Molecular and pharmacological studies into a mouse model (*Peromyscus maniculatus bairdii*) of obsessive compulsive disorder

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Abstract

There have been various attempts to develop animal models of obsessive compulsive disorder (OCD) in the hope that they may provide a means to better understand and treat this disorder. Given that the aetiology of OCD most likely involves the interaction of multiple genetic and environmental factors, animal models in which the behavioural pathology develops spontaneously may be particularly useful. With this in mind, the present study has set out to evaluate the predictive, construct and face validity of naturalistic stereotypic behaviour in the deer mouse (*Peromyscus maniculatus bairdii*). The diversity of stereotypy in deer mice, and its separation into different degrees of stereotypy in different animals, was validated by comparison to C57Bl mice, an animal not known to engage in stereotypy under standard laboratory conditions.

Given the selective response of OCD to serotonin reuptake inhibitors (SRIs) compared to noradrenaline reuptake inhibitors (NRIs), behavioural response to chronic administration of a SRI and a NRI was investigated. Validation studies were initiated by comparing untreated stereotypic deer mice to non-stereotypic C57Bl mice. Deer mice engaged in clear, stereotypic behavioural patterns such as backward somersaulting, repetitive jumping, and patterned running. Deer mice executed stereotypic behaviour to different extents within the population and could be classified as low stereotypic (LSB), high stereotypic (HSB), or non-stereotypic mice. C57Bl did not perform any stereotypic behaviour and comparison of the two mouse strains not only highlights the stereotypic behaviour of deer mice, but also contributes to the face validity of the model.

Given the dose-specific therapeutic effects of SRI's in OCD, predictive validity was evaluated by administering fluoxetine and desipramine to stereotypic deer mice at either low (10 mg/kg) or high (20 mg/kg) doses for 21 days. In view of the important role of serotonin (5-HT) and dopamine in the neurobiology of OCD, the response to 5-HT and dopamine receptor agonists, and whether the resulting behaviours can be modified by chronic fluoxetine or desipramine treatment was also studied. Subacute challenge studies with the 5-$HT_{2A/C}$ agonist, *meta*-chlorophenylpiperazin (mCPP) and the D_2 agonist, quinpirole (QNP) were administered at doses of 2 mg/kg and 5 mg/kg respectively for 4 days with and without high-dose (20mg/kg) SRI or NRI treatment. High and low dose fluoxetine, but not desipramine, significantly reduced stereotypic behaviour compared to

vehicle-treated animals in stereotypy animals, providing predictive validity. Subacute mCPP and QNP challenges evoked significant suppression of stereotypic behaviours, while high dose fluoxetine, but not desipramine, reversed the suppressive effects of mCPP and QNP in LSB and HSB mice. This distinct involvement of dopamine D_2 and 5-HT_{2A} and 5-HT_{2C} receptors in stereotypic behaviour in deer mice confers noteworthy construct validity to the model.

OCD has been associated with disturbances in signalling of the cyclic adenosine 3', 5'-cyclic monophosphate (cAMP) cascade. To this end, molecular data collected in the study have focussed on striatal and prefrontal cortex measurement of cAMP and the expression of phosphodiesterase 4 (PDE4) enzymes. Importantly, the degree of stereotypy could be linked to cAMP under basal conditions. High stereotypic mice had the highest cAMP levels compared to low and non-stereotypic and C57Bl mice. High stereotypy was also characterised by low PDE4 activity. Analysis of protein expression found an increase in PDE 4A1, 4A5, 4A8, and 4D1 in the prefrontal cortex of HSB mice as well as the striatum of LSB mice. The negative correlation between PDE4 and cAMP levels provides definitive confirmation for altered states of the cAMP signalling cascade in the cortico-striatal-thalamic-cortico (CSTC) circuit of these animals.

Differences in regional expression of PDEs are explained by the need of each region to express its own compliment of functionally relevant PDE4 isoforms. Darpp-32 is an important protein and marker for activity along the D_1/D_2 receptor pathway. The study has described the differential regulation of Darpp-32 phosphorylation on Thr34 and Thr75 and suggests an imbalance along the D_1/D_2 pathway in the CSTC circuit of deer mice. Construct validity of the model was explored by examining distinct molecular parameters linked to drug treatment. Chronic drug treatment and subacute challenges noticeably highlight the disparity between LSB and HSB mice. This discrepancy is seen at the molecular levels in HSB mice where the cAMP pathway is down-regulated following drug treatment or challenge. Accordingly, the severity of pre-existing behavioural and/or neurochemical abnormalities is not only a factor to be utilized for behavioural classification, but is also the basis for differential molecular responses. In conclusion, the present study provide behavioural and pharmacological evidence that spontaneous stereotypic behaviour in the deer mice presents with significant face, predictive and construct validity as an animal model of OCD.

Opsomming

Die afgelope paar dekades is gekenmerk deur die ontwikkeling van talle diermodelle vir obsessiewe-kompulsiewe steuring (OCD) met die doel om hierdie angssteurnis beter te verstaan asook om beter behandeling strategieë te ontwikkel. Aangesien die etiologie van OCD heel waarskynlik 'n breë interaksie tussen omgewings- en genetiese faktore is, sal 'n diermodel waarvan die gedrags afwyking spontaan ontstaan en wat naturalisties van aard is, baie waardevol wees. Die huidige studie het dit ten doel gestel om die voorspelbaarheid, fenomelogiese en konstruk (eng: construct) grondheid van spontane stereotipiese gedrag onder deer muise (*Peromyscus maniculatus bairdii*) te ondersoek en om dit sodoende as diermodel vir OCD voor te hou.

Gegewe die selektiewe en gunstige reaksie van OCD pasiënte teenoor selektiewe serotonien (5-HT) heropname inhibeerders (SRIs) in vergelyking met noradrenergiese heropname inhibeerders (NRIs), is daar besluit om ondersoek in te stel na gedrags veranderinge in deer muise deur gebruik te maak van kroniese toediening van fluoxetine (SRI) en desipramine (NRI). Die studie is van stapel gestuur deur onbehandelde, stereotipiese deer muise op gedrags sowel as molekulêre vlak te vergelyk met 'n nie-stereotiperende variant, naamlik C57Bl muise. Die mees algemene stereotipiese gedragspatrone wat deur deer muise uitgevoer word sluit in bolmakiesie spronge, vertikale spronge asook die uitvoer van herhalende hardloop-patrone (eng: pattern running). Hierdie stereotipiese gedrag word teen verskillende snelhede uitgevoer wat dit moonlik maak om deer muise te klassifiseer as sogenaamde lae stereotipiese (LSB), hoë stereotipiese (HSB) of nie-stereotipiese muise (NS). Alhoewel C57Bl bekend is daarvoor dat dit 'n aktiewe muis is met hoë lokomotoriese aktiwiteit, voer hierdie variant nie die kenmerkende stereotipiese gedrag van deer muise uit nie. Vergelyking van die twee variante dui daarop dat stereotipiese gedrag soos dit onder deer muise voorkom, maklik gemeet kan word en dat dit bydra tot die fenomelogiese grondheid van die model.

Die voorspelbare gegrondheid, of te wel, farmakologiese isomorfisme van die model is ondersoek deur stereotipiese muise vir 21 dae met 'n lae (10mg/kg) of hoë (20 mg/kg) dosis fluoxetine of desipramine te behandel. Subakute behandeling van stereotiperende deer muise met die 5-$HT_{2A/C}$ agonis, *meta*-chlorophenylpiperazin (mCPP, 2 mg/kg), of met die dopamien D_2 agonis, quinpirole (QNP, 5 mg/kg) het oor 4 dae gestrek, met of

sonder SRI of NRI behandeling. 20 mg/kg Fluoxetine, en nie desipramine, het stereotipiese gedrag betekenisvol verlaag in beide LSB en HSB deer muise. Subakute mCPP en QNP behandeling van deer muise het 'n aansienlike onderdrukking van stereotipiese gedrag tot gevolg gehad, terwyl fluoxetine, en nie desipramine, die onderdrukkende effek van mCPP en QNP verhoed het. Hiermee is die konstruk grondheid van die model bevestig, en terselfde tyd is die betrokkenheid van dopamien D_2 sowel as 5-HT_{2A} and 5-HT_{2C} reseptore in stereotiperende deer muise bevestig.

Om die gedrags data verder te gerugsteun, is daar op molekulêre vlak na sikliese adenosien 3', 5'-monofosfaat (cAMP) sowel as fosfodiesterase tipe 4 (PDE4) ensiem aktiviteit ondersoek ingestel. Data spreek ten gunste van die betrokkenheid van 5-HT_{1A}, 5-HT_{1B}, en 5-HT_{2C} in stereotipiese deer muise. Hierby toon proteïen uitdrukking analises 'n aansienlike toename in PDE 4A1, 4A5, 4A8 en 4D1 isovorm uitdrukking in die prefrontale korteks van HSB muise, sowel as in die striatum van LSB muise. Verskille in die anatomiese verspreiding van die PDE isovorme kan toe geskryf word aan die spesifieke funksie van elke anatomiese streek en nut van PDE4 aktiwiteit binne hierdie area. Die gevolgtrekking word dus gemaak dat PDE4 isovorme 'n anatomies spesifieke rol speel binne die cAMP kaskade van deer muise.

Die studie het verder getoon dat daar 'n uiteenlopende verskil is tussen LSB en HSB muise, en dat hierdie dispariteit duidelik sigbaar is op gedrags sowel as molekulêre vlak. In HSB muise word veral die cAMP kaskade aansienlik af-gereguleer deur fluoxetine behandeling. Die regulering van Darpp-32, 'n belangrike proteïen in die D_1/D_2 seinkasdade, is ook uiteenlopend in stereotipiese deer muise deur behandeling beïnvloed Hieruit kan aflelei word dat die graad van die voorafgaande gedrags afwyking gebruik kan word om deer muise mee te klassifiseer en dat dit bydrae tot die unieke molekulêre reaksie van deer muise teenoor geneesmiddel behandeling. Ter afsluiting, die huidige studie verstrek beide gedrags en farmakologiese data wat die voorspelbaarheid, fenomelogiese en konstruk grondheid van spontane steoreotipiese gedrag in deer muise ondersteun en dié model as diermodel vir OCD promoveer.

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1. Introduction

1 Introduction

Obsessive compulsive disorder (OCD) is a debilitating disorder marked by two distinct phenomena: recurrent, disturbing, intrusive thoughts (obsessions) and overt repetitive behaviours or mental acts (compulsions) that are performed to reduce distress caused by obsessions. The primary symptom characterizing these disorders is anxiety, which can manifest itself as panic, phobic avoidance, intrusive experiences, excessive worry, and/or difficulty controlling worry. OCD is currently considered as an anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and the primary basis for categorizing it as an anxiety disorder is the central role anxiety plays in the disorder (Tynes et al., 1990). Obsessions lead to a sense of mounting anxiety and engaging in compulsive behaviours or mental acts reduces anxiety.

OCD is a highly heterogeneous condition and it has been suggested that it is actually composed of several distinct subtypes (McKay et al., 2004). The behaviours associated with OCD have been linked to a wide variety of cognitive (Tallis, 1997) and occasionally non-cognitive deficits (Szechtman & Woody, 2004) such as memory, attention, and other neuropsychological factors such as set shifting ability, security motivation, and reward. Either the neuropsychological deficits associated with OCD may rest on structural or neurochemical abnormalities that drive obsessions or the deficits may be secondary to OCD symptoms. The different brain regions associated with the pathogenesis of OCD include the frontal lobe including the orbitofrontal, dorsolateral and anterior cingulated cortices, and the right mesial temporal areas (Alptekin et al., 2001). Subcortical regions are also the sites of frequently reported dysfunctions in OCD where the striatum is posited as a primary site of OCD pathology (Rauch et al., 1998).

Recent brain imaging techniques have been particularly convincing in suggesting that specific neuro-circuits are responsible for the mediation of OCD symptoms. The predominant hypothesis is that prefrontal-basal ganglia-thalamic-prefrontal circuits are particularly important (Insel, 1992). Dysfunction in these circuits may be associated with implicit processing deficits and intrusive symptoms related to OCD (Rauch et al., 1998). Furthermore, neurophysiological (Greenberg et al., 2000) and neuroimaging (Mataix-Cols et al., 2004) investigations consistently reveal functional abnormalities in brain circuitry underlying motor control and sensori-motor integration in patients with OCD. In addition,

pharmacological and neurobiological studies have implicated several central neurotransmitter systems in the pathophysiology of OCD. The strongest pharmacological evidence concerns the serotonergic system and the well-established efficacy of serotonin reuptake inhibitors (SRIs) in the treatment of OCD (Zohar & Insel, 1987). A growing body of evidence suggests that the pathophysiology of OCD is complex, and that despite the fundamental role played by serotonin (5-HT) in the pathogenesis of this disorder, a serotonergic dysfunction may explain no more than 50% of the variability of the disease (Goodman et al., 1990; Goodman et al., 1991). Other neurotransmitter systems such as dopamine, and second and third messengers such as cyclic adenosine 3', 5'-cyclic monophosphate (cAMP; Marazziti et al., 2000) may be important in the pathophysiology and aetiology of OCD.

There have been various attempts to develop animal models of OCD in the hope that they may provide a route for further understanding the neurobiology and treatment of this disorder. Given that the aetiology of OCD most likely involves the interaction of multiple genetic and environmental factors, animal models in which the behavioural pathology develops spontaneously may be particularly useful. With this in mind, the present study has set out to evaluate the predictive, construct and face validity of naturalistic stereotypic behaviour in the deer mouse (*Peromyscus maniculatus bairdii*). This represents a completely novel approach since the deer mouse and its unique behavioural phenotype has to date not been considered or studied for its potential value as an animal model of OCD. A concise statement of the objective of the study as well as justification for the present study is provided at the end of the literature review chapter (section 2.10).

In order to establish the authenticity of the stereotypy observed and measured in deer mice, deer mice were first compared to non-stereotypic C57Bl mice by monitoring a specific series of stereotypic behaviours that were first confirmed visually before characterisation using a digital animal activity monitor. Computerised tracking of behaviour subsequently identified a triad of stereotypic movements unique to deer mice but that were separate from general locomotor activity. These included clear, stereotypic behavioural patterns such as backward somersaulting, repetitive jumping, and patterned running. These stereotypy's were then used throughout the study. Disparity between untreated deer mice and C57Bl mice were compared at both the behavioural and molecular level in order to establish both face and construct validity. Deer mice

furthermore executed stereotypic behaviour at various rates and could accordingly be classified as low stereotypic (LSB), high stereotypic (HSB), or non-stereotypic mice. C57Bl did not perform any stereotypic behaviour characteristic of deer mice. Comparison of the two mouse strains not only highlights the spontaneous stereotypic behaviour of the deer mice, but also contributes to the face validity of the model. In this regard, deer mice stereotypy is not unlike that observed in OCD patients who often perform compulsions at different rates within the population.

Recent evidence implicate altered cyclic adenosine 3', 5'-cyclic monophosphate (cAMP)-protein kinase (PKA) function in OCD (Perez et al., 2000), and SRI's are known to modulate this signalling system via action on $5HT_{1A}$ receptors. With this in mind, the degree of stereotypy in deer mice was linked to the amount of cAMP under basal conditions in the cortico-striatal-thalamic-cortico (CSTC) circuit, and correlated with phosphodiesterase 4 (PDE4) activity and expression. HSB mice had the highest cAMP levels compared to LSB, non-stereotypic and C57Bl mice. HSB mice also were characterised by the lowest measured PDE4 enzyme activity.

Stereotypic deer mice furthermore expressed lower quantities of the short form PDEs namely PDE 4B4 and 4D1 as well as the noradrenergic associated PDE 4A4 and 4A8 isoforms compared to C57Bl mice. Untreated stereotypic deer mice also presented with increased levels of Thr75-Darpp-32 compared to C57Bl and non-stereotypic deer mice, which would suggest an imbalance along the D_1/D_2 pathway in the CSTC circuit of deer mice. Taken together, deer mice, particularly LSB and HSB mice, can be unmistakably distinguished from C57Bl at both the behavioural and molecular level.

Since there is abundant evidence for a dose response relationship in the selective response to SRI's in treating OCD (Zohar et al., 1987), predictive validity was evaluated by administering fluoxetine and desipramine to stereotypic deer mice at either low (10 mg/kg) or high (20 mg/kg) doses for 21 days. Since disturbances in 5-HT, especially 5-HT_1 and 5-HT_2 mediated events, are of importance in the development and treatment of OCD (Zohar & Insel, 1987), and there is furthermore strong evidence for the role of dopamine in OCD (Goodman et al., 1990; Goodman et al., 1991), construct validation studies were undertaken by subacute challenge with the 5-$HT_{2A/C}$ agonist, *meta*-chlorophenylpiperazin (mCPP) and the D_2 agonist, quinpirole (QNP), administered at

doses of 2 mg/kg and 5 mg/kg respectively for 4 days. The effect of said treatment on natural stereotypy of deer mice was thus studied. In addition, these subacute drug challenges were also undertaken with and without SRI or noradrenergic reuptake inhibitor (NRI) pre-treatment to establish whether these behaviours were amenable to reversal with an SRI or NRI, thus assessing predictive validity at a deeper level.

High and low dose fluoxetine, but not desipramine, significantly reduced stereotypic behaviour compared to vehicle-treated animals in both low and high stereotypy animals. Subacute mCPP and QNP challenges both evoked a significant suppression of stereotypic behaviours, while high dose fluoxetine, but not desipramine, reversed the suppressive effects of mCPP and QNP in stereotypic deer mice. These data are unanimous in the conclusion that stereotypic behaviour in deer mice is underpinned by both aberrant dopamine and 5-HT function, with the current study convincingly implicating hyposerotonergia, since heightened stereotypy in these animals was attenuated by $5HT_{1/2}$ receptor agonists.

The latter drug responses were also reversible with chronic SRI treatment, but not with an NRI, consolidating the prominent role of serotonergic pathways in regulating both serotonergic and dopaminergic responses in this animal model. This involvement of dopamine D_2 and 5-HT_{2A} and 5-HT_{2C} receptors in stereotypic behaviour provides important construct validity. The role of dopamine in deer mouse associated stereotypy was also corroborated by the Darpp-32 studies, given that the latter protein is an important and inseparable marker of dopaminergic activity in the brain (Svenningsson et al., 2004).

OCD has been associated with disturbances in signalling of the cAMP cascade. It is noteworthy that the receptors targeted by SRI's and alleged to underlie the efficacy of these agents in treating OCD, particularly 5-HT_{1A} and 5-HT_{1B} receptors, are linked to the synthesis of cAMP. To this end, molecular data collected in the study have focussed on striatal and prefrontal cortex measurement of cAMP and the expression of phosphodiesterase 4 (PDE4) enzymes. Importantly, the degree of stereotypy could be linked to cAMP under basal conditions. High stereotypic mice had the highest cAMP levels compared to low and non-stereotypic and C57Bl mice. High stereotypy was also characterised by low PDE4 activity. Analysis of protein expression found an increase in

PDE 4A1, 4A5, 4A8, and 4D1 in the prefrontal cortex of HSB mice as well as the striatum of LSB mice. The negative correlation between PDE4 and cAMP levels provides definitive confirmation for altered states of the cAMP signalling cascade in the cortico-striatal-thalamic-cortico (CSTC) circuit of these animals.

The present study provides new and important behavioural and pharmacological evidence that spontaneous, naturalistic stereotypic behaviour in the deer mice present with significant face, predictive and construct validity. Stereotypic deer mice are therefore a suitable animal model of OCD and will greatly contribute to furthering our understanding of the pathology of this disorder.

2. Literature Review

2 Introduction

2.1 Current classification of obsessive compulsive disorder

OCD is a debilitating disorder marked by two distinct phenomena: recurrent, disturbing, intrusive thoughts (obsessions) and overt repetitive behaviours or mental acts (compulsions) that are performed to reduce distress caused by obsessions. The most common obsessions include contamination concerns, pathological doubt, aggressive, religious and sexual thoughts, somatic concerns, and the need for symmetry and precision. The most frequent compulsions are checking, arranging, ordering, cleaning or washing, hoarding and counting (Rasmussen & Eisen, 1992). Although the predominant symptoms can change with time in any individual (Swedo et al., 1989) symptoms do not differ markedly between children and adults. Sub-clinical obsessive compulsive symptoms however are not uncommon and are seen during the course of normal development.

By definition, obsessions and compulsions in OCD must cause marked anxiety, be time consuming, and seriously interfere with daily functioning. Because of the powerful role obsessions and compulsions can play in a person's life, individuals with OCD often avoid those things or situations that trigger their obsessive and/or compulsive behaviours; thus avoidance behaviour is also a central feature of OCD (Rasmussen & Eisen, 1992).

OCD is currently categorized as an anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) along with such other anxiety disorders as panic disorder, posttraumatic stress disorder, and generalized anxiety disorder (American Psychiatric Association, 2000). The most important symptom characterizing these disorders is anxiety, which can manifest itself as panic, phobic avoidance, intrusive experiences, excessive worry, and/or difficulty controlling worry. The primary basis for categorizing OCD as an anxiety disorder is the central role anxiety plays in OCD (Tynes et al., 1990). Obsessions lead to a sense of mounting anxiety and engaging in compulsive behaviours or mental acts reduces anxiety.

The problem with this rationale, however, is that it is symptom focussed; OCD is conceptualized as an anxiety disorder because of the prominence of anxiety; however, anxiety is a relatively non-specific symptom that is associated with a number of other

psychiatric disorders (e.g. depression. bipolar disorder and schizophrenia). Recent thought is that, although conceptualizing OCD as an anxiety disorder may be useful from a diagnostic perspective, it does little to further the understanding of OCD and address questions related to the aetiology and treatment of this disorder (Bartz & Hollander, 2006).

At a phenomenologic or psychologic level, OCD therefore demonstrates a continuum with a broad range of other conditions, e.g. on a spectrum of reward related or affective disorders (Hudson & Pope, 1990; Black et al., 1995), on a spectrum of obsessive-compulsive disorders (Stein, 2000; Phillips, 2002; Baca-Garcia et al., 2005), and on a spectrum of stereotypic disorders (Ridley, 1994).

2.2 Clinical features of obsessive compulsive disorder

The obsessions and compulsions of OCD should not be confused with inflexible character traits that comprise obsessive compulsive personality disorder. Although the distinction between axis I (e.g. a disorder such as OCD) and axis II disorders (e.g. a personality disorder) is unclear at times, the obsessions and compulsions of OCD differ qualitatively from obsessive compulsive personality traits such as over conscientiousness and perfectionism (Stein, 2002). For patients with OCD, the disorder has a major impact on quality of life in several domains, including social functioning, employment, marriage and family relationships, and socioeconomic status (Calvocoressi et al., 1995; Hollander et al., 1997; Koran, 2000). OCD has a lifetime prevalence of 2-3% (Weissman et al., 1994) and is thought of be more common in women that men (Fineberg & Roberts, 2001).

The disorder is a highly heterogeneous condition and it has been suggested that it is actually composed of several distinct subtypes. As outlined by McKay (McKay et al., 2004), researchers interested in identifying OCD subtypes have used a number of approaches for example focusing on clinical descriptions while others have focused on family studies and laboratory tests. Accordingly, a number of subtypes have been suggested (table 2.1).

Table 2.1 Proposed OCD subtypes (adapted from McKay et al., 2004).

OCD subtype
early versus later onset OCD
OCD with *presence or absence of tics*
OCD with *presence or absence of disease*, such as streptococci-related autoimmune disorder
OCD with *presence or absence of psychotic or neurological features*, i.e. neuropsychological and information processing differences
OCD *according to symptom presentation* patterns e.g. washers versus checkers

Research to identify specific subtypes of OCD has focused primarily on symptom presentation with factor analyses producing symptom dimensions. Leckman and co-workers (Leckman et al., 1997) were the first to identify these dimensions and their analyses revealed four replicable factors: (1) OCD with obsessions and checking (2) OCD with symmetry and ordering (3) OCD with cleanliness and washing and (4) OCD with hoarding. Other subtype topologies followed, with cluster analyses revealing OCD in the midst of harming, hoarding, contamination, certainty and obsessionals (Calamari et al., 1999). It is apparent that the core symptoms of the identified groups are comparable. This division of OCD based on neuropsychological and information processing differences or those mentioned in table 1, might go a long way toward determining the utility of such subtypes.

