.* Seeman et al., 2002; 2007; 2009), no high affinity binding site could be observed (data not shown). Therefore, only dopamine D_1 receptor binding using [^{3}H]-SCH-23390 was investigated. Another key note to be made is, although specific ligands bind to the specific dopamine receptor subtypes not every compound is suitable for performing a receptor binding assay. For example, quinpirole is a prototypical selective dopamine D_2-like receptor agonist. Quinpirole has a 250-fold selectivity for dopamine D_2-like receptors over dopamine D_1-like receptors (Seeman and Schaus, 1991), however due to its high dissociation constant of *ca.* 5 nM it is vulnerable to inhibition by endogenous dopamine. Moreover, radiolabelled quinpirole showed high non-specific binding, indicating that this compound binds to other unidentified binding sites as well. Other radiolabels which have been used to selectively label the dopamine D_2 receptor include [^{3}H]-NPA (agonist, only high affinity state), [^{3}H]-spiperone (antagonist, high and low), [^{3}H]-raclopride (antagonist, high and low), [^{3}H]-domperidone (antagonist, only high), and [^{3}H]-PHNO (agonist, only high). However, their commercial availability is often limited. [^{3}H]-domperidone is found the best label to measure dopamine D_2 receptor binding and determine high and low affinity binding sites. However, the present research was limited by patent issues of [^{3}H]-domperidone for this type of research (Seeman, 2005). Additionally, preliminary experiments of the present study showed very low specific binding and high variation when using [^{3}H]-domperidone (data not shown), and therefore these experiments were discontinued.

Conclusion

The high and low affinity binding site hypothesis it is a very interesting theory with potential to better understand the receptor binding kinetics of a ligand, although, many attempts, including this study, failed to visualise the high and low affinity binding sites. Nicotine induced behavioural sensitisation does not affect the affinity of the tested dopaminergic ligands for the dopamine D_1 receptor. However, functional studies have now shown that behavioural sensitisation only has an effect on tissue taken 45 min post-challenge and further binding studies should include this additional time point.

Chapter 7. General Discussion

Abstract.
OBJECTIVES: The present study focussed on the behavioural, neurochemical and receptor alterations of the dopaminergic system as a consequence of nicotine induced behavioural sensitisation to gain a better understanding of its neurobiology. Herein it is hypothesised that understanding the mechanism underlying the phenomenon behavioural sensitisation can help to elucidate the responsible mechanism(s) underlying different neuropsychiatric disorders, and eventually could lead to improved (pharmaco) therapies. The present study investigated the working hypothesis that the dopamine receptor, specifically the dopamine D_1 receptor subtype, plays an important role in the mechanism underlying behavioural sensitisation.
CONCLUSIONS: The present study showed that selective antagonism of the dopamine D_1 receptor blocks the expression of nicotine induced behavioural sensitisation *in vivo*. From a mechanistic perspective, the results suggest that sensitisation induced by chronic nicotine administration occur as a result of neuroadaptations, *i.e.* the enhanced response of neurones in the dorsal striatum to dopamine D_1 receptor activation. Information from genetics, neuropathology, brain imaging, and basic neuroscience has provided insights into understanding the aetiology of neuropsychiatric disorders, however remains still not fully understood. This thesis presents a successful multidisciplinary approach which resulted in more information about the mechanism underlying behavioural sensitisation and showed an important role for the dopamine D_1 receptor. Furthermore, the described approach provides a successful framework to study behavioural sensitisation which also can be used for future studies and ultimately the elucidation of the mechanism(s) underlying neuropsychiatric disorders.

Keywords: cAMP, LC-MS/MS, nicotine, behavioural sensitisation, dopamine, release, voltammetry, receptor binding

7.1 DISCUSSION

Basic biomedical research is mainly directed toward the understanding of neurobiological mechanism(s) behind diseases. Better understanding of the neurobiology can improve drug discovery and eventually could lead to improved medications. Herein it is hypothesised that understanding the mechanism underlying the phenomenon behavioural sensitisation can help to elucidate the responsible mechanism(s) underlying many neurpsychiatric disorders. The present study investigated the working hypothesis that the dopamine receptor, specifically the dopamine D_1 recpetor subtype, plays an important role in the mechanism underlying behavioural sensitisation.