It is further suggested that although OCD may present with additional comorbid conditions, other distinct pathologies may emerge (be inserted) independently over time (Yaryura-Tobias et al., 2000). This supports the conceptualization of OCD as a

continuum, where additional diagnoses may be expected to occur in the time course of the condition. The value of viewing OCD as part of a so-called obsessive compulsive (OC) spectrum, as suggested by clinical researchers (Stein, 2000; Lochner & Stein, 2006) may be of significance and recent studies (Baca-Garcia et al., 2005) have started to investigate the continuum between compulsivity and impulsivity.

The notion of a spectrum of OC related disorders that is comprised of such disparate disorders as OCD, certain eating disorders, body dysmorphic disorder, and autism, is gaining acceptance. The fact that these disorders share OC features and evidence of similarities in patient characteristics, course, comorbidity, neurobiology, and treatment response raises the question of whether OCD is best conceptualized as an anxiety disorder or as part of an OC spectrum of disorders (Bartz & Hollander, 2006). Taken together, the majority of OCD patients are aware of the irrationality of their thoughts and behaviours, notwithstanding the subtype or dimension. OCD patients thus unwillingly partake in distressful behaviour even though it is recognized that the obsessions and impulses are a product of his or her own mind.

2.3 Neuropsychological and neuropathological attributes of OCD

2.3.1 Neuropsychology

The behaviours associated with OCD have been linked to a wide variety of cognitive (Tallis, 1997) and occasionally non-cognitive deficits (Szechtman & Woody, 2004). These findings are relatively inconsistent, born of differences in methodological approaches such as inclusion-exclusion criteria for subjects, as well as assessment measures (Schultz et al., 1999). Nonetheless, some patterns do emerge, suggesting that obsessive compulsive behaviours are in fact associated with neuropsychological deficits. Observations of neuropsychological performance deficiencies have variably supported three possibilities regarding the nature of the cognitive (and non-cognitive) impairment: a fundamental impairment of executive function ability, deficits of immediate and secondary memory and a spatial information processing disorder.

2.3.1.1 Memory

There is a substantial body of evidence to suggest that OCD patients show impaired performance on a number of different memory tasks. Additionally, aspects of the behaviour seen in people with OCD might be argued to be suggestive of memory problems, e.g. patients engage in repetitive checking behaviour. However, studies indexing reality monitoring and memory for self-actions in OCD patients have failed to find evidence of impairments in these areas (McNally & Kohlbeck, 1993). In general cognitive scientists have distinguished between three types of memory: episodic memory, semantic memory and procedural memory (Grusec et al., 1990). The distinction between types of memory processes is important because it is possible that certain processes are more important than others in perpetuating and maintaining the repetitive behaviours that characterize OCD.

In an up to date review, Muller and Roberts (Muller & Roberts, 2005) studied various memory functions in patients with OCD, highlighting the role of memory for experimentally presented verbal and non-verbal stimuli, memory of personal experiences, memory of actions and imagined actions, as well as confidence in memory and enhanced memory for threat-related stimuli. The authors concluded that although research has provided a fairly mixed and inconclusive picture concerning the possibility of an overall memory deficit in OCD patients, there is relatively strong evidence that OCD is associated with low memory confidence, as well as memory biases towards threatening information. Furthermore, there is broad agreement that deficits in memory, albeit visuospatial or nonverbal memory, do not result from memory impairment per se, but rather from impaired ability to use strategies efficiently (Savage et al., 1999; Deckersbach et al., 2000). The mixed pattern of results combined with limited statistical power emphasizes caution in concluding whether or not memory impairment exists in OCD.

2.3.1.2 Attention

OCD appears to be closely associated with attention and two distinct features of attention are of importance, namely basic attention and attentional bias. There is little evidence in the literature for dysfunctional basic attention in OCD patients, while performances on tasks that assess attention span as well as sustained attention appear to be unaffected in

OCD (Savage et al., 1996; Milliery et al., 2000). Literature also presents to some extent mixed support concerning attentional bias in OCD. Direnfeld et al. (Direnfeld et al, 2001) demonstrated that patients with OCD showed interference to threat-related stimuli and in addition that this interference significantly correlated with the total number of obsessive and compulsive symptoms. This was however not supported by another study (Kampman et al., 2002) which failed to replicate the general findings of bias toward fear- or threat-related stimuli. Despite these mixed results, similar attentional biases have been documented in a variety of anxiety disorders, including post-traumatic stress disorder (Buckley et al., 2000), generalized anxiety disorder (McNally & Kohlbeck, 1993) and social phobia (Heinrich & Hofmann, 2001).

It seems reasonable to suggest that such biases in selective attention could contribute to the development and maintenance of intrusive thoughts in OCD. Clinicians have long observed that individuals with OCD have difficulty inhibiting negative thoughts. Here, the term 'attentional inhibition' is important and refers to how an individual narrows down incoming information in order to selectively attend to the stimuli that are most relevant. Given the difficulties OCD patients have in controlling unwanted intrusive thoughts, deficits in attentional inhibition may play an important role in OCD (for a detailed review see Muller & Roberts, 2005).

2.3.1.3 Additional neuropsychological factors within OCD

Apart from memory and attention, other executive functions are also important to OCD and have been extensively studied. The term 'executive function' refers to higher-order cognitive functions such as volition, intended action, planning and self-monitoring of behaviour. The performance of OCD patients on set shifting tasks has been investigated repeatedly and the majority of these studies suggests that performance of OCD patients is comparable to those of healthy controls (Christensen et al., 1992; Deckersbach et al., 2000; Moritz et al., 2001). Impairment of set shifting ability in OCD remains highly debated owing to the different sensitivity levels of the tasks most commonly used to assess this ability. Fluency, mainly verbal fluency, has been examined in OCD but owing to the small number of studies as well as the marked differences between the studies, comparison is difficult (Muller & Roberts, 2005).

Additional neuropsychological factors that may perhaps be impaired to some extent in OCD, includes conceptual thinking and planning ability, especially if short comings seen here are associated with memory (Simpson et al., 2006). The results of these and other studies suggest that some cognitive deficits seem to be common in OCD and that further studies should be done using reliable testing procedures in sufficient groups of healthy controls and if possible, to include clinical controls to account for the specificity of the neuropsychological dysfunction in OCD.

2.3.1.4 OCD as a disturbance of security motivation

As mentioned before (section 2.2), OCD patients are well aware of external reality, generally recognize the irrationality of their obsessions/compulsions and prefer not to engage in them. Yet, despite this strong tie to reality they knowingly continue to perform such activity. Clearly, OCD is a cruel demonstration that normal control of behaviour can be over-ridden by some powerful non-cognitive-based system(s). OCD symptoms do possess a thread of continuity across patients and the content of most obsessional thoughts, ideas or actions revolves around the issue of security or safety, either of the self or of others (Salkovskis, 1985).

Given the universality of OCD symptoms and their circumscribed focus on concerns regarding self-preservation and preservation of the species, it is suggested that OCD constitutes the expression of a security motivational system (Szechtman & Woody, 2004). More specifically, it is hypothesized that symptoms of OCD stem from an inability to generate the normal 'feeling of knowing' that would otherwise signal task completion and terminate the expression of a security motivation system. In other words, OCD patients seem unable to switch off their security motivation system by means of a so-called terminator emotion. Major working characteristics of this system include that it is tuned to detecting potential danger, is orientated toward action, is readily activated and that it can be distinguished from other systems that protect the organism (such as the pain motivation system) (Szechtman & Woody, 2004).

To summarize, the security motivation system identifies as the core deficit a failure to put closure on an experience. The system furthermore constrains this failure to experiences invoked by biologically primal motivation for protection of self and others and considers

that failure to put closure on experiences does not stem from cognitive inability but from the breakdown in a satiety-like mechanism that normally generates a feeling of knowing.

2.3.1.5 Reward as a neurobiological correlate in OCD

Many aspects of the phenomenology of OCD suggest a dysfunctional reward system. The compulsions of OCD patients can be seen as driven by a goal-directed reward-seeking mechanism. In healthy individuals, the expectation of a natural reward is accompanied by an increase in dopamine in specific neuroanatomical areas (details to follow, section 2.4.1) (Kalivas, 2002; Wise, 2004), whereupon the signal then initiate appropriate cognitive and behavioural strategies. For OCD patients the ability to generate the normal 'feeling of reward' that would otherwise signal task completion and terminate the expression of goal-directed reward-seeking behaviour is absent.

The ensuing failure to attenuate reward-associated behaviour would result in repetitive, stereotyped behaviour that is typical of OCD. The reward hypothesis correlates strongly with the security motivation model, and as will be seen (section 2.4.3), share a number of neural underpinnings. Reward and security motivation can therefore be seen specifically as the inability to modulate goal-directed behaviour and adapt to changing environmental demands. These deficiencies would appear to represent deficient inhibitory control with respect to selected processes and, preliminary investigation has begun into the role of inhibitory control in OCD (Krikorian et al., 2004).

2.4 Neuropathology

2.4.1 Neuroanatomy central to OCD

The neuropsychological deficits associated with OCD may rest either on structural or neurochemical abnormalities that drive obsessions or the deficits may be secondary to OCD symptoms which can subsequently lead to interference with mental functioning (Otto, 1992). In this context the neuropsychological approach is a useful tool for assessing the potential role of different brain regions in the pathogenesis of OCD. The frontal lobe including the orbitofrontal, dorsolateral and anterior cingulated cortices and the right mesial temporal areas represent the cortical regions involved in the cognitive and neuropsychological manifestations of OCD.

The orbitofrontal cortex (OFC), which has been demonstrated by functional neuroimaging to be overactive in OCD (Alptekin et al., 2001; Lacerda et al., 2003), is a region mediating the active expression of emotional response to significant biological stimuli, as well as the inhibition of behavioural response (Rolls, 1999). The OFC seems to play a leading role in motivational aspects of decision-making. If this region is overactive the natural estimation of the consequences of immediate action might be increased, leading to uncontrolled thoughts and behaviours (Rolls, 1999; Alptekin et al., 2001). Such an event might mediate the cognitive generation of inappropriate 'error detection' signals that drive the feeling that 'something is wrong'.

Additionally, neuroimaging studies indicate that the anterior cingulated cortex (ACC) is involved in a variety of cognitive processes such as attention, motivation, reward and error detection, working memory, problem-solving and action-planning (Devinsky, 1995). The ACC therefore seems to be involved in the emotional evaluation of the consequences of action i.e. is responsible for error detection. This implies that over-activity of the ACC might be regarded as a result of OCD symptoms rather than as a factor involved in the genesis of obsessions (Carter et al., 1998).

To underscore this, and similar to predictions that OCD involves overactive error processing, significantly greater error-related activity has been noted in the ACC of patients with OCD (Fitzgerald et al., 2005). It was concluded that the differences in ACC activation between patients and healthy participants could represent a stable vulnerability factor for the development of OCD. Furthermore, there is a fairly direct route from the ACC to the motor cortex, with the ACC sending direct anatomical connections to the rostral cingulated motor area, which in turn projects to the motor cortex. The latter is of significance because OCD symptoms affect both cognition and motor behaviour.

Another large region, the dorsolateral prefrontal cortex (DLPC), plays a key role in the adaptation to changes in environment and in the control of behavioural responses (Dubois et al., 1994). Functional neuroimaging have shown decreased activity in the DLPC in patients with OCD (Baxter, 1999) which may explain the difficulty to 'stop' compulsive behaviours such as reward-seeking and security motivation. This may clarify OCD patients' reduced ability to think or concentrate on a particular stimulus and their indecisiveness.

Subcortical regions are also the sites of frequently reported dysfunctions in OCD where, despite the striatum being posited as a primary site of pathology in OCD (Rauch et al., 1998), structural neuroimaging studies of caudate nucleus in adult OCD patients have revealed contradictory findings. The striatum is the main input structure of the basal ganglia and is a key component of the motor system (Kelly, 1999). It is divided into the dorsal striatum, which includes the caudate and the putamen, and the ventral striatum that is mainly composed of the nucleus accumbens. The caudate-putamen and the nucleus accumbens show differences in their input and output projections. The caudate-putamen is mainly innervated by the primary motor cortex, the anterior premotor and cingulated areas, and the substantia nigra pars compacta. The caudate-putamen in turn projects to the globus pallidus and the substantia nigra pars reticulate and pars compacta (Kelly, 1999; figure 1).

The ventral striatum receives inputs from numerous prefrontal areas, limbic structures, such as the hippocampus and the amygdala; in turn, the ventral striatum sends projections to the ventral pallidum, the substantia nigra pars compacta and reticula, the ventral tegmental area and the hypothalamus (Kelly, 1999). Therefore, while the dorsal striatum appears more allied with voluntary motor functions and is involved in the initiation, production and sequencing of motor behaviour and in the development of addiction, the nucleus accumbens is more likely an interface between the limbic and the motor system and plays a major role in motivated and goal-directed behaviours as well as the development and expression of addiction (Kelly, 1999). The striatum together with other components of the basal ganglia, which includes the putamen and globus pallidus, have all been implicated in OCD (Cummings, 1996; Baxter, 1999). The most frequent findings indicate volumetric differences between OCD patients and healthy controls with reductions in volume sometimes associated with OCD symptom severity (Rosenberg et al., 1997).

Lastly, the thalamus and amygdala also seem to play a role in the derivation of OCD. The amygdala appears to play an important role in the expression of emotion and motivation and a dysfunction here might mediate the non-specific anxiety OCD patients feel relative to obsessive thoughts (Baxter, 1999). Increase in thalamic volume has furthermore been reported in paediatric OCD patients compared to controls (Gilbert et al., 2000) but this finding could be due to the specific drug therapy that were used. The expression of

emotion (recognition of cues of threat/danger) and motivation that engenders the non-specific anxiety symptoms in OCD is likely to involve the amydala (Baxter, 1999).

2.4.2 Neuro-circuits implicated in OCD

Recent brain imaging techniques have been particularly convincing in suggesting that specific neuro-circuits are responsible for the mediation of OCD symptoms. The predominant hypothesis is that prefrontal-basal ganglia-thalamic-prefrontal circuits are particularly important (Insel, 1992). Dysfunction in these circuits may be associated with implicit processing deficits and intrusive symptoms related to OCD (Rauch et al., 1998). Classically, the role of the basal ganglia is to integrate the various inputs arriving from the cortex and to use this information to select certain motor and/or cognitive programs (Cummings, 1996). Drawing upon functional neuroimaging data and earlier theorizing, a model was put forward that point to dysfunction in orbitofrontal-subcortical circuitry in the pathophysiology of OCD (Saxena & Rauch, 2000).

This circuitry is thought to be comprised of a direct and indirect pathway, both of which originate in the frontal cortex and project to the striatum (figure 2.1). From the striatum, however, the direct pathway projects to the globus pallidus interna/substantia nigra, pars reticulate (GPi) complex – the primary output location of the basal ganglia – and back to the cortex. This pathway is thought to facilitate complex motor programs by activating the thalamic system.

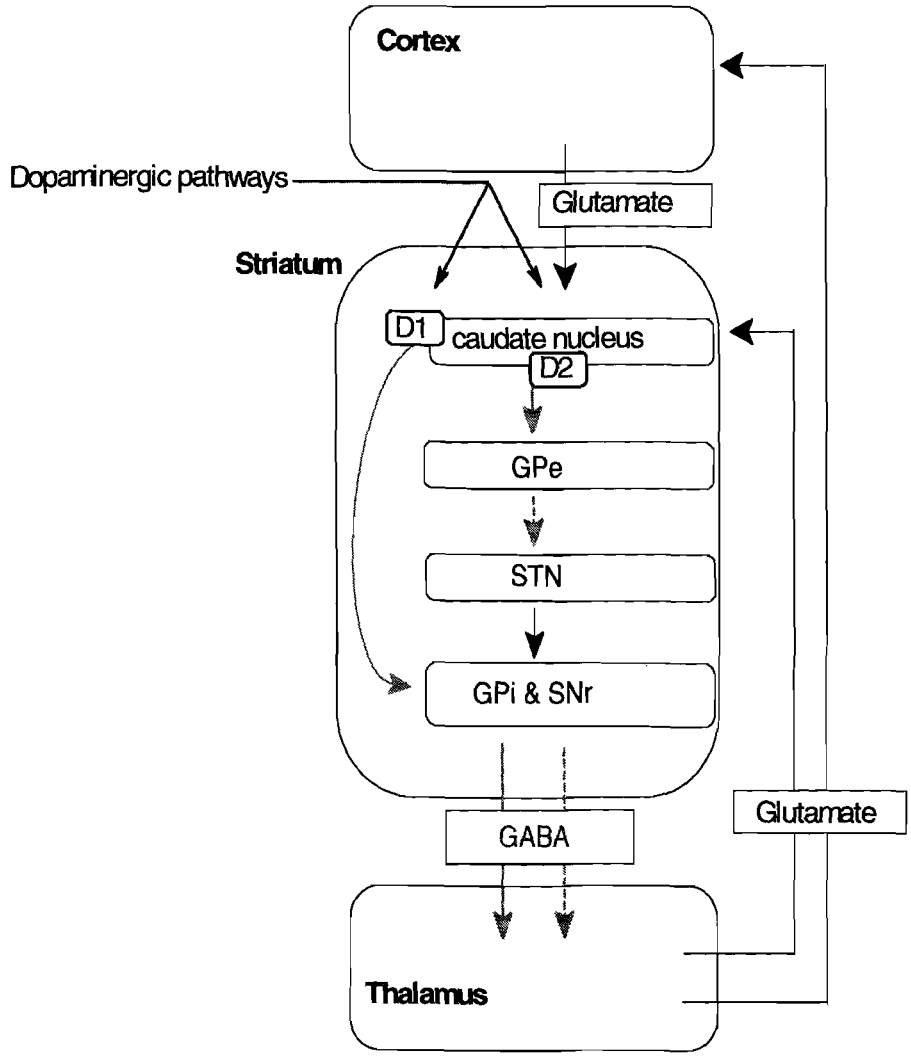


Figure 2.1 Neuro-circuits and neurochemicals underlying the cortico-striatal-thalamic-cortico (CSTC) circuit. The striatum receives both glutamatergic as well as dopaminergic afferents. Two loops, one a direct pathway via the globus pallidus internal (GPi) or substantia nigra pars reticulla (SNr) to the thalamus and the other an indirect via the globus pallidus external segment (GPe) and subthalamic nucleus (STN) arise from the striatum. Cortical input to the striatum results in activation of the thalamus through the direct pathway and a GABAergic inhibition of the thalamus through the indirect pathway. The CSTC is completed with the thalamus sending glutamatergic afferents to the cortex. Stippled lines indicate reduced neurochemical activity whereas solid lines indicate increased neurochemical activity. Red lines indicated GABA, black lines indicate glutamate and blue lines indicate dopamine signalling. GABA, gamma amino butyric acid (Korff & Harvey, 2006).

The thalamus serves as a gateway to the cortex and plays a significant role in consciousness, perception and integration of information before it reaches the cortex (Rosenberg & MacMillan, 2002). By comparison, the indirect pathway projects to the globus pallidus externa (GPe), the subthalamic nucleus, globus pallidus-substantia nigra pars reticula, thalamus, and back to the cortex. This pathway is thought to suppress complex motor programs by inhibiting activation of the thalamus, most likely through the actions of GABA.

It is theorized that these pathways balance each other out in healthy individuals, but that there is a bias in favour of the direct pathway in OCD patients, leading to increased activity in the OFC, ventromedial caudate, and medial dorsal thalamus, resulting in characteristic obsessions and compulsions (Saxena & Rauch, 2000). The imbalance is assumed to set prejudice toward executing well-prepared adaptive behaviours, whereas the ability to switch to new behaviours is weakened. Detailed anatomical analyses have demonstrated that the direct and indirect pathways can furthermore be distinguished based on their peptide content. The direct neuronal subpopulation contains substance P and dynorphin as co-transmitters, whereas the indirect neuron subpopulation contains enkephalin (Gerfen & Young, 1988).

Recent studies have confirmed that dysfunctional sensory gating mechanisms do exist in OCD (Rossi et al., 2005) and furthermore that decreased frontal-striatal functions underlie this disorder (Kathman et al., 2005; Van den Heuvel et al., 2005). Functional brain imaging studies have revealed increased brain activity of OCD patients in the OFC and the caudate nucleus during rest as well as during symptom provocation, thereby identifying possible disease related neuro-circuits.

Neurophysiological (Greenberg et al., 2000) and neuroimaging (Mataix-Cols et al., 2004) investigations consistently reveal functional abnormalities in brain circuitry underlying motor control and sensori-motor integration in patients with OCD. These abnormalities might account in part for the clinical picture of OCD, which is characterized by the failure to suppress repetitive movements, complex acts, and intrusive thoughts. Some motor 'intrusive' and reverberating behaviours in OCD might appear as a result of higher-than-normal level of motor cortex excitability and reduction of cortico-cortical inhibitory phenomena (Greenberg et al., 2000). This is partly a consequence of deficient inhibitory

control on motor behaviour and output from subcortical structures, such as basal ganglia and thalamus, which have been described as being hyper functioning in OCD (Alptekin et al., 2001).

A recent study (Rossi et al., 2005) would seem to indicate that the gating effects of motor commands are both spatially reduced and less effective in the modulation of sensory inputs during movements in OCD patients compared to control subjects. Furthermore, a tonic state of regional cortical hyper excitability in OCD patients might be responsible for the reduced ability to modulate sensory inputs during movements. This theory would reflect the notion that excessive, uncontrolled, and repetitive motor behaviours in OCD patients might be linked to frontal hyper metabolism (Alptekin et al., 2001), cortical hyper excitability and failure of inhibitory mechanisms (Greenberg et al., 2000) as result of deficient inhibitory control in circuits linking basal ganglia, thalamus, and supplementary motor and pre-motor areas (Cummings, 1996). The complex network that regulates motor control is known as the cortico-striatal-thalamic-cortico (CSTC) (Figure 1) circuit and dysfunction in this loop has been implicated in the genesis of OCD (Insel, 1992).

2.4.3 Neurochemical underpinnings of OCD

Pharmacological and neurobiological studies have implicated several central neurotransmitter systems in the pathophysiology of OCD. The strongest pharmacological evidence concerns the serotonergic system and the well-established efficacy of serotonin reuptake inhibitors (SRIs) in the treatment of OCD (Zohar & Insel, 1987). It was observed (Frenandez & Lopez-Ibor, 1967) early on that clomipramine and several other selective SRIs are effective anti-obsessional agents, while agents selective for noradrenaline are markedly less effective (Stein, 2002). The chronic administration of clomipramine or selective SRIs induces enhanced serotonin (5-HT) release in the OFC, probably as a result of the desensitization of the terminal 5-HT auto-receptors. This has been hypothesized to be the neurobiological basis for the efficacy of SRIs in the treatment of OCD as well as the purported serotonin hypothesis regarding the illness (Zohar & Insel, 1987).

A growing body of evidence suggests that the pathophysiology of OCD is complex and that, despite the fundamental role played by 5-HT in the pathogenesis of this disorder, a serotonergic dysfunction may explain no more than 50% of the variability of the disease (Goodman et al., 1990; Goodman et al., 1991). Although work on the role of the 5-HT system in mediation of OCD is important, to date relatively few abnormalities in this particular system has been identified as a cause of OCD. Most studies have indicated amongst other the 5-HT transporter (Torres & Caron, 2002; Lesch et al., 1996), as well as various 5-HT receptors (Enoch et al., 1998; Mundo et al., 2002) to be involved in OCD. The most widely accepted alternative neurochemical theory regarding the neurobiology and pharmacology of OCD, suggests that the dopamine (DA) system also plays an important role.

During preclinical studies, administration of dopamine agonists leads to stereotypic behaviour, whereas in human beings, such agents can exacerbate symptoms and tics of OCD (Goodman et al., 1990). Conversely, dopamine blockers are used in treatment of Tourette's syndrome, one of the spectrums of OCD. The 5-HT and dopamine systems interact extensively, particularly in the basal ganglia (Goodman et al., 1991), an area that is particularly important in the pathogenesis of OCD. Briefly, 5-HT is known to cause a decrease in dopamine activity/neurotransmission in the basal ganglia, a fact illustrated by the down regulation of dopamine activity when SRIs are administered (Zohar & Insel, 1987).

Other neurotransmitter systems and second and third messengers (Marazziti et al., 2000) may be important in the pathophysiology and aetiology of OCD. The role of anxiogenic neuropeptides, especially cholecystokinin (CCK), may also play a role in OCD since it is a neurotransmitter in the brain closely related to anxiety (Fekete et al., 1981). Although the precise mechanism of CCK-induced anxiety remains to be elucidated, evidence has accumulated that other neurotransmitter systems may modulate CCK actions and visa versa. CCK closely interacts with 5-HT (Rex et al., 1994) and GABA (Siniscalchi et al., 2003) pathways, while its co-localization and functional relationship with the dopaminergic system is well documented (Vaccarino, 1994).

Given the complex interactions between these various systems and assorted receptors in the brain, it is likely that a number of neurotransmitters and neuronal messengers are

involved in OCD. Considering the important role of the CSTC circuit (Cummings, 1996), and apart from 5-HT and dopamine, other neuroransmitters that warrant consideration include glutamate (Glu) (Carlsson, 2001), gamma aminobutyric acid (GABA), (Zai et al., 2005), and various other neuropeptides such as neuropeptide Y, tachykinins (i.e. substance P), and corticotrophin-releasing factor (McDougle et al., 1999). Glutamatergic afferents impinge on the caudate nucleus and thalamus with the caudate nucleus receiving a dense dopaminergic innervation from the substantia nigra (figure 2.1).

A delicate balance between dopamine and Glu cross-talk regulates the output of the thalamus. Here, dopamine may activate either D_1 receptors to directly activate GABA projections to the GPi, (direct pathway; figure 2.1), or D_2 receptors through sequential activation of GABA'ergic projection to the GPe and subthalamic nucleus (indirect pathway; figure 2.1). Glutamatergic projections from the subthalamic nucleus innervate the GPi, which sends GABA'ergic projections to the thalamus. The thalamus subsequently activates the caudate and cortex via ascending glutamatergic projections (Cummings, 1996).

Therefore, an imbalance between dopamine and glutamate input into the striatal complex may be critical in determining the degree of CSTC activation and the eventual output from the circuit. In summary, the discovery of the dual functions of the afore mentioned loops has led to the speculation that these two loops may represent the substrate for a process of evaluation that results in suppression of 'unwanted' and in facilitation of 'wanted' behaviour.

2.5 Genetics underlying OCD

The genetic study of OCD has made tremendous progress in the last decade. Twin studies have supported the genetic basis of OCD and family studies further support a genetic component for the disorder (Black et al., 1995). It is however, the pursuit of candidate genes that might ultimately provide clues to the underlying genetic basis and pathophysiology of OCD. To date, polymorphisms have been identified (Tot et al., 2003) which may be a factor in clinical severity or susceptibility of OCD, but none has been implicated as underlying cause of OCD. Candidate genes include the 5-HT transporter (Torres & Caron, 2002; Lesch et al., 1996) as well as receptor genes (Enoch et al., 1998;

Mundo et al., 2002), monoamine oxidase A (Karayiorgou et al., 1999), catechol-*O*-methyl transferase (Kararyiorgoi et al., 1997), dopamine receptor (Catalano et al., 1994) and transporter genes (Hemmings et al., 2004), and developmental genes such as transcription factors (Grados et al., 2003). Of particular interest here is the role of serotonergic and dopaminergic genes in OCD, since these two systems are heavily involved in circuits implicated in OCD (sections 2.4.2 and 2.4.3) as well as genes encoding for GABA-ergic and glutamatergic receptors (sections 2.9.3 and 2.9.4).

2.6 Treatment of OCD

For many patients, OCD is a lifelong illness extending from early childhood into adulthood. However, pharmacological and behavioural treatments at adequate doses and duration offer a majority of patients' improvement in symptoms and functioning. At the present time, there are a number of effective medications with relatively tolerable side effects (Pato & Phillips, 2003). Experimental treatment for the truly treatment refractory, such as neurosurgery, deep brain stimulation, and vagal nerve stimulation, are being developed and refined, and may ultimately offer new hope to patients who do not respond to traditional treatment.

2.6.1 Pharmacological treatment of OCD

The principal pharmacological agents used to treat OCD prominently affect the 5–HT system and include the SRIs listed in table 2.2. The SRIs are antidepressants that are effective for OCD in addition to depression and many other psychiatric disorders (Pato & Phillips, 2003). It appears that non-SRI antidepressants, such as the tricyclic antidepressant desipramine, which are effective for depression and other disorders, are generally not effective for OCD (Zohar & Insel, 1987). Based on the efficacy of SRIs, the anti-OCD effect appears to rest largely on the inhibition of the 5-HT reuptake process (El Mansari & Blier, 2006).

SRI treatment seems to alter metabolic activity in particular neural areas such as the OFC, head of the caudate nucleus, and the thalamus (Baxter, 1999). These changes appear to be adaptive changes induced by SRIs on 5-HT neurotransmission and include amongst other, changes in presynaptic and postsynaptic 5-HT receptors (El Mansari & Blier, 2006). SRI

treatment of OCD has been hypothesised to cause desensitisation of pre-synaptic terminal 5-HT_{1A} and 5-HT_{1D} receptors (Bergqvist et al., 1999) resulting in an increase in serotonergic activity in sub-cortical regions of the brain, as well as up-regulating 5-HT_2 receptor binding sites (Laakso et al 1996).

SRIs are first-line agents for treatment of OCD. However, 40 to 60 percent of patients do not respond adequately to SRI treatment and agents that alter other neurotransmitter systems, such as dopamine, norepinephrine, and second messengers systems, may play a role in the treatment of resistant patients (Goodman et al., 1990; Goodman et al., 1991). Various drugs, including antipsychotics, have been used (see table 2.2), but nonresponse to treatment more often than not is the norm when using non-SRIs. Common clinical observations also include the fact that high doses of SRIs are sometimes needed to obtain an anti-OCD effect and that the time course of treatment is much longer before any therapeutic effects are seen (Baxter, 1999). Trials often extend 10 to 12 weeks before the frequency and intensity of OCD symptoms are reduced.

2.6.2 Psychotherapy and neurosurgery in OCD

Behavioural therapy, especially exposure and response prevention (ERP) has been firmly established as the psychotherapy treatment of choice for OCD (Whittal et al., 2005) and up to 60 percent of patients experience a reduction in OCD symptoms after treatment (Stanley & Turner, 1995). OCD patients are exposed to anxiety-evoking stimuli and with constant staff supervision prevented from engaging in compulsions. The idea is that the unconditional responses that is said to reduce anxiety, is gradually replaced by neutral conditioned responses. Cognitive behaviour therapy (CBT) is also used to treat OCD, mainly to provide a less anxiety-provoking alternative to ERP and to target cognitions conceptualized as potential maintaining factors that remain unaddressed with behavioural treatment (Whittal et al., 2005).

Some patients have intractable OCD symptoms even with adequate medication, augmentation and psychotherapy. In these cases, more aggressive treatment may be warranted. Experimental treatments include neurosurgery and other procedures, such as deep brain stimulation and vagal nerve stimulation (Pato & Phillips, 2003). The

Table 2.2 Drugs used in the treatment of OCD.

SRIs	Antipsychotics	Other drugs
clomipramine	risperadone	oral morphine
fluoxetine	olanzapine	mirtazapine
fluvoxamine	quetiapine	pindolol
sertraline	aripiprazole	clonzapam
paroxetine		nicotine
citalopram		

neurosurgical procedures interrupt brain tracts involved in the 5-HT system and implicated in the pathophysiology of OCD. The surgical procedures used include anterior capsulotomy, cingulotomy, and limbic leucotomy, which all aim to interrupt the connection between the cortex and the basal ganglia and related structures (Mindus & Jenike, 1992). The general goals of treatment then are to reduce the frequency and intensity of symptoms, such as obsessions and compulsions, and to minimize interference in functioning caused by the symptoms (Pato & Phillips, 2003).

2.7 Contemporary animal models of OCD

2.7.1 Assessing the validity of animal models

Animal models are "experimental preparations developed in one species for the purpose of studying phenomena occurring in another species" (McKinney, 1988). Although there has been an expansion in the development and use of animal models in psychiatry and various other fields, there is still a lack of clarity regarding the terminology and classification of animal models and their validation criteria. McKinney and Bunney (1969) first suggested that the minimum requirements for an animal model are that the symptoms induced in the model be reasonably analogous to those seen in the modelled disease, and that treatment modalities effective in the modelled disease reverse the symptoms seen in animals.

McKinney (1988) specified two requirements why animal models are designed, namely to simulate a specific sign or symptom of the human disorder (behavioural similarity models), and also to permit preclinical drug evaluations (empirical validity models). The author also added a third type of animal model, those designed to evaluate specific etiological theory (theory-driven models). In short, the validity of behavioural models is judged by how closely the model approximates the human condition, and the validity of empirical validity (pharmacological isomorphism) models is evaluated by how well drugs that work in humans also work in the model and how well the effects of drugs in the model predict clinical effects (Mckinney, 1988).

The classification was developed further and grouped according to the criteria for assessing animal models into criteria used to establish face, predictive, and construct validity (Willner, 1991). Face validity refers to a phenomenological similarity between the model and the disorder it simulates. Ideally, the model should resemble the condition it models in its aetiology, symptomology, treatment, and physiological basis. Predictive validity entails that performance in the test predicts performance in the modelled condition. In principle, predictive validity can rely on aetiology, physiology, and response to treatment, but in practice, predictive validity is usually based on the latter (Willner, 1991). Construct validity means that the model has a sound theoretical rationale, and depends on the degree of homology between the behaviour that is being modelled and the

behaviour in the model, and on the significance of the modelled behaviour in the clinical picture.

An additional narrowing of the definition of face and predictive validity has recently been put forward (Geyer & Markou, 1995). Here, the term face validity is restricted to the phenomenological similarity between the behaviour in the animal model and the specific symptoms of the human condition. Predictive validity is defined as the degree to which performance in the model allows accurate predictions about the human condition. According to this definition, the identification of any variable that influences the animal model and the modelled phenomenon in similar ways strengthens the model's predictive validity. Based on this definition, the authors conclude that the only necessary and sufficient validation criterion for animal models in neurobiological research is predictive validity (Geyer & Markou, 1995).

While there is widespread disagreement on terminology and classification regarding animal models, there seems to be wide agreement that is impossible to develop an animal model that mimics a psychiatric syndrome in its entirety, and that therefore the criteria that an animal model must satisfy to establish its validity depend on the purpose of the model (Willner, 1991). In the context of neurobiological research, in which the aim of the animal model is to promote our understanding of the modelled condition, it is agreed that a common physiological basis of the model and the modelled condition contributes greatly to the model's validity (Geyer & Markou, 1995). Here, a critical component in the demonstration of a common physiological basis is the demonstration of a similar response to treatment, because the latter suggests similarity in the neurotransmitter systems involved. This makes pharmacological isomorphism an important factor in assessing the validity of an animal model of OCD.

2.7.2 Assessing the validity of animal models of OCD

Many efforts have been made to produce animal models of OCD. Each one in general satisfy at least one of the validation criteria described above (section 2.2), but ultimately, the more types of validity a model has, the greater its usefulness and relevance to OCD. Several points are important with respect to the assessment of predictive validity in

animal models of OCD, the latter being the 'only necessary' validity criterion that must be satisfied in neurobiological research.

First, although SRIs are, to date, the only effective pharmacological treatment of OCD, they are effective in several other psychiatric disorders, including depression, generalized anxiety disorder and social phobia (Vaswani et al., 2003). Animal models of OCD should therefore demonstrate both sensitivity to SRIs and insensitivity to other classes of drugs, which are not effective in OCD but are effective in these other conditions (e.g. non-serotonergic antidepressants such as desipramine) (Joel, 2006).

Second, SRIs are not effective in all OCD patients (Insel et al., 1994) and up to half of patients who have OCD do not respond to treatment with an SRI (Goodman et al., 1991). Therefore, a lack of effect of SRI in a model may suggest that it is a model of compulsive behaviour in the subgroup of OCD patients that do not respond to SRI treatment, rather than demonstrate that it is not a model of OCD. Such a model should however still demonstrate insensitivity to other types of pharmacological treatment, because there is currently no other effective monotherapy for this subgroup of OCD patients (Joel, 2006).

Third, SRIs are effective in patients only after several weeks of repeated administration. There is currently disagreement on the importance of demonstrating similarity in treatment regime (acute versus chronic) in the animal and the modelled disease. In the field of animal models of OCD, most models have used chronic rather than acute administration to establish predictive validity (Rapoport et al., 1992; Altemus et al., 1996; Szechtman et al., 1998), whereas more recently developed behavioural models, have mainly used acute administration (section 2.7.3.1).

2.7.3 Current animal models of OCD

2.7.3.1 Behavioural models

Stereotypy and compulsivity, inherent qualities of OCD, are invariably driven by obsessions, and this association represents a distinct drawback when attempting to model OCD (Korff & Harvey, 2006). Obsessions are of a cognitive nature and as such cannot be observed directly in animals (Man et al., 2004). For example, OCD is strongly associated with low memory confidence, as well as memory biases towards threatening information.

These deficits in memory are due more to an impaired ability to use strategies efficiently rather than memory impairment per se (Deckersbach 2000), and may underlie why patients, for example, engage in repetitive checking behavior.

There is also some support for an attentional bias towards fear/threat-related stimuli which could contribute to the development and maintenance of intrusive thoughts in OCD (Kampman et al., 2002). However, the cognitive and motor behavioural manifestations of OCD are intimately linked, with distinct evidence that the disorder is a condition where normal control of behaviour is over-ridden by non-cognitive-based systems (Kampman et al., 2002). This argues strongly that modeling compulsive behaviours is a meaningful surrogate endpoint for an OCD model.

Behavioural animal models, sometimes referred to as ethological animal models, include naturally occurring repetitive or stereotypic behaviours, such as tail chasing, weaving, and fur chewing (Stein et al., 1994), innate motor behaviours that occur during periods of conflict, frustration or stress (displacement behaviours) such as grooming, cleaning, and pecking (Insel et al., 1994), and natural behaviours that occur following some behavioural manipulation (adjunctive behaviours) (Insel et al., 1994) such as food-restriction-induced hyperactivity (FRIH) (Altemus et al., 1996) and rewarded alternation (Tsaltas et al., 2005). Although some of these models have good predictive validity in addition to face validity, many have not been used since the original publications.

To date only three behavioural models of OCD are in use, namely, the barbering, marble burying and signal attenuation models. Similarly to earlier behavioural models, barbering and marble burying have been suggested as potential models of OCD on the basis of behavioural similarity. The signal attenuation model on the other hand, is a theory-driven model of OCD, in which a compulsive-like behaviour is induced by simulating a deficient psychological mechanism hypothesized to underlie compulsive behaviours in OCD (Joel & Avisar, 2001).

2.7.3.1.1 Marble burying

Pharmacological evidence first suggested a possible relationship between the burying of glass marbles by laboratory rodents and OCD (Broekamp & Jenck, 1989). Careful

analysis of marble burying behaviour has led to the conclusion that it does not model anxiety, but rather is related to compulsive behaviours. In this model, burying begins as an appropriate, investigative activity but, after frustrated investigation of the non-reactive stimulus-object, begins to persist as a compulsive stereotypy. This suggestion is in line with the view that compulsive behaviours results from an inability to achieve a sense of task completion (Szechtman & Woody, 2004).

Marble burying can also be used as a model for hoarding, a subtype of compulsive behaviour frequently encountered in OCD. SRIs seem to decrease burying (Broekamp & Jenck, 1989), supporting the predictive validity of the model. There is however documented findings that drugs that do not have anti-compulsive activity, such as diazepam (e.g. Broekamp & Jenck, 1989) also reduce burying. The latter would seem to completely disappear when repeated administration is used (Ichimaru et al., 1995), arguing for predictive validity of the model and demonstrating the importance of chronic treatment in animal models of OCD.

2.7.3.1.2 Barbering

Hair pulling (trichotillomania) is a compulsive behaviour often found in OC spectrum disorders and OCD. Barbering, the plucking of fir or whiskers from cage mates or the animal itself, is a common abnormal, repetitive behaviour in laboratory mice. Although barbering is probably an abnormal behaviour, it is not a stereotypy. The drive to pluck hair from body locations is improperly repeated and the motor pattern involved are variable and goal directed (Sarna et al., 2000) during barbering, whereas in stereotypies the motor pattern is repeated identically without any apparent goal. Barbering has the important advantage over other models, in that it develops spontaneously and as pointed out by Garber and co-workers (Garner et al., 2004), may represent a refined and non-invasive model for studies of the complex genetic and environmental etiologies of OC spectrum disorders. Barbering currently lacks predictive and construct validity although it seems to have strong face validity as a model of trichotillomania.

2.7.3.1.3 The signal attenuation model

The signal attenuation model has been developed on the theory that compulsive behaviours result from a deficit in the feedback associated with the performance of normal goal-directed responses (Baxter, 1999) hence the model is a theory-driven model. In this model, the goal-directed behaviour is lever-pressing for food. The feedback associated with making a response is controlled using the following strategy: rats are first trained to lever-press for food, whose delivery is accompanied by a stimulus which had been previously paired with food (Joel & Avisar 2001). In this fashion the stimulus is established as a feedback cue which signals that the lever-press response was effective in producing food. The "signalling" property of the stimulus is then attenuated by repeatedly presenting the stimulus without food. Lastly, the effects of signal attenuation on lever-press responding are assessed under the extinction conditions, i.e., pressing the lever results in the presentation of the stimulus but no food (Joel & Avisar, 2001).

The behaviour of rats undergoing an extinction test preceded by a signal attenuation stage is compared to that of rats in an extinction session that is not preceded by signal attenuation. This is referred to as 'regular extinction' and some rats engage in a high number of excessive lever-presses even after non-reward. The model therefore provides the behavioural measure of 'compulsive' behaviour seen in OCD (Joel & Avisar, 2001). A central aspect of this model is the return (or more importantly, non-return) of rats after the lever-press behaviour to the magazine. Animals that do not return to the magazine is said to have uncompleted trials, and those that do, completed trails.

The critical behavioural measure therefore is the number of excessive lever-presses with uncompleted trials in the signal attenuation model, with completed trails merely reflecting the encounter of non-reward. SRI treatment demonstrated an 'anti-compulsive' effect in the model, i.e., resulted in a reduction in uncompleted trails, supporting the predictive validity of model (Joel & Doljansky, 2003).

2.7.3.1.4 Additional behavioural models

Acral lick dermitis and other models of excessive grooming present with symptoms similar to exaggerated cleaning rituals seen in OCD. Acral lick dermatitis is a disorder of

grooming seen a variety of mammalian species, particularly in large-breed canines (Veith, 1985), and is characterized by excessive licking and biting of extremities. The model has convergent face and predictive validity to OCD, since it responds to SRI treatment (Moon-Fanelli et al., 1999). Feather picking in birds overlaps somewhat with barbering and resembles compulsive skin picking and hair pulling in OCD, thus suggesting some face validity. Feather picking is however classified as an abnormal compulsive behaviour (Garner et al., 2006) and lack supportive predictive validity.

2.7.3.2 Pharmacological models

The foremost criterion for a pharmacological animal model of OCD is that the applied pharmacological challenge should induce behavioural and neurochemical disturbances similar to those found in the disorder, such as compulsive checking (Szecthman et al., 1998) or indecision and perseveration (Yadin et al., 1991). An important consideration for the investigation is that pharmacological studies often are not suited to long-term baseline studies because the animals need to be pharmacologically treated. Prudently selected drug-induced behaviours present with significant face, predictive, and construct validity, but even though these models may provide insight into the underlying pathophysiologic processes in disorders that include stereotypy or compulsivity, they may oversimplify complex neurological disorders (Korff & Harvey, 2006).

2.7.3.2.1 Compulsive checking models

Compulsive checking behaviour is one of the most distinctive behaviours evident in patients diagnosed with OCD (Henderson & Polland, 1988). Szechtman and co-workers developed a model of compulsive checking by chronic treatment of rats with the D_2/D_3 agonist quinpirole (Szecthman et al., 1998). The authors argue that the behaviour of quinpirole-treated rats is similar in several respects to compulsive checking in OCD patients. First, quinpirole-induced compulsive checking meets formal ethological criteria of OCD compulsive checking behaviour, such as a preoccupation with and an exaggerated hesitancy to leave the items of interest, dependence of checking behaviour on environmental context, and a ritual-like motor activity pattern (Szecthman et al., 2001).

Second, equivalent to the compulsions in OCD patients, the compulsive checking in drug-treated rats can be suspended, i.e. ceased or discontinued, for a period of time. Third, the anti-compulsive drug clomipramine has been found to transiently reduce quinpirole-induce checking (Szecthman et al., 2001). On the basis of the latter finding it has been suggested that quinpirole-induce checking may provide a model of only a subtype of OCD, namely, of the subgroup of patients that are less responsive to the effects of SRI treatment (Szecthman et al., 1998) just as marble burying can also be used as a model for hoarding (section 2.3.1.1). The partial reversal of checking behaviour with clomipramine offers some evidence for predictive validity along with strong face validity.

2.7.3.2.2 Spontaneous alternation models

Spontaneous alternation refers to the natural tendency of rats (or any other rodents) to explore novel places sequentially and in succession. Yadin and co-workers were the first to suggest that pharmacologically induced decrease in spontaneous alternation may serve to model indecision often found in OCD (Yadin et al., 1991). In short, food deprived rats were ran in a T-maze in which the two goal boxes were baited with flavoured milk. The mean number of choices made until alternation occurs was recorded, with spontaneous alternators scoring lower than perserverators. Spontaneous alternation was then reduced by acute administration of the non-selective 5-HT agonist 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeODMT) or the 5-HT1a agonist 8-hydroxy-2-(di-*n*-propylamino)-tetralin hydroxybromide (8-OHDPAT). This reduction was prevented by chronic administration of the SRI, fluoxetine (Yadin et al., 1991), but not by the antidepressant desipramine (Fernandez-Guasti et al., 2003).

It is not clear what the decrease in spontaneous alternation is a model of, because motor perseveration is common in neurological and psychiatric disorders other than OCD (e.g. schizophrenia, Parkinson's disease). The question of the importance of pharmacologically induced decreased alternation to OCD becomes even more decisive when considering the fact that spontaneous alternation is highly sensitive to neurochemical interference, with decreased alternation found following manipulations to all of the major neurotransmitter systems, including Glu, GABA, 5-HT, DA, acetylcholine and noradrenaline (Myhrer, 2003). Thus, many different physiological processes are influenced including attentional, emotional, sensory and motor when alternation is decreased. This makes it difficult to

establish if there is any dysfunction present in the model and how this is relevant to OCD pathophysiology.

2.7.3.2.3 Stereotypy models

Stereotypy represents a wide range of invariant and repetitive behaviours and can be provoked in otherwise nonstereotyping animals using specific drug treatment (Roffman & Raskin, 1997). That stereotypy is a primary construct of OCD makes it a particularly useful endpoint for pharmacological challenge studies. Investigations have focussed frequently on the influence of the dopamine system in the expression of these behaviours, for example, using psychostimulants or other dopamine agonists (Roffman & Raskin, 1997).