Among other reasons, acute animal models do not resemble, nor correlate with the clinical efficacy observe because the clinical symptoms are developed over a longer period of time (*i.e.* years). Therefore this study used a chronic treatment model and examines the expression of behavioural sensitisation. The used dose of nicotine (*i.e.* 0.4 mg/kg, s.c.) was chosen as the representative that was shown to be behaviourally active in rats and produced a maximal response (Clarke and Kumar, 1983; Ksir *et al.*, 1985; Ksir *et al.*, 1987) and in our own laboratory it was observed that higher dose (*i.e.* 0.6 mg/kg, s.c.) resulted in what is believed 'clenching' of the peritoneal musculature (data not shown). Moreover, the 0.4 mg/kg dose is used in other reported nicotine induced behavioural locomotor studies (*e.g.* Domino, 2001) and which therefore can be used for comparison with other literature. The present study showed that behavioural sensitisation to intermittent nicotine administration was developed within 5 days and reaching a plateau (Figure 2-2). This is in agreement with the complex pharmacokinetics of nicotine. Intermittent nicotine administration in the rat (0.03 mg/kg) showed increased brain concentrations of nicotine and its major metabolites (*i.e.* cotinine and nornicotine) by 2-, 12-, and 9- fold, respectively, reaching a plateau following the fifth injection (Ghosheh *et al.*, 2001).

Dopamine D_1-like agonists (*e.g.* A-68930, dihydrexidine) suppress acute locomotor activity and amphetamine induced hyperlocomotor activity, an effect which can be selectively reversed by dopamine D_1-like, but not D_2-like, receptor antagonists (Isacson *et al.*, 2004). This antagonism of hyperactivity produced by

amphetamine is traditionally interpreted as a sign of potential antipsychotic properties suggesting clinical implications for the dopamine D_1 receptor in the treatment of schizophrenia.
This study provides evidence that a dopamine D_1 antagonist can block the expression of nicotine induced behavioural sensitisation. From a perspective of therapeutics, it could imply that the dopamine D_1 receptor might be a valid target for treatment of neuropsychiatric disorders related to the behavioural sensitisation model.

The measurement of cAMP accumulation is a very robust and simple method with a high reproducibility and very usefull screen compounds for a certain receptor regarding their functional effect on a molecular level, altough, the physiological relevance compared to the *in vivo* situation is often very low. Receptor binding and cAMP accumulation as targets to study dopaminergic medications offers only modest predictive validity of *in vitro* to *in vivo* extrapolations. Especially for GPCR targets other than dopamine the predictive validity is very low. Therefore, one should know its limitations and the search for more advanced *in vitro* assays with high predictive validity, in order to predict efficacy *in vivo*, still goes on.

The potency of the dopaminergic ligands was determined by assessing their effect on cAMP accumulation. The agonists did increase cAMP accumulation whereas antagonists had no effect on cAMP accumulation. The potency of the agonists was decreased following chronic nicotine treatment *in vivo*, although examination of the antagonist (in the presence of 1 µM agonist) did not show any effect on potency.
The measured locomotor behaviour related to hyperdopaminergic activity was accompanied by changes in cAMP levels. It has been reported that methamphetamine administration does not change striatal cAMP levels directly, but systemic co-administration of rolipram (a cAMP phosphodiesterase type IV inhibitor) has been shown to antagonise methamphetamine induced behavioural changes and sensitisation (Iyo *et al.* 1995, 1996). This is in agreement with the findings of the present study where no changes on basal cAMP accumulation were observed and changes were only observed following cAMP stimulation by dopamine D_1 receptor agonists.

Dopamine D_1 receptors are generally coupled to Gs and can activate cAMP signalling, however *in vitro* and *ex vivo* studies have shown that they can also affect other pathways. For example the phosphatidyl inositol (IP) cascades which activate protein kinase C (PKC, EC 2.7.11.13, Von Euler and Von Euler, 1991; Undie and Friedman, 1990, 1992; Zhen *et al.*, 2005). Therefore it should be noted that the effects of ligands observed cannot simply be explained by a single pathway or route. Additionally, the results of the present study, together with previous research, suggest an important role of cAMP in behavioural sensitisation. However, cAMP is likely not the only player in the field, and additionally cGMP might be involved as well. The novel LC-MS/MS method presented in Chapter 4, was also validated to measure other nucleotides like cGMP (data not shown). The involvement of the dopamine D_1 receptor in behavioural sensitisation, together with the fact that the dopamine D_1 receptor (but not the D_2 receptor) is able to alter cGMP accumulation, suggests that this is an important area which needs to be further explored.

cAMP and cGMP are very important intracellular second messengers and moreover, they are also important extracellular neurotransmitters. Considering the effect of behavioural sensitisation on their intracellular accumulation, future research should investigate the role of these nucleotides in the extracellular space following behavioural sensitisation. The presently developed LC-MS/MS method and described pharmacological assays provides an excellent framework to asses the extracellular involvement of (cyclic) nucleotides. Preliminary experiments during the present study already identified their extracellular presence (data not shown).

Elucidation of the responsible intracellular pathways between the receptor protein and cAMP accumulation might include research into RGS (regulators of G-protein signalling), G-protein coupling, adenylate cyclase activity, and phosphodiesterases (PDE) activity. In the present study, the effect of basal and adenylate cyclase activation on cAMP accumulation was investigated. It was shown that behavioural sensitisation did not affect basal nor forskolin stimulated cAMP accumulation, suggesting adenylate cyclase was not affected by behavioural sensitisation. Furthermore, the *ex vivo* cAMP accumulation assay was performed in the presence of a high concentration IBMX, a non-selective PDE inhibitor. This

PDE inhibitor prevents the metabolism of cAMP into AMP. Although the present experiment cannot exclude a putative role of PDE in sensitisation, at least it can be concluded that if PDE is involved, it is not the only player.

Another player in the intracellular signalling networks is the accessory protein regulating G protein-coupled receptors (RGS). RGS inhibits GDP dissociation from the Ga subunit in a receptor independent manner, which returns the G-proteins back to their inactive form, and exert regulatory functions on intracellular effectors (Natochin *et al.*, 2000). It has been shown that dopaminergic receptor ligands also can regulate RGS, the study showed that dopamine D_1 receptor ligands (SKF-82958 and SCH-23390) and D_2 receptor ligands (quinpirole and haloperidol) can regulate the five most abundant RGS in the striatum (Taymans *et al.*, 2003). This study also showed a specific link between dopamine D_1-RGS_2-cAMP and between dopamine D_2-RGS_4-cAMP, and proposed a distinct function of RGS in dopaminergic signalling. Little is known about RGS in behavioural sensitisation, although Rahman and colleagues observed that $RGS_{9\text{-}2}$ was increased following chronic cocaine administration (Rahman *et al.*, 2003) and Schwendt and McGinty showed that RGS_4 was decreased in rat dorsal striatum and nucleus accumbens following the expression of amphetamine induced behavioural sensitisation (Schwendt and McGinty, 2007). Targeting RGS may introduce new possibilities of fine tuning the effects of GPCR activation by, for example, dopaminergic receptor ligands, although, future studies are required.

Furthermore, in addition to RGS, the identification of activator of G protein signalling (AGS) proteins as receptor-independent activators of G-protein signalling reveals unexpected mechanisms for the regulation of heterotrimeric G-protein activation and has opened up new areas of research related to the role of G proteins as signal transducers (Blumer *et al.*, 2005). Activator of G protein signalling 3 (AGS3) has been shown to play a putative role in the expression of cocaine induced sensitisation (Bowers *et al.*, 2004).

The affinity of the ligands was measured by radioligand (antagonist) binding. The agonists as well as antagonists both inhibited [^{3}H]-antagonist binding. No change in potency of either agonist or antagonist was observed following chronic nicotine treatment *in vivo* (Chapter 6). On a molecular receptor level, the effect of an antagonist in the presence of 1 µM agonist is the result of a competition between these two ligands for a specific binding-pocket. The use of an agonist radioligand

instead of an antagonist radioligand could provide additional information about the putative changes in receptor conformation following behavioural sensitisation.

The lack of effect on receptor binding following behavioural sensitisation, together with the data showing changes in cAMP accumulation, could suggests that behavioural sensitisation impaires specifically the intracellular functionality. However, as mentioned before, the lack of effect on the receptor binding could be due to the fact that only tissue taken 24 h post-challenge was used and not tissue taken 45 min post-challenge. However, it appears that changes in the dopamine D_1 receptor mediated signal transduction can occur in the presence or absence of changes in dopamine receptor number (Unterwald *et al.*, 1994).