As dopamine agonists produce stereotypic activity, drugs with dopamine-selective actions will have great value in studying the underlying neurobiology of OCD. Serotonergic agonists such as meta-chlorophenylpiperazine (mCPP) also evoke similar stereotypic responses (Goodman et al., 1991), however, and highlight the complexity of motor function. This phenomenon opens the dispute that stereotypy and other behaviour manifestations of OCD may represent a cross-talk between 5-HT and DA projections in the CSTC circuit (Goodman et al., 1991). Drug-induced stereotypy animal models will provide valuable insight into this particular neurobiological aspect of OCD.

2.7.3.3 Genetic animal models

Genetic animal models can be useful in identifying specific genes and neurobiological pathways thought to be important in OCD. It is important to note that the models currently in use are not genetic models in the sense that they were created on the basis of a known mutation in humans. Rather, these models are based on behavioural similarity, that is, the behaviour of genetically engineered mice was found to be similar in specific respects to that of OCD patients. A number of specific genetically engineered animal models of OCD have been developed, usually according to the research group's hypothesis concerning the underlying cause of OCD or OCD symptoms. Although transgenic models have great potential, an important question is whether the behaviour is

a direct result of the specific manipulated gene or caused by other targets lying downstream of the modified gene.

2.7.3.3.1 D1CT-7 transgenic model

The D1CT-7 transgenic mouse models engages in episodes of perseverance or repetition of a range of normal and abnormal behaviours, for example, repeated non-aggressive biting and skin pulling of cage mates during grooming (Campbell et al., 1999). The mice are engineered to express a neuro-potentiating cholera toxin transgene in a subset of dopamine D_1 receptor-expressing neurons that induce cortical and amygdalar glutamate output. The engineered mice were also shown to exhibit an increased basal level of anxiety compared to non-transgenic siblings (MacGrath et al., 1999.) In addition to good face validity, construct validity is illustrated by exacerbation of symptomatic behaviour by glutamatergic drugs (MacGrath et al., 2000). However, predictive validity of the model still requires study.

2.7.3.3.2 Sequential stereotypy owing to dopamine transporter knockdown

In this animal model, mice with a knockdown of the dopamine transporter (DAT) have been developed (Zhuang et al., 2001). The result is an impaired synaptic reuptake of dopamine and elevated levels of extracellular DA in the neostriatum resulting in spontaneous, over-rigid and serially complex behaviour in the form of grooming. Because the behavioural sequence of the mutant mice was more predictable and stereotyped, it is referred to as sequential super-stereotypy (Berridge et al., 2004). The model is yet to be tested for predictive validity, but good face and construct validity are present. The model differs from other genetic models in that the overly rigid and serially complex behaviour allows highly predictable stereotyped patterns that can be easily quantified.

2.7.3.3.3 Compulsive behaviour owing to 5-HT_{2C} receptor knockout

Mice with targeted gene disruption of the 5-HT_{2C} receptor have been developed that display increased compulsive-like behaviour such as clay and screen chewing and head dipping (the latter being head dipping into a hole located in the centre of an elevated board and a measure of novelty) (Nonogaki et al., 1998; Chou-Green et al., 2003). These

mice exhibit an increased responsiveness to novelty and increased sensitivity to the psychostimulant and reinforcing effects of cocaine as well as cocaine-induced increase in nucleus accumbens DA levels (Rocha et al., 2002). The behavioural and neural abnormalities may contribute to our understanding of OCD, but once more, predictive validity of this model remains to be determined.

2.7.3.3.4 *Hoxb8* model

Compulsive behaviour in OCD may have several features, including both perserverative tendencies and more rigid sequences of entire serial patterns. In the *Hoxb8* mutant model, mice with disruptions in *Hoxb8* show excessive and increased persistence in grooming (Greer & Capecchi, 2002). *Hoxb8* is expressed in the orbital cortex, the anterior cingulated cortex, the striatum and the limbic system, all of which are implicated in the pathophysiology of OCD (section 1.4.1). As is the case with regard to the D1CT model, the *Hoxb8* model is promising in that excessive grooming has face similarity to the symptoms observed in OC spectrum disorders (such as trichtillomania) and may involve neural systems similar to those involved in compulsive behaviour in patients. However, the model currently lacks predictive validity.

2.8 Stereotypic behaviour

Normal behaviour plays a key role in facilitating homeostasis, especially by allowing the subject to control and modify its environment. Captive environments may interfere with these behavioural responses, and the resulting stress may alter many physiological parameters (Garner, 2005). Abnormal behaviours, particularly abnormal repetitive behaviours (ABRs) indicate that the animal is unable to adjust behaviourally to its captive environment and, hence, may be expressing abnormal physiology. ABRs in captive animals appear to involve the same mechanisms as ABRs in human psychiatry, which reflect underlying abnormalities of brain function (Garner, 2005).

ABRs are defined as behaviours that are inappropriate, repetitive and unvarying in either goal or motor pattern and may be subdivided into two basic categories namely stereotypies and impulsive/compulsive behaviours (Garner, 2005). Stereotypic behaviour refers to the repetitive behaviour induced by frustration, repeated attempts to cope and/or

CNS (brain) dysfunction (Mason et al., 2007), and is said to lack any goal or function. Compulsive behaviours involve the repetition of an inappropriate goal with variable flexible goal-directed behaviour.

OCD is a neuropsychiatric disorder characterized by obsessions and compulsions (section 2.2). Obsessions refer to recurring thoughts, impulses or images that intrude into awareness, whereas compulsions are the need to repeat physical behaviours such as checking or mental behaviours such as counting things (Rasmussen & Eisen, 1992). Surveys on OCD indicate that an ethological approach in the study of compulsive rituals may reveal the structure of such behaviour, which could prove valuable in uncovering the underlying mechanisms. Indeed, studies of human compulsions frequently describe the profuse rate of performance of behavioural patterns using terms borrowed from ethology such as 'displacement activity' and 'stereotypy' (Insel, 1988). Altogether, the repetitive performance of motor rituals constitutes the focus of recent surveys (Eilam et al., 2006; Rapp & Vollmer, 2005), which highlight the fact that normal behaviour, cage stereotypies, drug-induced animal stereotypies, and psychopathological behaviour of OCD all feature similar spatiotemporal structure.

The term stereotypies and OCD are increasingly used as being synonymous (e.g. Luescher et al., 1991). However, there are overt behavioural differences. OCD is classified as an anxiety disorder (American Psychiatric association, 2000) and patients engage in compulsive behaviours to reduce the anxiety caused by the obsessions. It is true that OCD behaviours can be performed in a relatively invariant way and be repeated, but they lack the rhythmic repetitive character of stereotypies. In humans, at a cognitive level, patients suffer from their OCD and try to resist them, while stereotypies are often performed unconsciously or without resistance (Tynes et al., 1990). Nonetheless, even if it has not been proven that stereotypies and OCD are similar they share a number of mutually relevant characteristics such as morphological identity, compulsivity, and underlying neurochemicals (5-HT and dopamine).

2.9 Cellular signalling, neurochemicals and receptors: significance in OCD

2.9.1 Serotonin

Of the neurotransmitters, 5-HT is perhaps most implicated in the aetiology and treatment of various disorders of the central nervous system including anxiety, depression, OCD, stroke, obesity, pain, hypertension, vascular disorders, migraine, and nausea (Baskys & Remington, 1996; Bloom, 2001). 5-HT is synthesized in situ from tryptophan through the actions of the enzymes tryptophan hydroxylase and aromatic L-amino acid decarboxylase. Both dietary and endogenous 5-HT are rapidly metabolised and inactivated by monoamine oxidase and aldehyde dehydrogenase to the major metabolite, 5-hydroxyindoleacetic acid (Baskys & Remington, 1996). The functions of 5-HT are numerous and appear to involve control of appetite, sleep, memory and learning, temperature regulation, mood, behaviour, cardiovascular function, muscle contraction, and endocrine regulation (Baskys & Remington, 1996; Bloom, 2001).

As in the case with all chemical neurotransmitters, 5-HT is synthesized in brain neurons and stored in synaptic vesicles. Upon a nerve impulse, 5-HT is released into the synaptic cleft, where it interacts with various postsynaptic receptors, transporter proteins and autoreceptors. The actions of 5-HT are terminated by diffusion, metabolism, or uptake back into the synaptic cleft through the action of specific amine membrane transporter systems (Bloom, 2001).

The 5-HT receptors have been divided into seven subfamilies by convention and are characterized by overlapping pharmacological properties, amino acid sequences, gene organization, and second messenger coupling pathways. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel related to *N*-methyl-D-aspartate (NMDA), GABA, and nicotinic receptors, all of the 5-HT receptor subtypes belong to the groups of G-protein linked receptors (Raymond et al., 2001). The predominant neurobiologic hypothesis regarding the pathophysiology of OCD is that at least some aspects of the disorder are related to abnormal regulation of brain serotonergic function. Since 5-HT receptors are crucial for 5-HT function, key features of the 5-HT receptors are now discussed.

2.9.1.1 5-HT_1 receptor

There are five members of the 5-HT_1 receptor family, termed 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}. A receptor formerly termed 5-HT_{1C} receptor is no longer felt to be a member of the 5-HT_1 receptor family, having been reclassified as the 5-HT_{2C} receptor based on similarities to other 5-HT_2 receptors in structure and second messenger systems. The 5-HT_1 receptors couple primarily through $G_{i/o}$-proteins to the inhibition of adenylyl cyclase (AC) and a multitude of other signalling pathways and effectors (Raymond et al., 2001).

The 5-HT_{1A} receptor plays potential roles in neuro-endocrine function and thermoregulation (Seletti et al., 1995), memory (Edagawa et al., 1998), mood (Blier et al., 1997) and anxiety (Parks et al., 1998). The 5-HT_{1A} receptor couples to a broad range of second messengers and is reported to activate and inhibit AC, activates K^+ channels, stimulates extracellular signal-regulated kinase (ERK) and inhibits Ca^{+2} conductances (Raymond et al., 2001). Administration of a 5-HT_{1A} receptor agonist to rats induces a significant increase in repetitive behaviour (Seibell et al., 2003). Based on this evidence and other studies where 5-HT_{1A} agonists (Zohar & Insel, 1987) have been used, 5-HT_{1A} receptors may perhaps play a crucial role in the compulsive behaviour seen among patients with OCD (Seibell et al., 2003).

The 5-HT_{1B} receptor is widely expressed in brain tissue, probably in both presynaptic and postsynaptic locations. The 5-HT_{1B} receptor has been shown to inhibit AC, activate Akt kinase and ERK, and stimulate endothelial nitric oxide production. It also activates Ca^{+2} – dependent K^+ channels and activates phospholipase C (PLC). Several groups have demonstrated that presynaptic 5-HT_{1B} receptors also decrease 5-HT release (Raymond et al., 2001).

5-HT_{1D} receptor activation reduces adenosine 3', 5'-cyclic monophosphate (cAMP) levels through inhibition of AC, regulates K^+ and Ca^{+2} channels and stimulates DNA synthesis. 5-HT_{1D} receptors can inhibit 5-HT release in several brain regions including the hippocampus, frontal cortex and mesencephalic raphe (Raymond et al., 2001). Both 5-HT_{1B} and 5-HT_{1D} receptors mediate more or less the same behavioural responses, which is to increase locomotor activity. The 5-HT_{1D} is furthermore thought to play an important

role in OCD (El Mansari et al., 1995). The 5-HT_{1D} receptor appears to play an important role in OCD since terminal 5-HT_{1D} autoreceptors have been implicated in the anti-OCD effect of SRIs (El Mansari et al., 1995). Moreover, genetic studies have also shown that a specific polymorphism of the 5-$HT_{1D\beta}$ gene may be preferentially transmitted in patients with OCD (Camarena et al., 2004).

There are few details about signalling pathways linked to the 5-HT_{1E} receptor. It has been shown to inhibit AC at low agonist concentrations, although it can also stimulate AC at high concentrations of agonist (Adham et al., 1994). The 5-HT_{1E} receptor has not been demonstrated to stimulate PLC, the release of inositol phosphates or intracellular Ca^{+2} (Adham et al., 1994). The 5-HT_{1F} receptor couples negatively to AC and has been shown to either activate or inhibit PI or Ca^{+2} in a cell-specific manner (Adham et al., 1994). This receptor is mainly found in the neocortical layers and thalamic nuclei of the brain (Raymond et al., 2001).

2.9.1.2 5-HT_2 receptor

There are three members of the 5-HT_2 receptor family, termed 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} all of which couple consistently to the PLC-β second messenger pathway (Peroutka, 1995). Although the 5-HT_2 receptors are similar in structure, pharmacology, and signalling pathways, there are a few differences in their signalling properties. The 5-HT_{2A} receptor is found in many forebrain areas, particularly in cortical areas, caudate nucleus, nucleus accumbens and hippocampus (Pazos et al., 1985). The 5-HT_{2A} receptor activates PLC-β, resulting in accumulation of inositol phosphates and elevation of intracellular Ca^{+2}. The 5-HT_{2A} receptor does not typically regulate cAMP formation in most cells or tissues, but it can both stimulate and diminish cAMP accumulation in specific cell types (Berg et al., 1994). 5-HT_{2A} receptors also have a unique role in that it is internalized in response to both agonists and antagonists. This feature of 5-HT_{2A} may play an important role in signalling and in the actions of antipsychotic medication (Bhatnagar et al., 2000).

The distribution of the 5-HT_{2B} receptor is limited to the cerebellum, lateral septum, hypothalamus, and medial amygdala (Pazos et al., 1985). This receptor activates PLC, can stimulate cAMP accumulation, activates ERK and causes production of reactive nitrogen

species (Raymond et al., 2001). The 5-HT_{2C} receptor is found primarily in the chorid plexus and in the limbic areas of the brain (hypothalamus, hippocampus) and in regions associated with motor behaviour such as the substantia nigra and the globus pallidus (Frazer & Hansler, 1999). The 5-HT_{2C} receptor activates PLC, can regulate K^+ and Cl^- channels as well as nitric oxide levels. Activation of these receptors leads to hypolocomotion, anxiety and even hyperthermia (Frazer & Hansler, 1999). Both 5-HT_{2A} and 5-HT_{2C} receptor antagonism have been postulated to play a role in the generation of obsessive-compulsive-symptoms in patients with psychotic disorder and recently these receptors have been implicated in OCD (El Mansari & Blier, 2006).

While some evidence implicates the 5-HT_{2C} receptor in OCD, a definitive function has yet to be validated since 5-HT_{2C} agonists (Zohar & Insel, 1987) and antagonists (Ramasubbu et al., 2000) have been shown to produce and exacerbate symtoms of OCD. 5-HT_{2C} receptor knockout mice have been proposed as an animal model of compulsive behaviour (Chou-Green et al., 2003) and this model may provide a means to investigate the neurobiological underpinnings of compulsive-like behaviour seen in OCD (see section 2.7.3.3.3).

2.9.1.3 5-HT_3 receptor

The 5-HT_3 receptor is the only ligand-gated ion channel of the 5-HT family and is found predominantly in the dorsal vagal complex in the brain stem (Frazer & Hansler, 1999). Expression in the forebrain is low, but 5-HT_3 is found in areas such as the hippocampus, amygdala and the outer layers of the cerebral cortex. 5-HT_3 receptors facilitate the release of the neuropeptide, substance P, while 5-HT_3 receptor antagonists may have anxiolytic and antidepressant activities (Frazer & Hansler, 1999).

2.9.1.4 5-HT_4 receptor

The 5-HT_4 receptors are found in high densities in the striatum, substantia nigra and olfactory tubercle (Frazer & Hansler, 1999). 5-HT_4 receptors stimulate AC activity with most of its effects due to activation of protein kinase A (PKA) (Raymond et al., 2001). 5-HT_4 receptors also regulate a variety of channels including L-type Ca^{+2} channels as well as K^+ channels.

2.9.1.5 5-ht$_5$ receptor

Currently, little is known about 5-ht$_5$ receptors and, as functional 5-ht$_5$ receptors have not yet been identified in vivo, the lower case designation is used. At present, two related receptor subtypes, the 5-ht$_{5a}$ and the 5-ht$_{5b}$, have been cloned from rat and mouse, but only 5-ht$_{5a}$ has been cloned from human (Raymond et al., 2001). Functionally these receptors remain to be classified, although it is known that they are G-protein coupled receptors.

2.9.1.6 5-HT$_6$ receptor

5-HT$_6$ receptors are found primarily in the striatum, olfactory turbucles, nucleus accumbens, caudate nucleus and the hippocampus (Frazer & Hansler, 1999). The 5-HT$_6$ receptor stimulates AC and has high affinity for typical and atypical antipsychotics, including clozapine (Raymond et al., 2001). Surprisingly, 5-HT$_6$ receptors appear to regulate cholinergic (rather than dopaminergic) neurotransmission in the brain, implicating it as target for the treatment of learning and memory disorders (Brancheck & Blackburn, 2000).

2.9.1.7 5-HT$_7$ receptor

The 5-HT$_7$ receptor is the third 5-HT receptor subtype shown to couple to G_s (the other two being 5-HT$_4$ and 5-HT$_6$). It is highly expressed in the central nervous system, especially in the hippocampus, the hypothalamus, and the neocortex (Frazer & Hansler, 1999). 5-HT$_7$ receptors activate ERK stimulates vasorelaxation and activates AC and is thought to be involved in the control of circadian rhythm due to its expression in the suprachiasmatic nucleus (Frazer & Hansler, 1999).

2.9.1.8 Serotonin receptors and relevance to OCD

Potent SRIs are the only antidepressant agents thus far shown to be effective in the treatment of OCD (section 2.6). It has been shown that following chronic SRI treatment, 5-HT release is enhanced in the frontal cortex, hypothalamus and OFC structures of OCD patients (Bergqvist et al., 1999). This increase is attributable to a desensitization of,

amongst other, the terminal presynaptic 5-HT_{1D} autoreceptor (Bergqvist et al., 1999). The responsiveness of postsynaptic 5-HT receptors is also important and contributes to alter 5-HT transmission in OCD during SRI treatment. Subsequent to SRI treatment, postsynaptic 5-HT_{1A} as well as 5-HT_2 receptors are also desensitized (El Mansari & Blier, 2005). The 5-HT_2 receptors mediating the anti-OCD effect most likely includes the 5-HT_{2A} and 5-HT_{2C} receptors. Interestingly, observations using 5-HT_2 receptor agonists and antagoninsts indicate that by enhancing the activation of 5-HT_2 receptors with selective agonists, a beneficial effect in OCD might be obtained, whereas by decreasing transmission at these sites with high doses of antagonists, an exacerbation of OCD symptoms may occur in patients improved by a SRI (Bergqvist et al., 1999).

Although not all studies are consistent (Goodman et al., 1995; Khanna et al., 2001), the non-selective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist, mCPP (Barnes & Sharp, 1999), has been found to exacerbate obsessive compulsive symptoms in OCD patients (Zohar et al., 1987; Goodman et al., 1995), underscoring the role of 5-HT and the 5-HT_2 receptors in OCD. The intensifying of these key symptoms of OCD is supported by evidence that the 5-HT_{2A} receptor has been implicated in OCD (Andrade, 1998), that 5-$HT_{2A/2C}$ receptor antagonists are anxiolytic in animal models of anxiety (Brocco et al., 1998; Griebel et al 1997) and that mCPP is anxiogenic in these models (Griebel 1995; Griebel et al., 1997). Furthermore, the established efficacy of SRIs in the treatment of OCD has been hypothesised to be due to these drugs causing desensitisation of pre-synaptic terminal 5-HT_{1A} and 5-HT_{1D} receptors (Bergqvist et al., 1999; El Mansari & Blier 2006) resulting in an increase in serotonergic activity in sub-cortical regions of the brain, particularly the CSTC circuit (Korff and Harvey 2006).

2.9.2 Dopamine

Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. Dopamine also plays multiple roles in the periphery as modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility (Bloom, 2001).

Dopamine is synthesized in the body (mainly by nervous tissue and adrenal glands) first by the hydration of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase and then by the enzymatic decarboxylation of DOPA. In neurons, dopamine is packaged after synthesis into synaptic vesicles, and released in a Ca^{+2} –dependent manner in response to the presynaptic action potential (Bloom, 2001). The inactivation mechanisms of dopamine neurotransmission involve uptake via specific dopamine transporters, enzymatic breakdown, and diffusion into neighbouring neurons and tissue. Uptake back into the presynaptic neuron via the dopamine transporter occupies a major role in inactivating dopamine neurotransmission.

Dopamine has many functions in the brain. Most importantly, dopamine is central to the reward system where it has a role in prioritizing and learning of reward-directed behaviour. Dopamine is commonly associated with the pleasure system of the brain, providing feelings of enjoyment and reinforcement to perform certain activities. Dopamine is released by naturally rewarding experiences such as food, sex, use of certain drugs and neutral stimuli that become associated with them (Wickens et al., 2003). Dopamine is moreover released when unpleasant stimuli are encountered, and so motivates towards the pleasure of avoiding or removing the unpleasant stimuli. Dopamine furthermore affects the basal ganglia motor loop which in turn affects the way the brain controls movements. Reward is important in motor learning and the integration of reward into behaviour occurs where rewards-related neural signals meet circuits concerned with motor performance (Wickens et al., 2003).

Upon a nerve impulse, dopamine is released into the synaptic cleft where it interacts with various postsynaptic receptors as well as dopamine transporter proteins. Currently, five dopamine receptors have been structurally characterized and all are G-protein coupled receptors (Jaber et al., 1996). Dopamine receptors can be divided into two subtypes namely the D_1-type and the D_2-type of receptors. D_1-class receptors include D_1 and D_5 receptors and the D_2-class include D_2, D_3, and D_4 receptors (Jaber et al., 1996).

In recent years, evidence has accumulated showing that the dopaminergic system might be involved in OCD (Goodman et al., 1990). Preclinical evidence includes experimental animal models of induced stereotypies through increased dopaminergic transmission (Randrup & Munkvad, 1967). In fact, putative animal models for OCD depend primarily

on changes in the dopaminergic system, e.g. rats treated chronically with the selective $D_{2/3}$ receptor agonist, quinpirole develop compulsive checking (Szechtman et al., 1998). A brief description of dopamine receptors is now provided along with their relevance to OCD.

2.9.2.1 D_1 receptors

Activation of D_1-type receptors increases cAMP levels by stimulating AC activity via a G_s-dependent manner. Dopmine receptors are furthermore associated with G-proteins other than G_s and G_i and can affect multiple second messenger systems in a brain region-specific manner (Jaber et al., 1996). In addition to increasing cAMP, D_1 activation increases phosphatidylinositol (PI) turnover and may be involved in regulating intracellular Ca^{+2} levels (Jaber et al., 1996).

The activation of dopamine autoreceptors inhibits dopamine transmission by decreasing dopamine release, firing rate, and dopamine synthesis. In humans, the D_1 receptors are predominantly found in the basal ganglia followed by the substantia nigra and the cerebral cortex (Baskys & Remington, 1996). D_5 receptors are found in the hippocampus, hypothalamus and the cerebellum and it too is involved in the stimulation of AC and increasing cAMP levels.

2.9.2.2 D_2 receptors

D_2-type receptors decrease AC activity via G_i-type G-proteins and have been reported to increase PI hydrolysis (Jaber et al., 1996). D_2 receptors may also regulate amongst other, phospholipase A2, intracellular Ca^{+2} levels, and K^+ currents. Compared with postsynaptic D_2 receptors, D_2 autoreceptors have a 5- to 10-fold higher affinity for dopamine and certain dopamine receptor agonists. At low doses of dopamine receptor agonists, activation of D_2 autoreceptors leads to diminished dopamine function, whereas at higher doses postsynaptic D_2 receptors are also activated, resulting in enhanced dopaminergic transmission (Bloom, 2001).

Activation of D_2 autoreceptors has furthermore been shown to decrease locomotor activity, whereas activation of postsynaptic D_2 receptors slightly increases locomotion

(Missale et al., 1998). Since D_1 receptors are also implicated in locomotion, a close balance between D_1/D_2 receptors function is necessary to ensure normal locomotor behaviour (see figure 2.1). D_2 receptors are found in the caudate putamen, the nucleus accumbens and the olfactory tubercle, and are part of the mesolimbic, nigrostriatal and tuberoinfundibular pathways (Missale et al., 1998). D_3 receptors are found in the hypothalamus and the nucleus accumbens, while D_4 receptors are found mostly in the hippocampus, the thalamus and the cortex.