It has been reported that dopamine D_2 agonists alone do not block or reverse behavioural sensitisation (Li *et al.*, 2000). In this same study, Li and colleagues suggest that a combination of a dopamine D_2 agonist with a NMDA antagonist is sufficient. However, dopamine D_2 partial agonists have been shown to block the expression of nicotine induced sensitisation (McCreary *et al.*, unpublished data). Partial agonists are drugs that bind and fully occupy the receptor, yet have sub-maximal capacity to activate its associated functional signal transduction mechanisms. By binding to dopamine receptors, the partial agonist will modulate dopamine function by acting as an antagonist preventing binding of endogenous dopamine. But, in comparison to full antagonists, will not completely block dopamine transmission. It should be noted that considering the overall binding-affinity profile of current available ‘selective’ dopamine D_2 partial agonists, activation or blockade of other receptors could account for the observed effects as well. It could be proposed that strong activation of the dopamine D_2 receptor is not sufficient, but at the same time full receptor occupation is required to achieve maximal efficacy. Furthermore, major drawbacks of using dopamine agonists are the risk of overdose effects such as respiratory depression and overstimulation of the mesolimbic reinforcement system (or reward pathway), which is the prime driver behind eurphoria, craving and loss of control seen in addiction. The partial agonist thus decreases the unwanted symptoms of withdrawal, euphoria and respiratory depression.

An analogous hypothesis is the potential for compounds which possess the right dopamine D_1/D_2 receptor affinity ratio. This bi-directional control of

neuroadaptation might be crucial for potential drug therapies for neuropsychiatric disorders. Maggio and colleagues hypothesis that the antipsychotic actions of "partial agonists" may not reflect inefficient stimulation of dopamine D_2 and/or D_3 receptors, but block D_2/D_3 receptorhetrodimers that are "weakly" coupled to transduction mechanisms of the dopaminergic pathway (Maggio *et al.*, 2008).

This thesis focussed on the dopamine D_1 receptor, although there is evidence to suggest that other receptor subtypes, for example the dopamine D_2 receptor, also do play a role in behavioural sensitisation. An increasing body of evidence suggests that the dopamine D_1 and D_2 receptor families interact functionally in ways that may be of potential importance in the pharmacotherapy of neuropsychiatric disorders (Braun *et al.*, 1997 and references herein; for review see Pollack, 2004).
The dopamine D_1 and D_2 receptor are colocalised on the striatal neurones and where they both regulate cAMP accumulation (Stoof and Verheijden, 1986; Kelly and Nahorski, 1987a). Dopamine D_1-like and D_2-like receptors have been defined traditionally by their opposing effects on adenylate cyclase (Stoof and Kebabian, 1981), although, their effect on signalling systems other than adenylate cyclase have been confirmed, including voltage-activated calcium channels, potassium channels, and phosphatidylinositol hydrolysis (Jaber *et al.*, 1996). It has been shown that dopamine D_1 and D_2 receptor co-activation can activate a novel Phospholipase C (PLC) -mediated pathway (Lee *et al.*, 2004; Pollack, 2004), possibly required for heterooligomer formation (George and O'Dowd, 2007; Hasbi *et al.*, 2010). Many effects resulting from dopamine D_2 receptor stimulation are manifest only when the dopamine D_1 receptor is stimulated. Dopamine D_1 receptor stimulation by administration of a selective D_1 receptor agonists (*e.g.* SKF-38393) significantly increased the functional effects of selective dopamine D_2 agonists (*e.g.* quinpirole), as shown by electrophysiological and behavioural (stereotypy) effects (Hu and White, 1994). These qualitative and quantitative forms of dopamine D_1 and D_2 receptor synergism are abolished by chronic dopamine depletion which is suggested a result of supersensitivity of either D_1 or D_2 receptors, or the combination of both. This heterologous regulation of dopamine D_2 receptor sensitivity by dopamine D_1 receptors may be related to uncoupling of functional D_1 and D_2 synergism. For example, cocaine self administration is also suggested to be result of specific differential alterations in

functional dopamine D_1 and D_2 receptors, and their ability to modulate cocaine behaviour (Edwards *et al.*, 2007).
It could be proposed that behavioural sensitisation is a result of impaired dopamine D_1 and D_2 receptor synergism in the dorsal striatum, which is hypothesised to be a functional filter for neurotransmitter signalling. Therefore, further studies should include the examination of compounds having different affinity combinations for dopamine D_1 and D_2 receptor on functional activity, for example, determining cAMP accumulation or dopamine release. In addition to the example of synergism between dopamine receptors, many other receptors are suggested to play a role in behavioural sensitisation and could putatively depend on the dopamine D_1 receptor activity.