Both D_3 and D_4 receptors have been shown to inhibit AC and are positively coupled to K^+ channels (Jaber et al., 1996). Interestingly, there are two isoforms of the D_2 receptor: the D_2L (long form) and the D_2S (short form). The two isoforms are generated by alternative splicing from the same gene. To date, no pharmacological agents exist that are selective for a specific D_2 receptor isoform (Fetsko et al., 2003).

2.9.2.3 Dopamine receptors and relevance to OCD

Typically, D_1 and D_2 receptors exert opposing actions on intracellular signalling molecules and they often have disparate physiological effects. Recent evidence show that DA exerts concentration-dependent effects on cortical inhibition and of particular interest is the fact that low DA concentrations act via D_1 receptors and PKA to increase GABA release and that high DA concentrations act via D_2 receptors through a complex signalling pathways to reduce GABA currents (Trantham-Davidson et al., 2004). GABA is the major inhibitor neurotransmitter in the mammalian central nervous system (see section 2.9.4).

In a study by Denys and co-workers (Denys et al., 2004), abnormalities in the binding potential of the dopamine D_2 receptor was shown in OCD patients, which suggest an involvement of the dopaminergic system in the pathophysiology of OCD. Here, D_2 receptor binding in the left caudate nucleus was significantly lower in the patients with OCD than in healthy control subjects. D_2 receptors may also play a role in the anxiety component of OCD, since D_2 receptor antagonists reduce anxiety-like activity in rodents, suggesting a specific role for the D_2 receptor in mediating fear or anxiety-like responses (Pich & Samanin, 1986).

However, D_1 receptors are also implicated in the pathophysiology of OCD. As was pointed out in an earlier section (section 2.7.3.3.1), D1CT-7 transgenic mice that engage in episodes of perseverance or repetition, was engineered to express a neuro-potentiating cholera toxin transgene in a subset of D_1 receptor-expressing neurons (Campbell et al., 1999). Because excessive cortical-limbic stimulation of striatal motor pathways may play a critical role in causing compulsive disorders, a clear understanding of D_1 receptors involvement is essential. The relevance of the D_1 receptor in the D1CT-7 mouse model would seem to be that, although the major physiological and behavioural effects are mediated through D_2 receptors, D_1 receptors are very important in regulating D_2 function and output (Campbell et al., 1999).

In a recently developed animal model (the signal attenuation model, see section 2.7.3.1.3) developed on the basis that there is a deficiency in response feedback underlying obsessions and compulsions. D_1 receptor responses were implicated in the production of compulsive lever-pressing in this model and the authors were left to conclude that these receptors may be important in the development of compulsive behaviour seen in OCD (Joel & Doljansky, 2003). In this model, D_2 receptors may be important in regulating D_1 receptor function and output.

Several lines of evidence suggest that concurrent activation of D_1 and D_2 receptors is required for certain dopamine -mediated responses, particularly stereotyped behaviour (Fetsko et al., 2003). In certain cases, activation of the D_1 receptor has been shown to facilitate the effect of D_2 receptor stimulation through a synergistic phenomenon termed dopamine D_1/D_2 receptor synergism (Marshall et al., 1997). To illustrate this, the dopamine agonist-induced stereotypy and climbing can be blocked by either a D_1 or D_2 receptor antagonist (Moore & Axton, 1998). Thus, D_1/D_2 synergism represents an important aspect of dopaminergic function and may have important clinical application in OCD.

2.9.3 Glutamate

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate acts mainly post-synaptically on three families of ligand-gated ion channels receptors (Tapiero et al., 2002). Although permeable to cations primarily,

the permeability to Na^+ and Ca^{+2} varies according to the family and the subunit composition of the receptor (Bloom, 2001). Glutamate is synthesized from glutamine by glutaminase within mitochondria, but it is also synthesized from aspartate by transamination or via the tricarboxlic acid cycle from glucose (Tapiero et al., 2002). Subsequent to synthesis, glutamate is packaged into vesicles for future release into the synaptic cleft (Tapiero et al., 2002).

Upon release, glutamate is either bound to pre- and post-synaptic receptors, is actively taken back up into the neuron via a glutamate transporter and repackaged, or diffuses away from the cleft, or is internalised by glial glutamate transporters (Bloom, 2001). Glutamate release occurs via a Ca^{+2}-dependent mechanism that involves voltage-dependent calcium channels (Meldrum, 2000). The release of a single vesicle produces an excitatory post-synaptic potential (EPSP).

Glutamate is found throughout the mammalian brain, participates in many metabolic pathways and plays key roles in many physiological processes including learning and memory, central pain transduction, activity dependent plasticity as well as pathological processes such as neurodegeneration, epilepsy, amnesia, and cerebral ischemia (Bloom, 2001; Meldrum, 2000). Glutamate receptors are distributed throughout the brain with little selectivity for specific brain areas. Glutamate receptors are present in all nuclei of the basal ganglia, but the highest densities are found within the striatum (Albin et al., 1992). The striatum-nucleus accumbens complex receives the greatest amount of glutamatergic innervation not only from the neocortex but also from the hippocampus, the amygdala and the thalamus (Albin et al., 1992).

Glutamate receptors are classified functionally either as ligand-gated ion channel (ionotrophic) or as G-protein coupled (metabotropic) receptors. Ionotrophic glutamate receptors are further classified according to the identity of agonist that selectively activates each receptor subtype (Bloom, 2001). These receptors include α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and NMDA. Glutamate is also capable of activating metabotropic receptors that are coupled via second messenger systems to biochemical pathways and ion channels. Metaboptropic receptors

seem to play a modulatory role in CNS function, regulating both neuronal exitability and the release of neurotransmitters (Doherty & Dingledine, 2002).

2.9.3.1 NMDA receptors

The NMDA receptor has been studied extensively, especially once it was discovered that activation of this receptor underlies different forms of synaptic plasticity (Dingledine et al., 1999). The NMDA receptor channel preferentially permits Ca^{+2} entry and the kinetics of this channel are much slower than that of either AMPA or kianate channel (Hudspith, 1997). NMDA receptors are composed of assemblies of NR1 subunits and NR2 subunits. Both subunits are required to form functional channels (Dingledine et al., 1999).

The gating properties of the NMDA receptor channel are complex and subject to modulation at several sites. In addition to its binding site for excitatory amino acid such as glutamate, the receptor has a binding site for glycine which facilitates the actions of glutamate or NMDA, as well as for various agents/molecules such as polyamines, protons and redox agents (Dingledine et al., 1999).

Interestingly, in the resting (non-depolarized) state, the NMDA receptor channel is blocked by Mg^{+2} ions at a site deep within the channel itself and binding of NMDA or glutamate to the agonist binding site, even in the presence of glycine, does not result in Ca^{+2} entry through the postsynaptic membrane (Dingledine et al., 1999). Depolarization of the membrane however, removes the Mg^{+2}-mediated channel blockade thus allowing Ca^{+2} flux through the channel (Dingledine et al., 1999).

Studies making use of NMDA receptor agonists and antagonists yielded interesting findings, particularly looking at behaviour. When MK-801, a NMDA antagonist is given to rodents, it produces very strong stereotypic and perseverative behaviour and also enhances sniffing behaviour and reduces vertical activity (Schmidt & Kretschmer, 1997). Administration of NMDA itself does not induce catalepsy in rats, but at very high doses, it induces convulsions (Schmidt & Kretschmer, 1997). NMDA, when administered bilaterally into the striatum-accumbens induces behavioural depressant or no effects: and when injected into the anterodorsal striatum it reduces locomotion and rearing (Schmidt

& Kretschmer, 1997). In some cases it has no effect on stereotyped behaviour (Svensson et al., 1994).

2.9.3.2 AMPA receptors

AMPA receptors are responsible for most rapid excitatory transmission within the vertebrate brain. The affinity for glutamate at AMPA receptors is much lower than its affinity at NMDA receptors (Dingledine et al., 1999). The associated channels are rapidly activated and inactivated, and appear to be present on all neurons within the CNS, although there are regional density differences (Dingledine et al., 1999). There are two agonist-binding sites on each AMPA receptor, with activation of the channel occurring only following occupation of both sites by the ligand. Following binding of ligands, the protein conformation of AMPA receptor is altered, the ion channel opens, cations enter, and this leads to formation of an EPSP (Dingledine et al., 1999). In general, non-NMDA receptors exhibit little permeability to calcium unless the receptor lacks a particular (GluR2) subunit.

The AMPA receptor antagonists of the 2, 3-benzodiazepines group have some remarkable pharmacological properties of possible value in the treatment of various neurological disorders. Members of this AMPA antagonist group have been shown to be effective as anxiolytics, anti-psychotics as well as dopamine uptake inhibitors (Szenasi & Harsing, 2004). AMPA antagonists are also of possible clinical value in amyotrophic lateral sclerosis, Parkinson's disease and multiple sclerosis. Recent studies have identified that the activity and synaptic distribution of AMPA receptors is dynamically regulated and could be crucial for the short- and long-term modification of synaptic efficiency (Szenasi & Harsing, 2004).

Ampakines are a structurally diverse family of small molecules that positively modulate AMPA receptors, and thereby enhance fast excitatory transmission throughout the brain (Granger et al., 1993). Surprisingly, ampakines have discrete effects on brain activity and behaviour (Mattson, 2003). Ampakines bind to a site on the AMPA receptor but have no agonist or antagonist effect; instead they stabilize the receptor in its channel-open state following the binding of released transmitter (glutamate) (Granger et al., 1993). This prolongs current flow through the receptor and thus enhances synaptic responses. Because

the excitatory synaptic targets of ampakines mediate communication between cortical regions which serve as sites of memory encoding and regulating the production of growth factors (Mattson, 2003), these drugs have a broad range of potential therapeutic applications. Recent findings indicate that ampakines may be beneficial for schizopherenia (Johnson et al., 1999), attention-deficit hyperactivity disorder (Gainetdinov et al., 2001) and depression (Li et al., 2003).

2.9.3.3 Kainate receptors

As with AMPA and NMDA receptors, kainate receptors present with two agonist-binding sites with both low and high affinity receptors having been identified. Kainate receptors are widely distributed, with high expression levels in several forebrain areas (Tapiero et al., 2002). A particular neurotoxin, kainate, binds to a specific high affinity kainate receptor and activates a rapidly desensitisizing Na^+ channel. Kainate also binds to the AMPA receptor with lower affinity resulting in persistent, non-desensitizing activation of the AMPA receptor channel. Currently the distinction between AMPA and kainate receptors is somewhat blurred and is dependent on classification according to gating or ligand binding. However, activation of either receptor by endogenous glutamate results in rapid, although transient, EPSP (Dingledine et al., 1999).

2.9.3.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors describe receptors activated by glutamate and couple via second messenger systems to various subcellular biochemical pathways. Presently, eight metabotropic glutamate receptors can be identified and split into three different groups according to their second messenger systems, sequence homology and agonist/antagonist pharmacology (Dingledine et al., 1999). Group I metabotropic receptors (mGlu1, mGlu5) are coupled to PLC and intracellur calcium signalling, while group II (mGlu2, mGlu3) and group III (mGLu4, mGlu6, mGlu7, mGlu8) metabotropic receptors are negatively coupled to AC (Dingledine et al., 1999). Metabotropic receptors are not composed of subunits that form an integral ion channel but instead are composed of polypeptides that have a putative seven trans-membrane spanning domain (Pin & Acher, 2002).

When an agonist binds to the metabotropic receptor, activation of a variety of G-proteins occur, resulting in the modulation of a range of cellular functions, including current flow through voltage-gated ion channels (Pin & Acher, 2002). Activation of this class of glutamate receptors has been implicated in a variety of CNS functions, including different forms of synaptic plasticity as well as excitotoxicity (Dingledine et al., 1999).

2.9.3.5 Glutamate and OCD

Functional, structural and imaging studies have implicated dysfunction in either the direct or indirect loops of the CSTC circuit in OCD (Saxena & Rauch, 2000; also see section 2.4.2), where the predominant neurotransmitter is glutamate. Recent studies have suggested that OCD is a result of glutamatergic excess (Chakrabarty et al., 2005; Carlsson, 2001). It is also significant that excitatory inputs in these CSTC pathways are predominantly glutamatergic, whereas inhibitory ones are predominantly GABAergic. It has been hypothesized that overactivity of the direct pathway or underactivity of the indirect pathway leads to OCD (Saxena & Rauch, 2000). Considering the predominant neurotransmitters involved in these pathways, the direct pathway over-activity model of OCD suggests glutamatergic excess in certain brain regions in OCD, which is compatible with increased cerebrospinal fluid glutamate levels found in OCD patients (Chakrabarty et al., 2005). This finding is attractive however, one need to keep in mind the broad metabolic effect of glutamate and that increased cerebrospinal fluid glutamate may not necessarily the reflect increased glutamatergic activity in cortical and subcortical pathways.

The findings that glutamatergic dysfunction is related to the pathophysiology of OCD has been supported by findings from animal studies. MK-801, a noncompetitive NMDA receptor antagonist that indirectly stimulates cortical-limbic glutamate output, has been found to aggravate transgene dependent abnormal behaviour in a transgenic mouse model of Tourette's syndrome and OCD (McGrath et al., 2000). Despite the postulated role of glutamate in the pathogenesis of OCD, there have been few studies in which candidate genes in the glutamate system have been tested for association with OCD. A recent study did however indicate the association of a glutamate NMDA subunit receptor gene (GRIN2B) with OCD (Arnold et al., 2004). Here, specific variants for GRIN2B were found to be present in OCD patients.

Preliminary findings have provided evidence of glutamate abnormalities in the caudaute nucleus of OCD patients (Rosenberg et al., 2000). Caudate glutamatergic concentrations were significantly greater in treatment-naïve patients than in controls, but declined significantly after treatment with the SRI, paroxetine. In a recent study, anterior cingulated glutamatergic concentrations were also found to be significantly reduced in pediatric patients with OCD (Rosenberg et al., 2004) compared to healthy controls. These findings support the role of altered glutamatergic neurotransmission in the pathogenesis of OCD.

2.9.4 GABA

GABA is believed to be the most important inhibitory neurotransmitter in the mammalian CNS. GABA mediates inhibition within the cerebral cortex and between the caudate nucleus and the substantia nigra (Bloom, 2001). Presumptive GABAergic inhibitory synapses have also been demonstrated in neurons in the cerebellum, cerebellar cortex, hippocampus and trochlear motorneurons (Waagepetersen et al., 2003). GABA is synthesized from glutamate by glutamic acid decarboxylase. Following release, the majority of released GABA is transported back into the presynaptic nerve ending whereas a much smaller fraction is taken up by astrocytes surrounding the synapse (Waagepetersen et al., 2003).

There are two subtypes of GABA receptors namely, $GABA_A$ and $GABA_B$. It is increasingly being appreciated that GABA neurotransmission through both $GABA_A$ and $GABA_B$ receptors, is linked to distinct neuronal circuits and consequently serve distinct functions. GABA neurotransmission has been implicated in learning and memory (Yee et al., 2004), sedative and hypnotic actions (Rudolph & Antkowiak, 2004) as well as in anxiety (Atack et al., 2005). Furthermore, GABA has also been implicated in the regulation of different types of motor behaviour (Akiyama et al., 2004) and stereotypy (Karler et al., 1997).

2.9.4.1 $GABA_A$

$GABA_A$ receptors structure exhibit homology with $5\text{-}HT_3$ receptors in the regions where ligand binding occurs (Bowery & Smart, 2006). The receptor has a pentameric structure comprising a variety of possible combinations of protein subunits (Bowery & Smart, 2006). $GABA_A$ receptors include not only binding sites for GABA, but also for substances that modulate the actions of GABA. Notable among these are the benzodiazepine-binding and neurosteroid sites. There exist certain $GABA_A$ receptors however, that lack specific subunits which do not bind benzodiazepines (Bowery & Smart, 2006).

However, most forms of the receptor have affinity for benzodiazepines, which enable them to modulate the function of the $GABA_A$ receptor to modify the overall response to the transmitter. This functional potentiation provides the basis for the therapeutic action of this important group of anxiolytics and sedatives (Bowery & Smart, 2006; Rudolph & Antkowiak, 2004). Other compounds such as neurosteriods and ions, particularly Zn^{+2}, also modulate GABA function (Bowery & Smart, 2006). Neuroactive steroids exert potent effects on excitatory and inhibitory neurotransmission (Lambert et al., 2003). These include compounds that can modulate $GABA_A$ receptor function with a potency and efficacy similar to or greater than benzidiazepines (Majewska, 1992). In animal experiments neurosteroids have shown marked sedative/anesthetic (Norberg et al., 1987), anxiolytic (Bitran et al., 1991), and anticonvulsant properties (Devaud et al., 1995).

2.9.4.2 $GABA_B$

The $GABA_B$ receptor exists as a heterodimer with two dissimilar subunits ($GABA_{B1}$ and $GABA_{B2}$) comprising the functional receptor (White et al., 1998). The receptor proteins $GABA_{B1}$ and $GABA_{B2}$ are produced independently within neurons and each acts as a chaperone to the other to transport it to the plasma membrane. $GABA_{B1}$ provides the GABA-binding domain within its extracellular chain, while $GABA_{B2}$ provides the G-protein coupling. In addition, there is an allosteric site on $GABA_{B2}$ receptors which can modulate the response to agonist (White et al., 1998).

Baclofen, one of the first selective $GABA_B$ agonists, is used clinically to treat spasticity although its general use is hampered by its poor pharmacokinetic profile and sedative properties. Nevertheless, baclofen-like agonists continue to be widely used for an array of diseases ranging from gastro-eosphageal reflux disease to addiction (Vacher & Bettler, 2003).

2.9.4.3 GABA and OCD

As has been described earlier (section 2.2), OCD is characterized by recurring unwanted thoughts, forced repetition of unwanted behaviours and high levels of anxiety and fear (American Psychiatric Association, 2000), with neuroimaging data proposing a dysregulation in the neural loops linking the frontal cortex, basal ganglia, and thalamus as a cause of OCD (see section 2.4.1). GABA functions as the main inhibitory neurotransmitter in these anatomical areas (Waagepetersen et al., 2003) and it is also accepted that anxiety/anxiety-like behaviour is regulated by GABAergic neurotransmission (Lydiard, 2003). Despite all this data, relatively few studies have investigated the possible role of GABA in OCD. In a recent study, Zai et al. (2005) found a correlation between clinically relevant OCD phenotypes and a polymorphism of the gene coding for the $GABA_{B1}$ subunit. This may point towards a functional involvement of possible causative role for GABA in OCD.

2.9.5 Noradrenaline

Noradrenaline, together with dopamine and adrenaline are catecholamines formed along an enzymatic cascade that begins with tyrosine (Hoffman & Taylor, 2001). Noradrenaline is the principle sympathetic neurotransmitter in the periphery and is prevalent throughout the brain. There are relatively large amounts of noradrenaline within the hypothalamus and in certain zones of the limbic system, such as the central nucleus of the amygdala and the dentate gyrus of the hippocampus (Hoffman & Taylor, 2001). This monoamine is synthesized from dopamine by dopamine β-hydroxylase and stored in presynaptic vesicles. Following a stimulus and then released into the synaptic cleft via vesicle fusion, noradrenaline is rapidly returned to the synaptic terminals via the noradrenaline transporters (Weiner & Molinoff, 1994).

Within the CNS, noradrenaline-containing nerve cell bodies are primarily located within the locus coeruleus of the brain stem. Projections from the locus coeruleus innervate most of the cortical and subcortical areas such as the prefrontal and entorhinal cortices, the amygdala, the hippocampus, and the thalamus (Hoffman & Taylor, 2001). Noradrenaline mediate remarkably diverse effects and key to this is the adrenergic receptors. Adrenergic receptors (adrenoceptors) can be divided into three broad sub-catagories namely α_1, α_2 and β-adrenergic receptors (Docherty, 1998).

2.9.5.1 α-Adrenoceptors

α-Adrenoceptors have been one of the most widely studied family of receptors because of the major physiological importance of these receptors in control of blood pressure and blood flow, neural modulation, digestion, reproduction, endocrine and metabolic processes and in behaviour (Hoffman & Taylor, 2001). α_1-Adrenoceptors can be divided into α_{1A}, α_{1B}, and α_{1D}- adrenoceptors, all of which mediate contractile responses involving amongst other inositol phosphate. α_2–Adrenoceptors can be divided into α_{2A}, α_{2B}, and α_{2C}- adrenoceptors, all of which mediate contractile responses (Docherty, 1998).

All α_2–adrenoceptors have been shown to inhibit AC activity, although some systems have been shown to stimulate cAMP synthesis. α_2–Adrenoceptors furthermore activate K^+ channels, and inhibit Ca^{+2} channels (Tausig & Gilman, 1995). α_1-Adrenoceptors are widely distributed in the brain and are mainly located in the immediate vicinity of adrenergic nerve terminals in peripheral target organs. These receptors regulate multiple effector systems, one of which involves the mobilization of Ca^{+2} from endoplastic stores (Tausig & Gilman, 1995). α_1-Adrenoceptors can also augment the generation of cAMP and are coupled to stimulation of phospholipase C. Interestingly presynaptic α_2 receptors mediate inhibition of release of neurotransmitter other than noradrenaline, such as GABA and 5-HT, in the CNS and peripheral nervous systems (Tausig & Gilman, 1995).

2.9.5.2 β-Adrenoceptors

β-Adrenoceptors are the principle postsynaptic excitatory receptors mediating noradrenaline signal transduction and can be divided into β_1, β_2, and β_3-adrenoceptors.

All β-adrenergic receptors stimulate adenylyl cyclase and cAMP production from ATP (Tausig & Gilman, 1995). In addition, these receptors can enhance the activation of voltage-sensitive Ca^{+2} channels in the plasma membrane of skeletal and cardiac muscle (Tausig & Gilman, 1995). At the same time as α_1-adrenoceptors, β_1 receptors are widely distributed in the brain near adrenergic nerve terminals and are mainly associated with neurons and not glial or vascular elements.

2.9.5.3 Noradrenaline and OCD

Animal models utilizing a variety of physical and behavioural stressors have demonstrated acute changes in the noradrenaline system in response to stress (Tanaka et al, 2000). Pharmacological challenge studies with noradrenergic agents have furthermore demonstrated dysregulation in the noradrenergic system in patients with anxiety disorders (Sullivan et al, 1999). In this regard, noradrenaline has been postulated to play an important role in the pathophysiology and subsequent treatment of mood and affective disorders, as well as in OCD (Ressler & Nemeroff, 1999).

SRIs are first-line agents for treatment of OCD, however, 40 to 60 percent of patients do not respond adequately to SRI treatment and agents that alter other neurotransmitter systems, including noradrenaline, may play a role in treatment resistant patients (Goodman et al., 1990; Goodman et al., 1991). Recent studies compared venlafaxine, a noradrenergic reuptake inhibitor, with clomipramine, a SRI, and concluded that venlafaxine may be as efficacious as SRIs in the treatment of OCD (Albert et al., 2002a). However, despite suggestions that SRIs have greater efficacy than noradrenergic antidepressants in OCD, recent findings (Fontenelle et al, 2005) further support the fact that noradrenaline may play a key regulator/mediator in OCD.

2.9.6 Darpp-32

Dopamine- and cyclic AMP-regulated phosphoprotein of relative molecular mass 32 000 (Darpp-32) has a key role in many neurotransmitter pathways throughout the brain (Greengard, 2001). Darpp-32, is a chemical involved in signalling pathways that range from the psychostimulant action of caffeine (Lindskog et al., 2002) to the biochemical

effects of antidepressants (Svenningsson et al., 2002b) and has also been linked to the psychiatric illness, schizophrenia (Albert et al., 2002b). Since its discovery over 20 years ago (Walaas et al., 1983), Darpp-32 has been established as a crucial mediator of the biochemical, transcriptional, and behavioural effects of dopamine. It furthermore plays an essential integrative role in the actions of various other neurtranmitters (Svenningsson et al., 2004).