Three important aspects involved in the pathophysiology of neuropsychiatric disorders have been proposed. The first is cell migration, the second is unbalanced excitatory-inhibitory networks, and the third is synapse formation and pruning. This all can be shorten to synaptic plasticity. Synaptic plasticity, the ability of a synapse to change in strength, can be intrinsic (*i.e.* as a result of its own activity) or extrinsic (*i.e.* induced by activity in another pathway). Mechanisms underlying synaptic plasticity include changes in neurotransmitter release and the efficacy of cells responding to these neurotransmitters. Behavioural sensitisation is putatively a result of synaptic plasticity as differences have been observed between nicotine induced sensitisation in adolescent (still developing) and adult rats (Schochet *et al.*, 2004; Belluzzi *et al.*, 2004). Long-term potentiation (LTP) is a persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse. Studies of LTP are often carried out in slices of the hippocampus, an important organ for learning and memory. Previous literature suggests that this form of synaptic plasticity is facilitated by D_1/D_5 dopamine receptors (*e.g.* Huang and Kandel, 1995; Otmakhova and Lisman, 1996). Activation of phosphatases is a key reaction in both long-term depression (LTD) and depotentiation (*i.e.* the reversal of LTP) (Mulkey *et al.*, 1994; O'Dell and Kandel, 1994; Staubli and Cheun, 1996), and there is a well established pathway by which cAMP activates PKA and subsequently inhibit the activity of protein-phosphatase-1 (PP1). This suggests that dopamine, which can modulate the cAMP pathway, could affect the storage of information that arrives before dopamine (Otmakhova and Lisman, 1998).

It was shown that dopamine D_1 receptor agonists increased cAMP accumulation in a concentration response manner. Moreover, in addition to this concentration dependent effect, a time-dependent effect was observed depending on the time of tissue preparation post-challenge, *i.e.* only an effect was seen after 45 min but not after 24 h post-challenge. This is in agreement with nicotine impaired long-term potentiation of GABAergic synapses (Niehaus *et al.*, 2010).

The pharmacological tools used in the present studies have been widely used to characterise the functional role of the dopamine D_1 receptor, both *in vitro* as well as *in vivo* paradigms (O'Boyle *et al.*, 1989; Gessa *et al.*, 1991; Lewis *et al.*, 1998). The used prototypical compounds might not be superior to other current available ligands, although, these compounds can easily be compared to the ones used in the existing literature. In addition to their selectivity for receptor subtypes, agonists can also be selective for different intracellular signalling pathways coupled to a single receptor subtype. This process is know as 'functional selectivity' (Berg and Clarke, 2006; Urban *et al.*, 2007) and should be taken in consideration when interpretating results from *in vitro* functional data.
In the literature, SKF-38393 is often referred to as a partial agonist for the dopamine D_1 receptor. However, signal amplification of second messengers (like adenylate cyclase) could also be responsible for the observation of partial agonism. It is previously reported that greater receptor occupancy is necessary to achieve half-maximal activation of G proteins compared to that of downstream effectors, such as described for the adenosine receptor A_{2C}-Gi-adenylyl cyclase and the serotonin receptor 5-HT_{2C}-Gq- phospholipase C signalling pathways (in CHO cells, Umland *et al.*, 2001; Cussac *et al.*, 2002a,b). Taken together, these results suggest that SKF-38393 could probably exhibit full agonist properties at the level of adenylyl cyclase in our cell (tissue) system.
As the adenylate cyclase isozymes differ in the parameters that define their stimulation and inhibition characteristics, and as various tissues, brain areas, and cell types are known to vary in their repertoire of adenylate cyclase isozyme populations (Mons and Cooper, 1995), it is likely that dopamine could have different regulatory effects on adenylate cyclase activity depending on the tissues examined.

As previously discussed in Chapter 2, SCH-23390 is generally used as a prototypical 'selective' dopamine D_1 receptor antagonist. However, it also has high affinity for the serotonin $5HT_2$ receptor and was found agonist using [^{3}H]-phophotidylinositol (PI) accumulation (K_i 9.3 nM, EC_{50} 2.6 nM, Millan *et al.*, 2001). The serotonin $5HT_2$ receptor is functionally coupled to PLC and will results in diacylglycerol activation, arachidonic acid and inositol synthesis, but not directly to cAMP synthesis. In consensus with previous literature, the serotonin $5HT_2$ agonist DOI (2,5-dimethoxy-(4-chlorophenyl)-isopropylamine) was tested in the present study and had no effect on cAMP accumulation in the striatum (data not shown). Therefore present study is not able to make conclusions about the involvement of the serotonin $5HT_2$ receptor, however showed that SCH-23390 is a dopamine D_1 antagonist and that the potency of dopamine D_1 agonists is altered after nicotine sensitisation. This suggests a role for the dopamine D_1 receptor in behavioural sensitisation. Nonetheless, this selectivity fact cannot be neglected, as activation of serotonin $5HT_2$ receptors by SCH-23390 may contribute to its functional properties both in animals and in humans, and therefore need further research.

Nicotine induced behavioural sensitisation is a complex phenomenon which occurs as a consequence of neuroadaptive changes in a number of brain systems. Historically, research into behavioural sensitisation was focused solely on adaptive changes in dopamine and dopamine receptor signalling. Recent literature showed that the behavioural effects of cocaine are a result of the impact of cocaine on many different neural receptor systems (*i.e.* opiodergic, GABAergic, serotonigeric, glutamatergic). Furthermore, evidence suggests that the cocaine induced changes in behaviour also result from the cocaine's impact on many ubiquitous intracellular neuronal molecules.

The present study focused on the dopaminergic system and showed that dopamine receptors play an important role in behavioural sensitisation. However, additional systems might be necessary to support the dopamine system in its role. In particular, but not limited to, the serotonergic system is involved in dopaminergic neurotransmission. For example in frontal cortical regions were less dopamine transporters are available or in striatal areas were less dopaminergic neurones are present as a result of neuropathological disorder (*e.g.*

Parkinson's disease), the serotoneric and noradrenergic systems play a key regulatory role in dopaminergic neurotransmission.
Activation of NMDA receptors, localised on dopaminergic cell bodies in the ventral tegmental area, may play an important role in the development of behavioural sensitisation (Karler *et al.* 1991; Wolf *et al.* 1994; Kalivas, 1995; Cador *et al.* 1999). The behavioural changes induced by amphetamine and its development of behavioural sensitisation mainly occur via pre and/or post-synaptic dopaminergic systems (Robinson and Becker 1986; Kalivas and Stewart 1991; Wolf *et al.* 1994). FK506 is a potent calcineurin (Proteinphosphatase2B, PP2B) inhibitor and acts on these dopaminergic systems. It can enhance dopamine release by inactivating calcineurin, which inhibits dopamine release at presynaptic sites by inactivating synapsin I (Iwata *et al.* 1997; Snyder *et al.* 1998). Moreover, it was shown that FK506 attenuated the development of methamphetamine induced sensitisation (Tsukamoto, 2001). Additionally, chronic nicotine administration produced functional upregulation of ionotropic glutamate receptors mediating striatal dopamine release (Risso *et al.*, 2004).

The present findings that SCH-23390 shows a differential effect on nicotine induced dopamine release (Figure 3-11) correlates with the *in vivo* data showing SCH-23390 blocks nicotine induced behavioural sensitisation (Figure 2-6). *In vivo* voltammetry and microdialysis are two complementary methods, and both methods can even be combined in the same animal (Yang and Michael, 2007). However, in some cases *in vivo* voltammetry is the only or at least the best option to answer the questions related to the effects of drugs or their mechanisms of action. Furthermore, the combination of simultaneous *in vivo* voltammetry with locomotor activity measurements to study behavioural sensitisation can provide more information about the real-time activation pattern of the different neurotransmitters and in different brain areas during the different stages of behavioural sensitisation (*i.e.* development, withdrawal and expression).

The FCV technique also has been proven successfully in a wide range of species, *e.g.* rats, mice, guinea pigs, (for review see Michael and Borland, 2007), zebrafish (Njagi *et al.*, 2010), and humans (Paul Garris *et al.*, unpublished data). It could therefore also be applied to investigate the effect of dopamine following behavioural sensitisation in one of these species which will give more insight in

the variety and differences in behavioural sensitisation and role of dopamine between species. Also a correlation can be made between the role of dopamine in preclinical (*i.e.* animal) and clinical (*i.e.* human) sensitisation.
However not described in this thesis, in parallel a novel FCV system (PGSTAT) was developed by the author of this thesis in collaboration with EcoChemie B.V. (Utrecht, the Netherlands). Recently, the development and validation of the PGSTAT within Abbott was finished (manuscript in preparation). Details of this system will not be discussed here, however it is noteworthy that the novel PGSTAT system has many advantages compared to the currently available systems. Therefore, future pharmacological experiments as suggested in this thesis should be considered to be performed using this new system.