Biochemical and a variety of structure-function studies have indicated that Darpp-32, once activated by phosphorylation, is a potent inhibitor of protein phosphatase-1 (PP-1), one of the major serine/threonine phosphatases in the CNS (Hemmings et al., 1984). The inhibition of PP-1 by phospho-Darpp-32 occurs via a multi-site interaction involving the binding of phospho-Thr34 to the active site of PP-1, and the interaction of residues 6-11 of Darpp-32 with a region of PP-1 removed from the active site (Kwon et al., 1997).

Darpp-32 is localized, with few exceptions, to regions that receive dopaminergic innervation. Studies have shown that the highest levels of Darpp-32 are found in caudate putamen, nucleus accumbens (together they form the striatum), and portions of the amygdaloid complex (Ouimet et al., 1984). Moderate levels of Darpp-32 are also found throughout the neocortex and in several subregions of the hypothalamus. The medium-sized spiny neurons, which constitute the major cell type in the striatum, are inhibitory and utilize GABA as their major neurotransmitter (Yoshida & Precht, 1971) and it has been demonstrated that Darpp-32 is found in a great majority of these medium-sized spiny neurons in rodents as well as primates (Ouimet et al., 1992). Darpp-32 is furthermore expressed in both direct striatonigral neurons and in indirect striatopallidal neurons (Anderson & Reiner, 1991).

Darpp-32 is evenly distributed within neurons and is found in the soma and dendrites of medium spiny neurons as well as in the axons and axon terminals of these projection neurons (Ouimet et al., 1992). In addition, neurochemical regulation, particularly glutamatergic regulation of Darpp-32, is very complex. It appears that the immediate and principal glutamate signalling is mediated through NMDA- and AMPA receptors and that other glutamateric dependent pathways, by both positive and negative feedback mechanisms (Nishi et al., 2002), modulate this pathway.

2.9.6.1 Regulation of Darpp-32

The activity of medium spiny neurons is primary regulated by the actions of three major neurotransmitters, glutamate, dopamine and GABA. Medium spiny neurons receive inputs from glutamate-containing corticostriatal nerve terminals and dopamine-containing nigrostriatal nerve terminals (Freund et al., 1997) as well as inputs from GABA-containing nerve terminals via recurrent collaterals (Yoshida & Precht, 1971). There is also moderate serotonergic innervation of the striatum, which is denser in the nucleus accumbens than in the caudate putamen (Saxena & Rauch, 2000). The enrichment of Darpp-32 in medium spiny neurons has suggested a key role for this phosphoprotein in integrating the effects of dopamine and other neurotransmitters on neuronal excitability.

Darpp-32 function is primarily regulated by altering the phosphorylation state of one or more of its regulatory threonine (Thr) or serine (Ser) residues (Greengard et al., 1998). To date, a number of different neurotransmitters and neuromolecules have been found to influence phosphorylation of Darpp-32 (Svenningsson et al., 2004), additionally this is accomplished through different signalling pathways. The physiological state of cells, including that of neurons, is controlled by signal transduction mechanisms which regulate the balance between protein kinase and protein phosphatase activities (Hunter, 1995). Darpp-32 is in the unique position that, depending on which particular amino acid residue is phosphorylated, it can function either as a kinase or as phosphatase inhibitor (Bibb et al., 1999). A summary of the effects of these neuromolecules as well as the effects of neurotransmitters on Darpp-32 phosphorylation is provided in table 2.3.

2.9.6.1.1 Regulation of Darpp-32 by dopamine

The nigrostriatal dopamine system plays a crucial role in the regulation of movements, whereas the mesolimbicortical system mediates the cognitive and rewarding effects of dopamine (Kalivas, 2002; Wise, 2004). Furthermore, the basal ganglia processess sensorimotor, associative and motivational information to produce appropriate motor behaviours. To date, five different dopamine receptors have been cloned (Jaber et al., 1996; sections 2.9.2), all of which are metabotropic and all of which alter cAMP signalling (section 2.9.7). Regulation of dopamine signalling is thus of critical importance. D_1-like and D_2-like dopamine receptors have opposing effects on the state of

Darpp-32 phosphorylation. Dopamine has been shown to stimulate the phosphorylation of Darpp-32 at the Thr34 residue by activation of a cascade involving stimulation of D_1 receptors, activation of adenyl cyclase, increased cAMP formation, and increased activity of cAMP-dependent protein kinase (PKA)(Walaas & Greengard, 1984).

Activation of D_1 receptors furthermore decreases the phosphorylation state of Darpp-32 at Thr75 by a process that likely involves the PKA-dependent activation of a specific isoform of PP-2A (Nishi et al., 2000). Thus, enhanced dopaminergic transmission via D_1 receptors leads to a decreased phosphorylation of Thr75-Darpp-32, which reduces inhibition of PKA and thereby facilitates signalling via the PKA/Thr34-Darpp-32/PP-1 cascade. Activation by dopamine of the D_2 receptors causes the dephosphorylation of Darpp-32 through two synergistic mechanisms, namely inhibition of cAMP formation (through G-proteins to decreased adenyl cyclase activity) and by an increase in intracellular Ca^{+2}.

Both processes activate the Ca^{+2}-dependent protein phosphatase-2B (PP-2B) and in turn PP-2B dephosphorylates Darpp-32 at Thr34 (Nishi et al., 1997). Several lines of evidence also indicate an important role for Darpp-32 in mediating the effects of dopamine long-term changes in neuronal excitability. Studies in Darpp-32 knockout mice showed that Darpp-32 plays a crucial role in the induction of both long-term depression and long-term potention (Calabresi et al., 2000), two opposing forms of synaptic plasticity.

Table 2.3 Regulation of Darpp-32 phosphorylation by various factors. (table adapted from Svenningsson et al., 2004). ↑/↓ indicate increase/decrease in phosphorylation state of particular amino acid residue; -- indicate no change.

Neurotransmitter/ neuromolecule	Receptor	Signalling pathway	Phosphorylation state		
			Thr34	Thr75	Ser137
Dopamine	D_1	cAMP/PKA	↑	↓	--
	D_2	cAMP/PKA	↓	↑	--
Serotonin	5-HT_2	PLC/CK1	--	--	↑
	5-$HT_{4/6}$	cAMP/PKA	↑	↓	--
Glutamate	NMDA	Ca^{+2}/PP-2B	↓	↓	--
	AMPA	Ca^{+2}/PP-2B	↓	↓	--
	mGlu	cAMP/PKA	↑	--	--
GABA	$GABA_A$	Ca^{+2}/PP-2B	↑	--	--
Adenosine	A_{2A}	cAMP/PKA	↑	↓	--
Nitric oxide		cGMP	↑	↑	--
Neurotensin		D_1/cAMP/PKA	↑	--	--
Cholecystokinin		NMDA/Ca^{+2}	↓	--	--

2.9.6.1.2 Regulation of Darpp-32 by serotonin

5-HT is implicated in the regulation of complex sensory, motor, affective, and cognitive functions (Saxena & Rauch, 2000). Several 5-HT receptors, i.e., 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_4, and 5-HT_6, have been found on medium spiny neurons in the nucleus accumbens and caudate putamen (see section 2.9.1). Detailed studies have shown that 5-HT causes an increase in phosphorylation of Darpp-32 at Thr34 and Ser136 and a decreased phosphorylation at Thr75 (Svenningsson et al., 2002a).

The actions of 5-HT in regulating Darpp-32 phosphorylation at Thr34 and Thr75 are mediated primarily via activation of 5-HT_4, and 5-HT_6 receptors, whereas the regulation at Ser137 is mediated primarily via 5-HT_2 receptors (Svenningsson et al., 2002a). The three pathways appear to inhibit PP-1 through synergistic mechanisms. Although the pattern of Darpp-32 phosphorylation induced by elevated serotonergic neurotransmission is similar to that induced by elevated dopaminergic neurotransmission, it is largely independent of altered dopaminergic neurotransmission (Svenningsson et al., 2002a).

2.9.6.1.3 Regulation of Darpp-32 by glutamate

Glutamate, released at glutamatergic nerve terminals, activates NMDA- and AMPA-type ionotrophic glutamate receptors, leading to a decrease in Darpp-32 phosphorylation on Thr34 (Nishi et al., 2002). The effects of NMDA and AMPA receptors on Thr34 phosphorylation are mediated via PP-2B. Activation of these glutamate receptors also results in a decrease in Darpp-32 Thr75 phosphorylation. Surprisingly, the effects of NMDA and AMPA receptors on Thr75 are not mediated through Ca^{+2} –dependent activation of PP-2B, but rather are mediated via a Ca^{+2} –dependent activation of PP-2A, which involves a mechanism that is not yet understood (Nishi et al., 2002).

It has furthermore been reported that activation of metabotropic glutamate receptors stimulates Darpp-32 Thr34 phosphorylation (Paolillo et al., 1998). The effect of metabotropic glutamate receptors, specifically subtype 5, on Thr34 phosphorylation is dependent on activation of adenosine A_{2A} receptors by endogenous adenosine, but not on D_1 receptors by endogenous dopamine (Paolillo et al., 1998). Thus, under some

conditions, glutamate may be able to potentiate dopamine/D_1 receptor/PKA/phosphor-Thr34 Darpp-32 signalling.

2.9.6.1.4 Regulation of Darpp-32 by GABA

GABA was shown to significantly potentiate Darpp-32 phosphorylation on Thr34 (Snyder et al., 1994). These effects are most likely mediated via activation of $GABA_A$ receptors, increased Cl^- influx, decreased neuronal excitability, decreased Ca^{+2} influx, and inactivation of PP-2B, since bicucullin, an direct antagonist of $GABA_A$ receptors, prevented Thr34 phosphorylation (Snyder et al., 1994).

2.9.6.1.5 Regulation of Darpp-32 by other neuromolecules

Apart from the neurotransmitters that regulate Darpp-32 phosphorylation, a number of other neuromolecules are also known to have regulatory effects on this phosphoprotein. Various neuropeptides such as opioids, cholecystokinin, and neurotensin have direct effects on Darpp-32 phosphorylation along with neuromodulators such as adenosine and nitric oxide and steroids such as progesterone and estrogen (Svenningsson et al., 2004). Darpp-32 phosphorylation is also regulated by cyclin-dependent kinase 5 (Cdk5), a notable member of proline-directed serine/threonine kinases (Bibb et al., 1999).

Darpp-32 is phosphorylated at Thr75 by Cdk5, and evidence have shown that phosphorylation at this residue inhibits PKA activity and thereby reduces the efficacy of dopamine signalling (Bibb et al., 1999). Recently, Cdk5 was shown to be an important regulator of neuronal death and survival (Cheung & Ip, 2004). Given the pivotal role of Darpp-32 in the brain and its ability to modulate the activity of numerous kinases and phosphatase, notably PP-1 and PKA, and its recent implication in schizophrenia (Albert et al., 2002b), Darpp-32 is likely to be implicated in various neurologic and psychiatric disorders.

2.9.7 Cyclic AMP

Although academic and pharmaceutical research has clearly characterized the gene or receptor implicated in numerous pathologies, a great number of diseases remain unresolved, inasmuch as they have multi-factorial origins. Downstream of receptor regulation, intracellular signalling plays a major role by governing normal and pathological cell responses. Alterations in intracellular signalling may be one clue toward addressing unresolved diseases. cAMP and guanosine 3', 5'-cyclic monophosphate (cGMP) are ubiquitous nucleotides that have been described as the first second messengers (Sutherland and Rall, 1958). In particular, cAMP in concert with intracellular calcium and inositol 1,4,5-triphosphate (IP3), occupies a pivotal role in orchestrating intracellular signalling.

cAMP is a ubiquitous second messenger produced in cells in response to hormones, neurotransmitters and various growth factors (Daniel et al., 1998). The binding of ligands such as neurotranmitters to their receptors located in the plasma membranes of cells activates GTP-binding proteins (G-proteins) that are coupled to adenylyl cyclase, which converts adenosine triphosphate (ATP) to cAMP (Daniel et al., 1998). The cAMP so formed activates PKA, which phosphorylates numerous proteins. Typically, the cAMP signal is transitory. Ligand-associated receptors are rapidly down-regulated by desensitization (Lohse, 1993), and the cAMP signal is mediated and modulated by various phosphodiesterases (PDEs), inhibitors of PKA and phosphatases (Daniel et al., 1998).

The PKA holoenzyme is a tetrameric complex containing two catalytic subunits bound to a homodimer of two regulatory subunits (Taylor et al., 1990). On binding cAMP, active catalytic subunits are released and activated. PKA kinase activity is specific for the phosphorylation of serine and threonine residues within proteins. The very large number of neurotransmitter receptors coupled to the cAMP signalling pathway and the extensive cross-talk with other pathways suggest that most signalling pathways are connected at some point with the cAMP-dependent pathway (Popoli et al., 2001). The brain is enriched with PKA and it contains a higher number of PKA substrates than any other organ, including ion channels, various receptors, as well as enzymes involved in

neurotransmitter synthesis, and proteins that regulate presynaptic neurotransmitter release (Popoli et al., 2001).

However, cAMP does not act exclusively through PKA and a number of direct molecular targets have been documented such as protein kinase G, cAMP-regulated guanine nucleotide exchange factors, and cyclic nucleotide-gated ion channels (Kopperud et al., 2003). It has also been recognized that the cAMP signalling cascade plays a central role in mediating responses of a number of monoaminergic, e.g., 5-HT, dopamine and norepinephrine, receptors implicated in the psychobiology of mood disorders (Bloom, 1976), as well as in the therapeutic effects of antidepressants (Popoli et al., 2001) and antipsychotics (Turalba et al., 2004).

PKA represents a key component in the cAMP signalling pathway and is responsible for the transduction of most cAMP-mediated physiological effects in the CNS (Popoli et al., 2001). Recent evidence has furthermore shown that cAMP-stimulated PKA activity is significantly lower in patients with OCD (Perez et al., 2000). Patients with OCD have significantly higher levels of the regulatory subunits and significantly lower levels of the catalytic subunit of PKA with respect to healthy controls. These findings suggest that dysfunctions of not only PKA, but also cAMP signalling activity may contribute to the pathophysiology of OCD and other affective disorders (Tardito et al., 2001).

2.9.8 Cyclic nucleotide phosphodiesterases

cAMP and cGMP play a central role in signal transduction and regulation of physiologic responses. Cyclic nucleotide phosphodiesterases (PDEs) are enzymes responsible for the hydrolysis of cyclic nucleotides and play an important and highly regulated role in controlling the resting state levels of cAMP and cGMP intracellularly. Eleven distinct PDE families (or types, e.g. PDE1, PDE2, PDE3 etc.) are differentiated functionally according to the genes of which they are products, their biochemical properties, regulation, and their sensitivity to pharmacological agents (Beavo, 1995). Most of the families include more than 1 gene product (subtype, designated with a letter following the family number – e.g., PDE 4D). Multiple splice variants (isoforms, designated with a number following the subtype letter – e.g., PDE 4D1), each with different functional and regulatory characteristics, may exist for any given gene product (Bolger et al., 1997).

Cyclic nucleotides are degraded by PDE-catalysed hydrolytic cleavage of the 3'-phosphodiester bond, resulting in formation of the corresponding inactive 5'-monophosphate (Beavo, 1995). The substrate specificities of the PDE families include cAMP-specific, cGMP-specific, and dual-specific PDEs. Interestingly, PDE inhibitors block the hydrolytic cleavage activity, causing accumulation of cyclic nucleotides corresponding to the type-specificity of the inhibitor used, e.g. accumulation of cAMP when inhibiting a cAMP-specific PDE such as PDE4 (Beavo, 1995).

The precise mechanism and contribution of the various PDE isoforms in modulating intracellular signalling remain to be established, but their role in intracellular signalling has designated them as potential new therapeutic targets (Houslay et al., 2005). An overview of the classification and primary tissue distribution of PDEs is given in table 2.4. Consequently, a number of selective and non-selective PDE inhibitors are currently used in clinical medicine, including sildenafil (PDE5), rolipram (PDE4) and milrinone (PDE3). A summary of various PDE inhibitors in clinical and basic research is given in table 2.5.

PDEs are composed of 3 broad functional domains: a regulatory N-terminus, a central catalytic domain, and a regulatory C-terminus (Xu et al., 2000, figure 2.2). The N-terminus is involved in membrane targeting and allosteric regulation (calmodulin, cyclic nucleotide, and phosphorylation sites as well as dimerization motifs) (Shakur et al., 1993). The C-terminus may be involved in dimerization and possess docking sites for PDE-specific kinases (MacKenzie et al., 2002). The catalytic domain, which constitutes the core of PDE, is a highly conserved region and includes consensus metal binding domains for Zn^{+2} and Mg^{+2} (Francis et al., 2001). The catalytic domain is encoded by related, but distinct genes, with each gene encoding multiple protein products generated by alternative splicing and/or the use of multiple promoters. The end result is that more than 50 different PDE proteins are probably produced in mammalian cells (Michell & Scott, 2002).

This multiplicity of PDE proteins also contribute to establishing local gradients of cyclic nucleotides by being localized to subcellular compartments and by being recruited into multiprotein signalling complexes (Michell & Scott, 2002). This contributes to the temporal and spatial specificity of cyclic nucleotide signalling by regulating the ability of

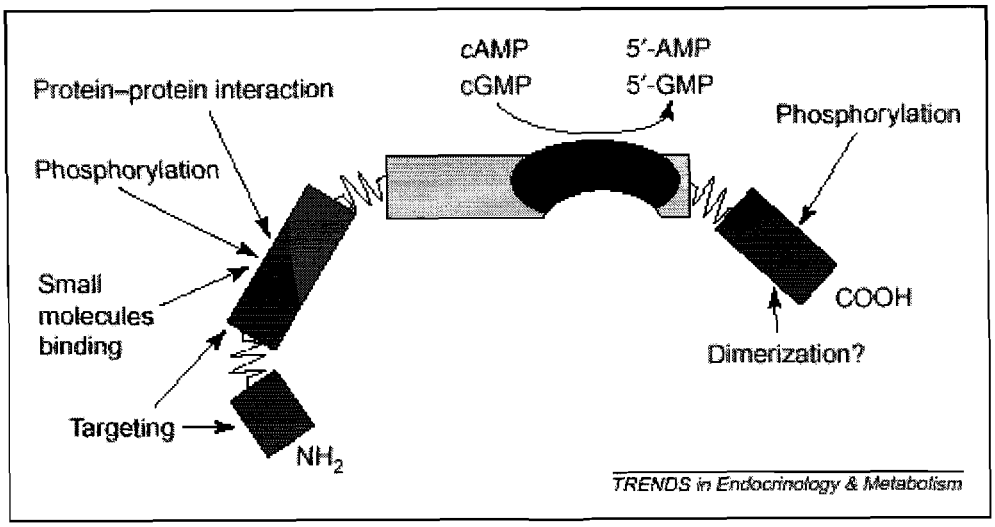


Figure 2.2 Functional and catalytic domains present in phosphodiesterase. PDEs share a common structure, with a conserved catalytic domain (light green) flanked by regulatory domains (dark green), the N-terminus and the C-terminus. Cyclic nucleotides are hydrolysed by the catalytic domain and a range of regulatory domains are found on the PDE peptide such as phosphorylations sites, binding sites for small ligands such as Zn^{+2} and Mg^{+2}, membrane targeting domains and protein-protein interaction domains (Mehats et. al., 2002).

Table 2.4 Phosphodiesterase enzyme family enzymes.

PDE family	Substrate	Characteristic	Primary tissue distribution
PDE1	cAMP, cGMP	Ca-calmodulin-activated	Brain, heart, lung, smooth muscle
PDE2	cAMP, cGMP	cGMP-activated	Adrenal gland, heart, lung, liver, platelets
PDE3	cAMP, cGMP	cGMP-inhibited	Heart, lung, liver, adipose tissue, immunocytes
PDE4	cAMP	cAMP-specific, cGMP-insensitive	Brain, liver, lung, Sertoli cells, immunocytes
PDE5	cGMP	PKA/PKG-phosphorylated cGMP-specific	Lung, platelets, smooth muscle
PDE6	cGMP	Transducin-activated, cGMP-specific	Photoreceptors
PDE7	cAMP	High-affinity cAMP-specific, Rolipram-insensitive,	Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes
PDE8	cAMP	cAMP-selective, IBMX insensitive, Rolipram insensitive	Testes, ovary, eye, brain, liver, heart, T lymphocytes
PDE9	cGMP	cGMP-specific, IBMX insensitive	Brain, kidney, liver, lung
PDE10	cAMP, cGMP	cAMP-sensitive	Testes, brain
PDE11	cAMP, cGMP	cGMP-sensitive	Skeletal muscle, prostate, kidney,

Table 2.5 Phosphodiesterase inhibitors and examples of their clinical use (table information adapted from Lunier, 2006).

PDE family	Specific inhibitor	Clinical application
PDE1	nimodipine, vinpocetine	Both used to implicated PDE1
PDE2	EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine)	reverse hypoxic pulmonary vasoconstriction in rat model
PDE3	cilostamide, milrinone	milrinone used for acute treatment of heart failure
PDE4	rolipram, roflumilast, piclamilast	rolipram is an antidepressant, piclamilast is anti-inflammatory
PDE5	zaprinast, sildenafil	zaprinast is a vasorelaxaxant, sildenafil improves endothelium-dependent vasodilatation, erectile dysfunction
PDE6	zaprinast, sildenafil	sildenafil enhance early memory consolidation
PDE7	BRL 50481, ICI242	Both used to implicated PDE7
PDE8	dipyridamole (PDE8A specific)	used to implicated PDE7
PDE9	zaprinast	used to implicated PDE7
PDE10	papaverine (PDE10A specific)	none tested
PDE11	none developed	none tested

cAMP/cGMP to their effectors. cAMP is generated by adenylyl cyclases at the plasma membrane and subsequently diffuses throughout the cell to sites where it can access its effector proteins. Ligand binding to various G protein-coupled receptors activates adenylyl cyclases in their proximity and generates pools of cAMP in that specific cellular location (Sunhara et. al., 1996).

A principle effector of cAMP is PKA, which, if unregulated, indiscriminately phosphorylates a myriad of substrates (Shabb, 2001). The cAMP-PKA pathway is one of the most common and versatile signal pathways in eukaryotic cells and is involved in regulation of cellular functions in almost all tissues in mammals (Tasken & Aandahl, 2004). A kinase anchoring proteins (AKAPs) have been shown to target PKA to specific substrates and distinct subcellular compartments providing spatial and temporal specificity for mediation of biological effects channeled through the cAMP/PKA pathway (Tasken & Aandahl, 2004).

AKAPs also serve as scaffolding proteins (proteins that stabilize cellular architecture) and contribute to the specificity of the cAMP-PKA pathway by assembling multi-protein signal complexes allowing signal termination by PDEs and cross-talk between different signalling pathways in close proximity to their substrates (Michell & Scott, 2002). For signal termination, PDE is bound to and forms part of the cAMP-PKA complex, PDE isoforms are also found in various cellular locations. The targeting of PDEs to different compartments can be via targeting domains (Manganiello & Degerman, 1999) or more commonly, protein-protein interactions (Yarwood et al., 1999). PDEs therefore contribute to establish local gradients of cyclic nucleotides by being localized to subcellular compartments and by being recruited into multi-protein signal complexes.

PDEs serve as important homeostatic regulators of nucleotide signalling and for this reason its activity need to be tightly regulated. Two feedback mechanisms regarding PDEs have been described. The first is a 'short term regulation' which involves phosphorylation of pre-existing PDE protein and occurs within minutes of the addition of hormonal stimulus (Oki et al., 2000). The second, termed 'long term regulation', requires protein synthesis and develops in hours or days (Mehats et al., 2002). Although other families have been implicated, PDE4s are the form most frequently involved in long-term regulation (see section 2.9.8.2).