Methodological considerations
In vivo locomotor activity showed that there is a certain natural variation within the same treatment group. As seen in *ex vivo* cAMP experiments, it was impossible to use only a single rat for an $n = 1$ *ex vivo* experiment. To overcome this issue, the dissected brain areas from at least four animals within the same treatment group were pooled. However, using *ex vivo* FCV this is not possible as only a single brain slice from one animal can be measured at the time. One solution to overcome this issue is to challenge and measure locomotor activity of one animal at the time.

Herein it is hypothesised that the dorsal striatum is a functional, multidimentional, frequency-dependent, signalling filter which, in the case of a disordered system (*e.g.* a neuropsychiatric disorder or behavioural sensitisation), becomes imbalanced. Secondly, it is hypothesised that the dopaminergic system is necessary in behavioural sensitisation, and moreover, that its mechanism is not limited to a specific dopamine receptor subtype but underlies receptor synergy (*e.g.* dopamine D_1/D_2 or D_1/D_3 combinations). Thirdly, behavioural sensitisation affects the frequency-dependent filtering of the striatum, resulting in a dopamine release deficiency and receptor synergism.
One challenge of investigating behavioural sensitisation in humans is the very difficult diagnosis of (early) development of behavioural sensitisation in the clinic, as the symptoms are just to become visual when sensitisation is already developed. To date, there are no biomarkers for the development of behavioural

sensitisation available yet. Therefore we are currently limited to treatment of symptoms and therefore this study focused on the expression of sensitisation.
Recent evidence suggests that the cognitive symptoms of nicotine withdrawal and the cognitive symptoms of attention deficit hyperactivity disorder (ADHD) may share neural correlates. One could suggest that therapeutics ameliorates ADHD symptoms may also ameliorate nicotine induced withdrawal symptoms. This hypothesis suggests they share a common mechanism, putatively behavioural sensitisation. It is suggested that behavioural sensitisation might be the trigger of the hypofunctionality of the mesolimbic dopamine system (ventral tegmental area → nucleus accumbens) following chronic psychostimulant administration and can be used as a model for (drug) addiction. A better understanding of behavioural sensitisation could then improve anti-addiction therapies and improve drug discovery by excluding addictive properties of new medications. Moreover, investigating changes that persist after drug discontinuation could be fundamental to understand the mechanisms underlying craving, relapse and withdrawal symptomes.
Preclinical data suggested a putative role for dopamine D_1 receptor mediated medication, however, when considering potential dopaminergic drug therapies for neuropsychiatric disorders a major concern is the liability for abuse of these pharmacotherapies by themselves. Besides that behavioural sensitisation can help to understand several disorders, it also could improve current medications. For example, the current treatment of Parkinson's disease is based on chronic L-DOPA administration. It is known that after a few years the L-DOPA will induce dyskinesia. In rodents, chronic L-DOPA treatment has been shown to induce behavioural sensitisation (*e.g.* Kalda *et al.*, 2009) and affects the cAMP pathway by increasing cyclic AMP response element-binding (CREB) protein phosphorylation in striatal neurones (Cole *et al.*, 1994; Oh *et al.*, 2003). By understanding the mechanism underlying behavioural sensitisation, also currently used pharmacotherapies can be improved and negative side effect can be excluded.
It is hypothesised that sensitisation to life events is the core feature of depressive disorders (Patten, 2008; Myin-Germeys *et al.*, 2005). This could be explained by the phenomenon of cross-sensitisation. Previous literature showed that cross-sensitisation can occur when a challenge is give by a compound different to the compound by which the sensitisation was developed, *i.e.* other factors can trigger

the 'disorder'. This is also suggested in case of life events triggering depression and therefore, it is extremely difficult to target the original cause(s).