2.9.8.1 Functional and physiological roles of phosphodiesterases

To illustrate the important and ubiquitous role of PDEs in the body, a select group of examples are presented. Insulin secretion, for example, is regulated by PDE3 through the PDE3B mediated control of insulin-like growth factor release (Zhoa et al., 1997). This is presumably accomplished by the modulation of intracellular pools of cAMP that is said to regulate insulin secretion.

PDE5 was first implicated in vasorelaxation since the specific inhibition of PDE5 by zaprinast (table 2.5) was shown to induce an increase in cGMP associated with vasorelaxing effect (Lungier et al., 1986). The potentiation of a PDE5 inhibitor relaxing effect obtained on the aorta containing functional endothelium or treated with nitric oxide donors (Martin et al., 1986) suggested that PDE5 mediates the nitric oxide/cGMP relaxing effect. PDE5 has been implicated in penile erectile function (Chuang et al., 1998), and a number of studies have demonstrated the utility of stimulating the nitric oxide/cGMP pathway by inhibition of PDE5 for enhancing penile erection and improving male erectile impotence (Chuang et al., 1998). The high level of PDE5 encountered in the lung, as well as the observation that PDE5 was activated in pulmonary hypertension (Hanson et al., 1998), has contributed to propose also PDE5 as a new target for the treatment of pulmonary hypertension and respiratory distress.

PDE6 plays a major role in phototransduction. The PDE6 cascade activation is initiated when the protein rhodopsin absorbs a photon. Each activated rhodopsin activates thousands of transducin (a G-protein) by catalysing the exchange of GDP for GTP. Transducin with GTP bound, activates the catalytic PDE6 subunits, thus allowing cGMP hydrolysis (Pugh & Lamb, 2000). The main function of the rod PDE is to rapidly reduce the concentration of cGMP in response to light stimulus. This decrease in cGMP concentration causes the closure of cationic channels and generates cell membrane hyperpolarization (Pugh & Lamb, 2000).

PDE7 induction on the other hand has been implicated in T-cell activation (Li et al., 1999). Regulation of T-cell activation is a complex process, requiring the coordinated occupation of the T-cell receptor CD3 and one or more stimulatory receptors. As PKA

signalling is thought to be one inhibitory pathway that must be overcome to produce T-cell proliferation, PDE7 modulation may provide such a mechanism.

The important role of cyclic nucleotides as second messengers in the CNS means that their concentrations must be tightly regulated by the rate of synthesis, and equally by the rate of degradation. The super family of PDEs is responsible for this important regulatory step and hence PDEs are key to a number of physiological functions. PDE10A is a newly identified PDE that is highly expressed by the medium spiny neurons of the striatum (Fujishige et al., 1999a), which function as the principal input site to the basal ganglia (Kelly, 1999). The basal ganglia are a series of interconnected subcortical nuclei that integrate widespread cortical input with dopaminergic signalling to plan, facilitate, and execute relevant motor and cognitive patterns while suppressing unwanted or irrelevant patterns (Cummings, 1996; Alptekin et al., 2001).

Striatal dysfunction is implicated in a number of disorders including Parkinson's disease, schizophrenia and OCD (Cummings, 1996; Baxter, 1999). Recent findings suggest that PDE10A is involved in regulating striatal output, possibly by reducing the sensitivity of medium spiny neurons to glutamatergic excitation (Siuciak et al., 2006). It follows then that pharmacological inhibition of PDE 10A may be a novel therapeutic approach to the treatment of diseases in which striatal hypofunction is a prominent feature. This PDE family was also recently shown to be associated with the progressive neurodegenerative disease Huntington's disease (HD), since PDE 10A2 mRNA decreases prior to the onset of motor symptoms in transgenic HD mice expressing exon 1 of the human huntington gene (Hebb et al., 2004).

2.9.8.2 Phosphodiesterase 4

2.9.8.2.1 PDE4 structure and isoform sub-categories

PDE4, a cAMP-specific PDE, is mainly present in the brain (Houslay et al., 1998), inflammatory cells (Tenor & Schudt, 1996), cardiovascular tissues (Stoclet et al., 1995), and smooth muscle cells but is absent in platelets. PDE4 is specifically inhibited by rolipram and Ro-201724 and is insensitive to regulation by cGMP (Lugnier et al., 1986). Presently, the PDE4 family represents the largest PDE family, constituted by 4 genes (PDE4A, PDE4B, PDE4C, and PDE4D) with various alternative mRNA splices encoding long PDE4 and short PDE4 isozymes (Sullivan et al., 1994).

Functional PDE4 isoforms can be divided into three major categories: long, short and super-short (Houslay, 2001). The long isoforms are characterized by two modules that are conserved in all PDE4 subfamilies, namely upstream conserved regions 1 and 2 (UCR1 and UCR2). In contrast with long isoforms, the short forms lack UCR1, whilst the super-short forms not only lack UCR1, but have a truncated UCR2. These two modules are joined together by linker region 1 (LR1), the composition of which differs in the four PDE4 subfamilies. LR2 connects UCR2 to the catalytic domain of PDE4.

Recent functional studies (MacKenzie et al. 2000) have established that UCR1 and UCR2 provide the molecular machinery that confers key regulatory functions on the PDE4 catalytic unit. UCR1 interacts with UCR2 via electrostatic interaction (Beard et al. 2000) to form a discrete module. The first indication that UCR1/2 plays a regulatory role came from studies where the removal of N-terminal portion of UCR2 led to an increase in PDE4 catalytic activity. This led to the notion that UCR2 may exert a constitutive inhibitory effect on the activity of PDE4 (Beard et al., 2002).

In addition to the UCR1/2 interaction, rolipram, the prototypic PDE4 inhibitor, binds to PDE4 in two sites, termed low-affinity (LARBS) and high-affinity rolipram binding sites (HARBS, Schneider et al., 1986) respectively. It has been proposed that anti-inflammatory effects are associated to LARBS whereas emesis is related to HARBS (Barnette et al., 1995). Furthermore, with PDE4s being metallohydrolases like other PDEs, it has been shown that the LARBS PDE4 conformer corresponds to the apoenzyme

(metal ion-free), whereas the HARBS corresponds to the holoenzyme (metal ion-associated, Laliberté et al., 2000; Liu et al., 2001).

2.9.8.2.2 PDE4 activity regulation

PDE4 activity is regulated, in the short term, by phosphorylation, association to protein or endogenous mediator, as well as proteolysis. The presence of an acceptor site in UCR1 for PKA-mediated phosphorylation allows a rapid change in PDE4 activity. In vivo, this increase in PDE4 activity is due to a prolonged elevation of cAMP resulting from hormonal stimulation, and it was proposed and shown to be a feedback mechanism allowing cAMP levels to return to basal levels (Oki et al., 2000). Long PDE4 isoforms from all four subfamilies can be activated through PKA phosphorylation of a single serine residue (Sette & Conti, 1996) found at the extreme N-terminal end of UCR1.

PKA phosphorylation of UCR1 also disrupts the interaction between UCR1 and UCR2 through a conformational change that is distinct from that which causes activation (Beard et al., 2000). The mechanism involves disruption of H-bond interaction at the target serine residue which in turn causes a small increase in the sensitivity of PDE4 to inhibition by rolipram (Hoffmann et al., 1998). PKA phosphorylation also enhances the sensitivity of PDE4 (specifically isoforms PDE 4D3 and PDE 4A4) to stimulation by Mg^{+2} (Sette et al., 1996). PKA phosphorylation of PDE isoforms thus leads to activation, altered Mg^{+2}-sensitivity, altered rolipram-sensitivity and an ability to re-programme the functional output of ERK phosphorylation of PDE4 enzymes (see below).

ERK phosphorylation sites on PDE4 are located at the carboxy-terminal end of the catalytic region and it has been shown that ERK2 phosphorylation induces the activation of PDE4D short forms whereas it induces the inhibition of long forms (MacKenzie et al., 2000). The various PDE4 isoforms thus provide a panel of enzyme that can either be activated or, alternatively, inhibited to different extents by ERK2 phosphorylation. In addition, as a number of PDE4 isoenzymes appear to be regulated to specific intracellular sites, differential regulation by ERK may offer the potential either to raise or lower cAMP levels in discrete intracellular domains and further hint at a key role for integrating the ERK and cAMP signalling pathways (MacKenzie et al., 2000).

In addition to short term regulation of PDE4 due to post-translational modifications, the expression of PDE4 is regulated at the transcriptional level (long term regulation). In both PDE4B and PDE4D, a cAMP-regulated intronic promoter has been identified (Vincini & Conti, 1997). Hormonal stimulation as well as pharmacological manipulation of intracellular cAMP causes large increases in PDE4D and PDE4B mRNA (Swinnen et al., 1991). An interesting example is that fluoxetine treatment up-regulates PDE4A, but down-regulates PDE4B and PDE4D protein and mRNA levels (Miro et al., 2002).

2.9.8.2.3 Functional and physiological roles of PDE 4

PDE4 enzymes are the principal phosphodiesterases responsible for the hydrolysis of cAMP. PDE4 is widely distributed and found in many tissues ranging from brain (Houslay et al., 1998), inflammatory cells (Tenor & Schudt, 1996) to cardiovascular tissues (Stoclet et al., 1995). As the predominant class of PDE enzymes in immune and inflammatory cells (Dent et al, 1991; Peachell et al., 1992), in which most of the effects of cAMP are inhibitory in nature (Alvarez et al., 1995), PDE4s play a key role in the regulation of a number of active processes, such as cell trafficking, release of inflammatory mediators, and immune cell proliferation. The functional consequences of elevating cAMP in both airway smooth muscle and inflammatory leukocytes also suggest that PDE4 inhibition is a potential novel therapy in asthma as well as chronic obstructive pulmonary disease (Torphy, 1998).

The brain cAMP signal pathway has been shown to be involved in the neurobiology of depression and in the therapeutic action of antidepressants (D'Sa & Duman, 2002). Since PDE4 is the major PDE hydrolysing cAMP in the mammalian brain it will play a pivotal role in regulating neuronal cAMP levels and that evoked by antidepressants (Houslay, 2001). Chronic, but not acute, treatment with antidepressant has been found to upregulate the cAMP second messenger system (Duman, 1998). Also, chronic antidepressant treatment has been reported to increase the expression of PDE4 in frontal cortex and nucleus accumbens (Takahashi et al., 1999). It is not surprising that rolipram, a PDE4 specific inhibitor, produces antidepressant-like effects in preclinical tests (Wachtel & Schneider, 1986; Zhang et al., 2002).

Studies of PDE4 function using rolipram indicate that PDE4s are involved in neural processes such as learning and memory (Barad et al., 1998; Zhang, 2000), as well as physical dependence and tolerance to morphine (Mamiya et al., 2001). A recent study also hinted at PDE4-mediated regulation of cAMP that could further underlie reward behaviour (Thompson et al., 2004) and synaptyic plasticity (D'Sa & Duman, 2002). A role for PDE4 in various other physiological functions have also been implicated such as bone mass formation and osteoporosis (Waki et al., 1999), immune and inflammatory cell function and hence rheumatoid arthritis (Francischi et al., 2000), as well as the pathogenesis of stroke through atherosclerosis (MacIntyre, 2004).

2.9.9 Neurochemical communication in the brain: relevance to OCD

Neurotransmitters are neither static nor isolated in their distribution in the brain. It is through these interactions that the CNS performs it vital role in sustaining life. All physiological, biochemical, pharmacological and behaviour is governed by neurochemical activity. Effective communication is however, achieved through neurochemical interaction and this allows neurochemicals to modulate, regulate and mediate each others activity as well as extracellular and intracellular levels. In the next few sections (2.9.9.1 – 2.9.9.5), interactions between neurotransmitters and neurochemicals implicated in OCD will be highlighted.

2.9.9.1 Serotonin-dopamine interactions

The dopaminergic and serotonergic neurotransmitter systems are thought to play a critical role in the regulation of emotion and mood and have been implicated in spectrum of neuropsychiatric disorders, including OCD. The nucleus accumbens, which has been implicated in reward and motivation, is one brain region where such interaction may take place (Sasaki-Adams & Kelly, 2001). Although early studies have indicated an inhibitory role of 5-HT on dopaminergic activity (Korsgaard et al., 1985), more recent evidence has also suggested a facilitator effect on dopamine (Yadin et al., 1994).

Although studies tend to suggest a potentiating influence of serotonergic drugs on dopaminergic tone, the picture is often complicated by the existence of multiple subtypes of 5-HT receptors having effects on multiple dopamine receptors. For example, 5-HT_{1B}

and 5-HT_3 agonists tend to facilitate dopaminergic effects (De Deurwaerdere et al., 1998), whereas 5-HT_{2C} agonists have been reported to inhibit such effects (Walsh & Cunningham, 1997). Dopamine has also been shown to regulate 5-HT activity, but conflicting results have been mentioned. Dopamine neurons send excitatory axons to serotonergic nuclei and evidence have shown that drugs which excite dopamine neurons trigger 5-HT release from amongst other the dorsal raphe (Boye et al., 2001).

In a comprehensive study by Thorre and co-workers (1998), D_2 mediated inhibition of nigral 5-HT release was highlighted, indicating that dopamine released from dendrites is not only implicated in the self-regulation of dopamine neurons, but is also responsible for the transfer of information to serotonergic systems. Dopamine and serotonin have also been shown to play an important role in the neurocircuitry of the CSTC, a circuit of great importance in the pathophysiology of OCD (Saxena & Rauch, 2000). In addition, extensive serotonin-dopamine interactions are thought to underlie the therapeutic efficacy of SRIs in the treatment of OCD (Yadin et al., 1994).

2.9.9.2 Serotonin-GABA interactions

5-HT is a powerful modulator of emotional processes in the CNS, particularly in the prefrontal cortex (PFC), a brain region associated with high-levels of serotonin innervation and required for complicated goal-directed behaviour (Baxter, 1999; Dubois et al., 1994). The PFC is composed of two major neuronal populations: glutamatergic pyramidal principal neurons and GABAergic interneurons. Serotonergic projections have been shown to target both types of PFC neurons (Smiley & Glodman-Rakic, 1996), with an inclination towards interneurons. For example, 5-HT has been shown to reduce postsynaptic GABA receptor currents through activation of 5-HT_2 receptors in PFC neurons and the inhibition of $GABA_A$ mediated currents (Feng et al., 2001).

2.9.9.3 Dopamine-Glutamate interactions

Dopaminergic and glutamatergic transmission have long been known to interact at multiple levels. One such level includes the basal ganglia where said interaction modulates motor and cognitive functions (Cummings, 1996). The nature of dopamine-glutamate interaction is complicated by the heterogeneity of the receptors activated by the

two neurotransmitters as well as the brain area considered. The facilitatory nature of glutamate modulating both presynaptic and dendritic dopamine release has clearly emerged from *in vitro* studies (Rosales et al., 1997).

Some results however, would indicate that, at least in the striatum and in the nucleus accumbens, glutamate-mediated inhibitory effects may also occur (Taber et al., 1996). *In vitro* and *in vivo* experiments in the striatum and midbrain dopaminergic areas mainly depict dopamine as an inhibitory modulator of glutamate release (Morari et al., 1998). This inhibitory effect is thought mainly to be through the D_2 dopamine receptors since infusion of D_2 receptor antagonist, sulpiride into rat striatum inhibits evoked glutamate release (David et al., 2004).

The nature of dopamine-glutamate interactions however is still under lively discussion since two ways of thinking have emerged mainly from behavioural and functional (locomotor activity) experiments. Some of these studies support the hypothesis that there is a synergistic interaction between dopamine and glutamate, while other data argue for an antagonistic relationship (David et al., 2004). Be that as it may, dopamine-glutamate interactions play a major integrative role in areas implicated in OCD, specifically the CSTC circuit (Saxena & Rauch, 2000; figure 2.1) as well as the striatum (Baxter, 1999).

2.9.9.4 Dopamine-GABA interactions

Considerable evidence supports the hypothesis that the frontal cortex modulates the release of neurotransmitters in subcortical structures such as the striatum (Murase et al., 1993; Taber et al., 1996). For the most part, dopamine in the cortex activates a cortical GABAergic system (Karler et al., 1997), resulting in an inhibitory effect on dopamine release. An important aspect of the dopamine-GABA interaction in the frontal cortex is that it may constitute the neurochemical basis of stereotypy (Karler et al., 1997).

Anatomical studies have shed light on the synaptic organization of monoamine-containing axons and GABAergic interneurons in the cortex, and it has been shown that dopamine axons form synaptic specializations with interneurons in the PFC (Sesack et al., 1995). Consistent with this anatomical arrangement dopamine acts through D_2-like receptors to increase extracellular GABA levels in the prefrontal cortex of the rat (Grobin

& Deutch, 1998). Dopamine-GABA interaction would be important, particularly since GABAergic interneurons is abundant in the prefrontal cortex, and the latter is one of the areas implicated in OCD (Carlsson, 2001).

2.9.9.5 Neurochemical interaction with Darpp-32

As has been described earlier on (section 2.9.6.1), Darpp-32 plays an essential integrative role in the actions of various other neurotranmitters (Svenningsson et al., 2004). In the CNS, Darpp-32 is localized, with few exceptions, to regions that receive dopaminergic innervation such as the caudate putamen, nucleus accumbens, and portions of the amygdaloid complex (Ouimet et al., 1984) as well as in medium-sized spiny neurons of the striatum (Ouimet et al., 1992). Protein phosphorylation has been proposed as a molecular mechanism by which striatal neurons integrate these converging inputs (Greengard et al., 1998), and a central role has been proposed for Darpp-32 in particularly in the striatum. Since abnormalities in dopaminergic neurotransmission have been implicated in several neurological and psychiatric disorders, including OCD (Goodman et al., 1991), malfunction and/or perturbations in Darpp-32 and its inter-connecting pathways with 5-HT, dopamine, GABA, and glutamate within the CSTC circuit may have great value in understanding the neurobiology of this disorder.

2.10 Research Question

2.10.1 Concise statement

The objective of the present study is to validate spontaneous stereotypic behaviour of the deer mouse (*Peromyscus maniculatus bairdii*), as a potential animal model of OCD. With this in mind, the study set out to evaluate the predictive, construct and face validity of naturalistic stereotypic behaviour in the deer mouse model. Subsequent to validation of predictive validity, construct validity will be explored by examining distinct molecular parameters linked to drug treatment, including changes in cAMP levels, PDE4 enzyme activities, PDE4 protein expression and Darpp-32 phosphorylation. Altered cAMP-PKA function has been described in OCD while SRIs are known to modulate this signalling system via action on 5-HT_{1A} receptors. An important question is to what extent cAMP and PDE4 inter-activity is evident, and whether PDE4, the principle PDE isoform responsible for cAMP hydrolysis in the brain, is dysregulated in these animals. Further, is

there evidence for altered dopaminergic activity inherent in naturalistic stereotypy? This question will be addressed by studying DARPP-32, an important protein marker of dopaminergic activity in the brain. Since serotonin and dopamine play important roles in OCD, the focus on these two neurochemicals throughout various aspects of the study will contribute much to answering the central question of this thesis, whether naturalisitc deer mice represent a new and effective animal model of OCD.

2.10.2 Justification

During the last three decades, there have been many attempts to develop animal models of OCD. Most of these animal models have been abandoned during the years; some have persisted, while some new models have emerged. Yet, none of the currently available animal models of OCD seems to fully capture and address various issues regarding OCD pathology, one of paramount importance is the issue of naturalistic sterotypy as opposed to induced stereotypy. Development of the deer mice model for OCD is justified since a well-validated deer mouse model may address many of the current limitations and considerations described in the literature review. Examples of previous difficulties include the lack of reproducibility of models and obtained results, the use of drug-induced behavioural alteration to study spontaneously occurring symptoms, the lack of construct validity in models and the lack of, or difficulty in measuring behavioural and molecular parameters.

In addition, none of the currently used models allows separation of individuals into groups according to symptom severity or subtype. Given that the aetiology of OCD most likely involves the interaction of multiple genetic and environmental factors, an animal model in which the behavioural pathology develops spontaneously and where individuals may be separated according to phenotypic/behavioural differences, may be particularly useful because they may mimic more fully the range of genetic and environmental aetiologic factors in the modelled condition.

2.10.3 Why it is worthwhile to answer this question

OCD is a complex neuropsychiatric illness that presents with diverse neurologic manifestations together with severe anxiety. Neuroimaging studies in OCD have

consistently implicated various regions in the brain in the pathophysiology of obsessions and compulsions, for instance the OFC, striatum, and thalamus. However, the nature of dysfunction in these regions and the relation between their malfunction and the disturbance in the neurotransmitter systems postulated to be involved in OCD is still unknown. For obvious reasons, the understanding and treatment of OCD must rely heavily on appropriate animal models that closely mimic their behavioural and neural manifestations.

Validation studies during the course of this project have set out to compare stereotypic deer mice to a non-stereotypic strain, namely C57Bl. Comparison of the two mouse strains will not only highlight the stereotypic behaviour of deer mice, but will also contribute to the face validity of the model. Pharmacological isomorphism (predictive validation) will be evaluated through the differential response of the model to drugs of known efficacy in OCD, namely fluoxetine, and a drug known to be ineffective in OCD, namely, desipramine. Construct validity will be evaluated through examination of distinct molecular parameters linked to drug treatment, including changes in cAMP levels, PDE4 enzyme activities, PDE4 protein expression levels and regulation of Darpp-32 phosphorylation.

By satisfying these definite criteria, deer mice may be considered an effective animal model of OCD. The focus of the effective animal model will be on specific behavioural, neurochemical, and structural anomalies that are analogous to OCD. Validation of these correlates in the deer mice will allow meaningful and accurate interpretation of preclinical data with direct implications for understanding the neurobiology of OCD.

3. Materials and methods

3.1 Animals

Deer mice (*Peromyscus maniculatus bairdii*) develop high rates of persistent, spontaneously emitted stereotypy which continue at stable levels when housed under standard laboratory conditions (Powel et al., 1999). Since stereotypic behaviour in deer mice becomes evident early in life, being most notable at about 20 days of age and becoming well developed by 30 days of age, the study was performed on adult mice older than two months (56 days). Deer mice were obtained from the *Peromyscus* Genetic Stock centre, University of South Carolina, USA. Since stereotypy develops to similar degrees in both male and female deer mice (Powell et al., 1999), mice of both sexes were used in the study. As control, a species that do not develop spontaneous stereotypic behaviour under laboratory conditions namely CB57 Black (C57Bl) mice were bred (male and female). C57Bl are a commonly used general-purpose inbred strain with high locomotor activity. C57Bl mice were bred in the Animal Research facility, North-West University (NWU), Potchefstroom campus.

Mice were group-caged (four same sex mice per cage) in standard (40 cm x 25 cm x 20 cm) laboratory cages. Rodent chow and water was freely available and temperature was maintained at 21 °C. Mice were maintained on a 12-h light/dark cycle, with lights off at 6:00 p.m. All procedures were performed in accordance with the guidelines stipulated by the Ethics Committee for use of experimental animals at the NWU (ethics # 04D09).

3.2 Drugs

Drugs were dissolved in 0.9% saline and were administered intraperitonealy (i.p.) to a maximum volume of 300 µl/25g per animal (0.012 ml/kg). Importantly, all mice used for pharmacological as well as behavioural studies were naïve. For the initial chronic fluoxetine (for supplier see table 3.4) and desipramine studies aimed at assessing their efficacy at attenuating inherent stereotypy in the deer mice, both drugs were administered at low and high doses of 10 mg/kg and 20 mg/kg respectively for a period of 21 days. Subacute challenge studies with mCPP (5-$HT_{1/2}$ agonist), quinpirole (QNP, D_2 agonist) and SKF 38393 (SKF, D_1 agonist) was administered at doses of 2 mg/kg, 5 mg/kg and 10 mg/kg respectively for 4 days while Control animals received an equivalent volume of

vehicle. In the subacute mCPP and QNP-challenges preceded by chronic fluoxetine and desipramine administration, the latter two drugs were administered at high dose (20mg/kg). Doses were selected based on previous chronic fluoxetine (Monleon et al., 2002; Vinet et al., 2004) and desipramine (Crowley et al., 2004; Vinet et al., 2004) administration and subacute mCPP (Gleason & Shannon, 1998), QNP (Ralph-Williams et al., 2003) and SKF 38393 (SKF; Bernaerts & Tirelli, 2003) administration.