Neuropsychiatric disorders like schizophrenia, psychosis, depressive disorders, and bipolar disorders are major health and socioeconomic problems. Despite the extensive research and drug discovery efforts currently available (pharmaco-) therapies offer only modest efficacy. The main difficulty challenging drug discovery is the unknown mechanism(s) responsible for these neuropsychiatric disorders. Psychostimulants, for example amphetamine, cocaine and methylphenidate, are widely used as pharmacotherapies in medical treatments and abused as recreational drugs. The chronic administration of these drugs are known to cause addiction, psychosis and other neuropsychiatric disorders, as will be described in more detail below. Moreover, many of the currently used medicines also induce behavioural sensitisation and the development of severe side effects including addiction. However, these medicines are still in use as no better medication is available at this time. A few examples of these prescript clinical medicines are amphetamine for the treatment of attention deficit hyperactivity disorder or ADHD, traumatic brain injury, and narcolepsy, methylphenidate (Ritalin) for the treatment of ADHD, morphine as an analgesic, methamphetamine for the treatment of obesity and depression. Moreover, chronic treatment with for example, classical antipsychotic agents (*i.e.* dopamine receptor antagonists, used to treat diseases like schizophrenia and Parkinson's) has been associated with the undesirable complication of tardive dyskinesia (an involuntary hyperkinetic syndrome) and anhedonia (a mood disorder like depression) and withdrawal from the treatment often results in incurring relapse psychosis.

The many research efforts over the last few decades to elucidate the mechanisms underlying neuropsychiatric disorders are pointing towards a common mechanism called behavioural sensitisation. Behavioural sensitisation is hypothesised to underlie many neuropsychiatric disorders including, but not limited to, Pakinson's disease (PD), schizophrenia, psychosis and mania (Segal *et al.*, 1981; Fibiger, 1991; Post, 1992), stimulant induced psychosis (Angrist, 1983; Tadokoro and Kuribara, 1986), dyskinesia, attention deficit hyperactivity disorder (ADHD), obsessive compulsory disorder (OCD: Szechtman *et al.*, 2001), bipolar disorders (Post, 1992), depressive disorders (Patten, 2008), (emotional-)

pain, and drug addiction (Robinson and Berridge, 1993). While behavioural sensitisation is of great interest, its mechanism of action is not yet fully understood.

7.2 CONCLUSION

Information from genetics, neuropathology, brain imaging, and basic neuroscience has provided insights into understanding the aetiology of neuropsychiatric disorders, however remains still not fully understood. After the discussion of the neurochemical, behavioural and clinical perspectives the overall conclusion suggests a putative common mechanism with behavioural sensitisation. The integration of methodologies from multiple disciplines such as behavioural neuroscience, molecular pharmacology, and neurochemistry, enables a powerful approach for investigating complex neuropsychiatric disorders. This thesis presents a successful multidisciplinary approach which resulted in more information about the mechanism underlying behavioural sensitisation and showed an important role for the dopamine D_1 receptor. Furthermore, the described approach provides a successful framework to study behavioural sensitisation which also can be used for future studies and ultimately the elucidation of the mechanism(s) underlying neuropsychiatric disorders.

The data presented here provides biochemical evidence that dopaminergic receptors exist on cells in the dorsal striatum that possess receptors for neuromodulators known to be important in regulating basal ganglia activity, as inducing and antagonising locomotor activity sensitisation. This information from behavioural and subsequent *ex vivo* biochemical studies, combined with clinical pharmacological studies might ultimately be useful in describing a role for dopaminergic neurones in behavioural sensitisation and their relation to disease states resulting from basal ganglia dysfunction. To the extent that behavioural sensitisation reflects underlying neuroadaptations that are related to neuropsychiatric disorders, present findings suggest that pharmacotherapies using dopamine D_1 ligands could be effective in the treatment of behavioural sensitisation related diseases as, for example, psychosis, bipolar diseases and schizophrenia.

In conclusion, the present results suggest that selective antagonism of dopamine D_1 receptors can block the expression of nicotine induced behavioural sensitisation. From a mechanistic perspective, the results of the present study suggest that sensitisation induced by chronic nicotine administration occurred as a result of neuroadaptations, the enhanced response of neurones in the dorsal striatum to dopamine D_1 receptor activation. This suggests that the mechanism involved in the expression of behavioural sensitisation to nicotine is the same to those involved in amphetamine or cocaine induced behavioural sensitisation. However, additional studies will be required to examine other putative neuroadaptations and elucidate the exact mechanism. The results of the present study, along with previous literature, suggest that a dopamine D_1 ligand pharmacotherapy has potential utility in the treatment of neuropsychiatric disorders. Although the exact mechanism is still not fully understood, the accumulating findings will elucidate the exact molecular mechanism of behavioural sensitisation.

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