3.3 Determination of stereotypic behaviour

3.3.1 Apparatus

Behavioural studies were performed using a Digiscan Animal Activity Monitor (DAAM; AccuScan Instruments, Columbus, Ohio, USA). This method provides automated and continual computerized monitoring of the animal that is more sensitive than simple observation and is without the risks of investigator bias (Sanberg et al., 1983, 1987). The cages employed in these observations are surrounded by a series of horizontal infrared light beams (16 beams spaced 2.5 cm apart), with one set of beams at ground level and a second set 10 cm above the first. This array of infrared beams enables the computerized collection of all stereotypic and locomotor activity by a digital analyzer that effectively determines the position of the animal 100 times/second. This high speed analysis provides a dynamic picture of all aspects of the animal's activity throughout the observation period. The interruption of any beam is recorded as an activity score while interruption of two or more consecutive beams is recorded as a movement score. Stereotypical behaviour was recorded by monitoring a number of parameters namely horizontal movements which represent patterned running (i.e. the interruption of consecutive horizontal beams), vertical activity which represent repetitive jumping (i.e. the interruption of two consecutive vertical beams), and vertical movements which represent backward somersaults (i.e. the interruption of two vertical beams with at least a one second break between successive interruptions).

3.3.2 Behavioural testing

In this study animal stereotypical behaviour was recorded by monitoring a number of parameters namely, horizontal movements (running), vertical activity (jumping), and vertical movements (backward somersaulting). Table 3.1 lists the topographies and

operational definitions of stereotyped behaviour that were observed and applied during the entire study. The most common topographies of stereotypy in our colony were repetitive jumping, patterned running, and backward somersaults. Each animal received a stereotypy score, or in the case of C57Bl, locomotor score that represents the average behavioural score per hour (using each of three 1- hour assessment sessions). Each assessment session for an individual mouse was at least 6 days apart to avoid stressing the animals. Mice that generated less than 1000 counts per hour (Cph) were classified as non-stereotypic (NS) mice and animals that generated more that 1000 Cph were classified as stereotypic mice.

Earlier studies have demonstrated that deer mice exhibit low or high levels of stereotypic behaviour (Powell et al., 1999). For the purpose of this study, a further distinction was therefore made between low stereotypic (LSB; Cph of 1000 – 2000) and high stereotypic animals (HSB; Cph > 2000). Animals exhibiting for example high levels of running were grouped together with animals exhibiting high levels of repetitive jumping. Thus no distinction was made between different stereotypic *topographies*, but only between different *rates* of stereotypic behaviour. Briefly, individual mice were gently transferred from the laboratory cage to the DAAM cage and allowed 30 minutes to recover from the stress of handling. Subsequent to this adjustment period, behaviour was monitored for 60 minutes. Since mice are nocturnal, data were collected during the dark cycle of the animals.

Table 3.1 Spontaneous stereotyped behaviour in deer mice in standard caging.

Repetitive jumping	Rearing in one of the four corners of the DAAM cage and repeatedly jumping on its hind paws
Backward somersaulting	A somersault in a backward direction with or without assistance from the cage top or side
Patterned running	Repetitive route tracing or circling of the cage in clear pattern

3.4 Brain tissue preparation

3.4.1 Preparation of tissue for cAMP analysis

Following drug treatment or behavioural testing, mice were killed by decapitation and their prefrontal cortex and striata were dissected on ice, immediately frozen in liquid nitrogen, and stored at -80°C. Immediately prior to cAMP determination, brain tissue was thawed on ice and deproteinized. Deproteinization was performed since very low levels of cAMP were present in our samples. Briefly, thawed samples were suspended in 2 ml ethanol, mixed and left to stand for 5 minutes at room temperature. Samples were then homogenized (10-12 strokes) with a Teflon homogenizer. A small aliquot amount of sample (150 µl) was saved for protein determination (section 3.5) and the remaining sample was centrifuged (3 minutes at 2000 x *g*). Supernatant was transferred to a new tube and used for cAMP determination.

3.4.2 Preparation of tissue for PDE4 activity analysis

Mice were killed and prefrontal cortex and striatal tissue dissected and stored as described above (section 3.4.1). Immediately prior to PDE4 activity assay, tissues were homogenised in a sample buffer containing 20 mM Tris-Cl (pH 7.2), 1 mM EDTA and 250 mM sucrose using a Teflon homogenizer (15 strokes, 4°C). The homogenates were sonicated on ice (30 seconds/ml) and the pH adjusted to 6.0 with 1 M acetic acid. A small aliquot amount of sample (150 µl) was saved for protein determination (section 3.5) and the sonicated homogenates were centrifuged at 1000 x *g* for 15 minutes. PDE4 activity was measured in the supernatant.

3.4.3 Preparation of tissue for western blotting

3.4.3.1 Sample preparation for PDE immunoblots

Mice were killed and prefrontal cortex and striatal tissue dissected and stored as described above (section 3.4.1). Samples were thawed and the striata or prefrontal cortex of 10 mice were pooled and homogenized in modified RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.25g sodium-deoxycholate and 1% SDS, 200 mM NaF, 100 mM EDTA, 200 mM PMSF, 0.5 ml sodium orthovanadate, 100 µl Pepstatin,

100 µl Aprotonin) using a Teflon homogenizer (15 strokes, 4°C). Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid protein assay method (section 3.5). Tissue aliquots were mixed with an equal volume of sample buffer (10% glycerol, 250 mM Tris-Cl pH 6.8, 4% SDS, 2% β-mercaptoethanol, 0.06% bromophenol blue), vortexed for 15 seconds and frozen in liquid nitrogen and stored at -20°C until immunoblotted. Immediately prior to immunoblotting, sample were thawed and boiled for 6 minutes and loaded into acrylamide gels.

3.4.3.2 Sample preparation for Darpp-32 immunoblots

Following drug treatment or behavioural testing, mice were killed by decapitation and their prefrontal cortex and striata were dissected on ice and immediately frozen in liquid nitrogen and stored at -80°C. Samples were thawed and the striata or prefrontal cortex of 10 mice were pooled and homogenized in 1% SDS and boiled for 10 minutes. Small aliquots of the homogenate were retained for protein determination. Tissue aliquots were mixed with an equal volume of sample buffer (10% glycerol, 250 mM Tris-Cl pH 6.8, 4% SDS, 2% β-mercaptoethanol, 0.06% bromophenol blue), vortexed for 15 seconds and frozen in liquid nitrogen and stored at -20°C until immunoblotted. Immediately prior to immunoblotting, sample were thawed and boiled for 10 minutes and loaded into acrylamide gels.

3.5 Protein determination

Protein content for each sample was determined using the Bicinchronic (BCA) protein assay (Smith *et al.*, 1985). 50 µl sample was transferred in triplicate to a 96-well plate. Also, 50 µl of endotoxin free BSA (bovine serum albumin) standards made in double distilled water containing 0.0626 to 2 mg of BSA per 50 µl was added to the outer wells of a 96-well plate in triplicate. 200 µl of the BCA reagent was added to all the wells and incubated for 20 minutes at room temperature. The absorbance was measured at 630 nm using the Genesis Multiscan plate reader, and the total protein for each sample was quantified using the BSA standard curve prepared on the day.

3.6 cAMP measurement

Use was made of the Amersham Biosciences cyclic AMP [^{3}H] assay system (acquired from Amersham Biosciences, see table 3.4). The assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample.

Separation of the protein bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on to coated charcoal, followed by centrifugation. An aliquot of the supernatant is removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated and expressed as pmol/mg protein. Briefly, samples were thawed on ice, prepared as described above (section 3.4.1) and following the manufacturers instructions, cAMP level for each individual mouse (both in prefrontal cortex and striatum) was determined.

3.7 PDE4 activity assay

PDE4 activity was assayed using a modification of the method of Torphy & Cielinksi (1990). Briefly, following sample preparation (section 3.4.2), the reaction was initiated by adding pre-warmed enzyme-containing extract (10 µg protein) into standard reaction mixture containing (final concentrations): 40 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 1.25 mM *B*-mercaptoethnol, 0.75 mg/ml bovine serum albumin, 100 000 counts per minute (c.p.m.) [^{3}H]cAMP, and 2.5 µM authentic cAMP. After incubation at 34°C for 10 minutes, the reaction was stopped by addition of an equal volume of ice-cold stop buffer (40 mM Tris-Cl, pH 7.4, and 10 mM EDTA). Samples were then boiled for 2 minutes at 94°C and transferred to an ice bath for 3 minutes. An excess amount (100 µl of a 100 mg/ml stock solution) of nucleotidases in the form of snake venom (*Cortalus atrox*) was added and incubated for 15 minutes at 34°C. The reaction was stopped by adding 2 ml ice-cold ethanol.

The reaction mixture was subjected to anion-exchange chromatography by adding anion resin (AG 1-X8, 100-200 mesh, formate form) to the test tube. The tubes were briefly vortexed and then incubated for 10 minutes at room temperature followed by centrifugation for 3 minutes at 500 x *g*. Supernatant (1 ml) was transferred to scintillation vial and 4 ml liquid scintillation fluid (Ultima gold XR fluid) was added to the 5'-monophosphate. Radioactivity (c.p.m.) was measured by scintillation spectroscopy (TRI-CARB 2100TR liquid scintillation analyser). PDE4 activity was defined as total PDE activity in the absence of PDE4 specific inhibitor, rolipram, minus the PDE activity in the presence of 100 μM rolipram in the standard reaction mixture and expressed as pmol/min/mg protein.

3.8 Western Blotting

3.8.1 SDS-PAGE Electrophoresis

Immediately prior to immunoblotting, prepared samples (section 3.4.3.1 and 3.4.3.2) were thawed and boiled. Proteins in samples were separated on a 0.75 mm discontinuous vertical slab mini-gel in an electrophoresis cell (Mini Protean II, Biorad). A number of 8% acrylamide resolving gels were made by mixing 13.45 ml of ultra pure water, 8.0 ml of 30% acrylamide/bisacrylamide solution, 7.5 ml of Tris-Cl (1.5 M; pH 8.8) and 0.75 ml of 10% SDS. Polymerisation of the resolving gel was then initiated by adding 0.3 ml of 10% ammonium persulphate (APS) and 20 μl of N'N'N'N- Tetramethyletylenediamine (Temed). Immunoblotting of Darpp-32 required a 10% acrylamide resolving gel. In this instance 12.0 ml ultra pure water and 10.0 ml bisacrylamide solution was used.

The monomer was gently stirred and applied between the glass plates filling 2/3 of the gel sandwich. The gel solution was then immediately coated with a thin layer of water-saturated isobutanol to assist in polymerisation and evenly spread the resolving gel. After leaving the gel to polymerise for approximately 1 hour, the isobutanol was carefully soaked with a filter paper, without disturbing the resolving gel. A 4% acrylamide stacking gel was prepared by mixing 9.15 ml ultra pure water, 1.95 ml of 30% acrylamide/ bisacrylamide solution, 3.75 ml of Tris-Cl (0.5 M; pH 6.8), 250 μl of 10% SDS, 75 μl of 10% APS and 20 μl of TEMED. To remove any traces of isobutanol that might be present, the surface of the polymerised resolving gel was rinsed with double distilled

water. The gel sandwich was then filled to the top with stacking gel. A 0.75 mm Teflon comb was inserted into the stacking solution to allow well formation and the gel was left to polymerise at room temperature.

Following polymerisation, the Teflon comb was removed and the wells rinsed with electrophoresis tank buffer (0.1% SDS, 0.25 M Tris base, 1.92 M glycine, pH 8.3). Equal amount of protein was loaded by adjusting volume applied to each well. 50 μg of samples mixed with loading buffer were then loaded into the wells and the gel was run for approximately 90 minutes at 200 volts with initial current of 60 mA/gel. To facilitate detection of proteins, a protein marker was loaded into the outer well of each gel. Electrophoresis was terminated when the tracking dye front had just reached the bottom of the resolving gel.

3.8.2 Immunoblotting and Chemilumenescence

The gels were removed from the electrophoresis cell and equilibrated in transfer buffer (39 mM glycine, 48 mM Tris-Cl, 0.0375% SDS and 20% methanol) for 30 minutes. Proteins were transferred onto a polyvinylidene diflouride (PVDF) membrane using a semi-dry electrophoretic transfer cell (Trans-Blot SD, Biorad) for 30 minutes at 20 Volts. The PVDF membranes were incubated in blocking buffer solution (5% w/v non fat dried milk in wash buffer containing 10 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 2 hours at room temperature to prevent any non-specific binding of antibodies to the membrane. The membranes were then washed 4 times with wash buffer (a primary 15 minute wash followed by three 5 minute washes) by gentle agitation on a rotary shaker, and subsequently incubated with a specific primary antibody (table 3.2) prepared in blocking buffer containing 5% milk.

Incubations with primary antibody were carried out at room temperature for 1 hour. Following incubation, each membrane was washed for 15 minutes in wash buffer followed by 3 washes of 5 minutes. The membranes were then incubated with the secondary antibody (goat anti-rabbit horseradish peroxidase (HRP)-labelled antibody) in blocking buffer containing 5% milk for 1 hour at room temperature on a rotary shaker. After thorough washing (30 minutes), the membranes were placed onto cling film and

incubated in ECL Advance detection solution (0.125 ml/cm^2 membrane) for 1 minute at room temperature. The solution was drained and the membrane wrapped with cling film, avoiding any trapped air bubbles, and exposed to films (Hyperfilm^{TM} ECL) for 1-2 min in a darkroom. The films were immediately developed using Premix developer and fixed using Premix fixer. Each primary PDE4 antibody detects a number of different isoforms (table 3.3). The identity of each individual isoform was confirmed by initial pilot studies. In each instance, sample was incubated with a blocking peptide specific for each isoform, and an immunoblot performed. The absence of the specific PDE4 isoform from the immunoblot confirmed its identity.

3.8.3 Chemilumenescence using gel documentation system

Immediately following x-ray film development, PVDF membrane with still active ECL Advance detection solution is rapidly transferred onto the transilluminator platen of the ChemiDoc XRS gel documentation system. The ChemiDoc XRS system consists of a universal hood on top of which is mounted a CCD camera for capturing images. The camera comes with a motorized zoom lens that allows remote adjustment of lens control functions such as focus, zoom and iris. The universal hood is designed to capture fluorescence and chemiluminescence images without using a photographic darkroom. The enclosure has built-in white light epi-illumination and an ultraviolet transillumination. Both camera and hood is connected to a computer with one full version of Quantity One® software.

To capture an image on the ChemiDoc XRS system the following steps were followed once the PVDF membrane was placed on the transilluminator platen. The main switch of both the hood (including the camera) and computer was turned on and the software started. In the Quantity One acquisition window, the *chemiluminescence* application is selected and *Live/Focus* mode is selected. The latter option will display the PVDF membrane via the camera in the Quantity One window on the computer screen.

The Epi-illumination button to turn on Epi White lights is pressed and the camera lens, iris, zoom and focus adjusted. Once a satisfactory image is seen, *Freeze* is selected and the Epi White lights are switched off. Finally an image is acquired by selecting *Live*

Acquire. For chemiluminescence of PDE and Darpp-32 samples, exposure was set to collect an image every 30 seconds for a period of 15 minutes. Bands began to develop after about 1 minute. For permanent records and general data collection, all images captured after a 5 minute exposure was further analysed.

Table 3.2 Standardized antibody dilutions prepared for western blot analysis

Primary rabbit polyclonal IgG	Primary antibody dilution used	Secondary antibody dilution used
PDE4A	1:2500	1:25,000
PDE4B	1:1500	
PDE4D	1:5000	
phospho-DARPP-32 Thr34	1:1500	
phospho-DARPP-32 Thr75	1:1500	

Table 3.3 PDE isoforms identified by polyclonal antibodies

PDE4 (subtype)	Isoform identified by primary antibody	Protein size (kDa)
PDE 4A	PDE 4A5	109
	PDE 4A8	106
	PDE 4A1	66
PDE 4B	PDE 4B1	107
	PDE 4B3	100
	PDE 4B4	66
PDE 4D	PDE 4D4	105
	PDE 4D1	68

3.9 Statistical analyses

3.9.1 Statistical analysis of behavioural data

All behavioural data was collected by the DAAM analyser and expressed as counts per hour (Cph), and graphically expressed as mean ± standard error of the mean (SEM). The computer software Microsoft Excel 2002® and GraphPad Prism 4.01® were used to process all experimental data. Statsoft Statistica (Statsoft Inc, 2001: version 7) data analysis software was employed for statistical analysis of all data. For the initial predictive validation, baseline data was compared to post-drug data by paired Student's t-test and expressed as the mean ± SEM. In all instances, statistical significance was defined at the 95% ($p < 0.05$) levels. For construct validation, data was analysed by one-way ANOVA followed by Bonferonni or Dunnetts test.

3.9.2 Statistical analysis of neurochemical and protein expression data

Statsoft Statistica (Statsoft Inc, 2001: version 7) data analysis software was use for the statistical analysis of all data. Data was analysed by one-way ANOVA followed by Tukey's post hoc tests. Statistical significance was defined at the 95% ($p < 0.05$) levels.

Table 3.4 List of reagents and drugs used during the study

Reagent/Drug	Supplier
Acetic acid glacial	Merck
Acrylamid/Bis solution (30%)	Bio-Rad
[2,8-^{3}H] Adenosine 3':5'-cylic phosphate	Amersham Biosciences
Adenosine 3':5'-cyclic monophosphate (cAMP)	Sigma
Ammonium persulfate	Sigma
Aprotonin	Sigma
Cling wrap	Local retailer
Cyclic AMP (^{3}H) assay system	Amersham Biosciences
BCA protein assay kit	Pierce
Blocking peptide	Santa Cruz
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Fluka
DARPP-32 phospho-Thr34 primary antibody	Santa Cruz
DARPP-32 phospho-Thr34 primary antibody	Santa Cruz
Desipramine	Sigma
ECL Advance western Blotting detection kit	Amersham Biosciences
Ethanol absolute	Merck
EDTA	Merck
Extra thick blotting paper	Bio-Rad
Fluoxetine	Aspen Pharmacare (South Africa)
Glycerol	USB
Glycine	Sigma
goat anti-rabbit IgG Horseradish peroxidase conjugate	Santa Cruz
Hybond-P PVDF membrane	Amersham Biosciences
Hydrochloric acid 32%	Merck
Hyperfilm X-ray	Amersham Biosciences
magnesium chloride ($MgCl_2$)	Fluka
2-mercaptoethanol	Merck
meta-chlorophenylpiperazine (mCPP)	Tocris
methanol	Sigma

Table 3.4 (continue)

Milk powder (non-fat)	Local retailer
Nonidet P-40	Roche
PDE4A rabbit polyclonal IgG	Santa Cruz
PDE4B rabbit polyclonal IgG	Santa Cruz
PDE4D rabbit polyclonal IgG	Santa Cruz
Pepstatin A	Sigma
phenylmethylsulfonyl fluoride (PMSF)	Sigma
resin (AG 1-X2, chloride form)	Bio-Rad
Premix Developer	AGFA
Premix Fixer	AGFA
Protein standard (Precision Plus All Blue)	Bio-rad
Quinpirole	Sigma
Rolipram	Sigma
SKF 38393	Sigma
snake venom (Crotalus atrox)	Sigma
sodium chloride (NaCl)	Sigma
sodium-deoxycholate	Sigma
Sodium dodecyl sulphate (SDS)	Bio-Rad
sodium fluoride (NaF)	Sigma
sodium orhtovanadate (Na_3VO_3)	Sigma
TEMED (N'N'N'N'-Tetramethylenediamine)	Sigma
Tracker tape	Amersham Biosciences
Trizma (Tris) base	Sigma
Trypsin inhibitor	Sigma
Tris-hydrochloride (Tris-Cl)	Sigma
Tween-20 (10% Solution)	Bio-Rad
Ultima gold XR liquid scintillation fluid	Perkin-Elmer

4. Results – Behaviour

4.1 Introduction and experimental design

The study was initiated with a baseline screening of deer mice and C57Bl mice. Animals were assembled into groups according to their stereotypic score. Stereotypic deer mice (LSB and HSB) were randomly assigned into three separate groups. For chronic drug studies aimed at assessing efficacy in attenuating inherent stereotypy in the model, fluoxetine and desipramine were administered at low and high doses of respectively 10 and 20 mg/kg for a period of 21 days. A control group of each stereotypic set received saline. For subacute challenge studies with mCPP (a non-selective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist) and quinpirole (QNP; a D_2 selective agonist), drugs were administered at doses of 2 mg/kg and 5 mg/kg respectively for 4 days. In the subacute mCPP and QNP-challenges preceded by chronic fluoxetine and desipramine, the latter two drugs were administered at high dose (20mg/kg) for 21 days. Upon completion of the treatments, stereotypic behaviour was determined as described in section 3.3. Behaviour was also collected following subacute mCPP or QNP challenges. Deer mice were sacrificed 12 hours following the last behavioural assessment and the striatum and frontal cortex was rapidly dissected and frozen at -80°C for further neurochemical and molecular analysis (see results, chapter 5 & 6).

mCPP was selected to explore the involvement of 5-HT in deer mice since 5-HT has been is implicated in the pathophysiology of OCD (section 2.4.3). QNP was chosen to explore the involvement of dopamine. In a study by Denys and co-workers (Denys et al., 2004), abnormalities in the binding potential of the dopamine D_2 receptor was shown in OCD patients, which suggest an involvement of the dopaminergic system in the pathophysiology of OCD. Here, D_2 receptor binding in the left caudate nucleus was significantly lower in the patients with OCD than in healthy control subjects. D_2 receptors may also play a role in the anxiety component of OCD, since D_2 receptor antagonists reduce anxiety-like activity in rodents, suggesting a specific role for the D_2 receptor in mediating fear or anxiety-like responses (Pich & Samanin, 1986). Since a number of studies have explored the involvement of D_1 receptors in deer mice (Presti el al., 2003; Presti et al., 2004), this study chose to further explore D_2 receptors and their involvement in stereotypy.

4.2 Validation of stereotypic behaviour and relevance to OCD

The validation of deer mice as an animal model for OCD requires that essential criteria be met namely face, predictive, and construct validity. Face validity refers to a phenomenological similarity between the model and the disorder it simulates. Ideally, the model should resemble the condition it models in its aetiology, symptomology, treatment, and physiological basis. Predictive validity demands that performance in the test predicts performance in the modelled condition. In principle, predictive validity can rely on aetiology, physiology, and response to treatment. In practice, predictive validity is usually based on response of the model to pharmacological treatment (Willner, 1991). Construct validity means that the model has a sound theoretical rationale, and that it should confirm current rationale and/or elucidate new underlying mechanisms responsible for the disorder.

4.2.1 Determination of basal stereotypic behaviour: face validity

An important aspect of the study was to separate animals according to degree of behavioural manifestations by assessing basal (untreated) stereotypy of each animal involved in the study and thereafter to perform pharmacological challenges in the separate stereotypic deer mice populations. For this reason a baseline screening was performed and animals were assembled into groups according to their stereotypic score as described in section 3.3 (figure 4.1). Three groups of deer mice were distinguished, namely high stereotypic (HSB; Cph > 2000), low stereotypic (LSB; Cph of 1000 – 2000) and non-stereotypic (NS; <1000 Cph). For the initial chronic fluoxetine and desipramine studies aimed at assessing their efficacy at attenuating inherent stereotypy, only LSB and HSB mice were used. To broaden face validity of the deer mouse as a model of stereotypy, the behaviour of deer mice was compared to behaviour of a species that do not develop spontaneous stereotypic behaviour under standard laboratory conditions, namely C57Bl mice.

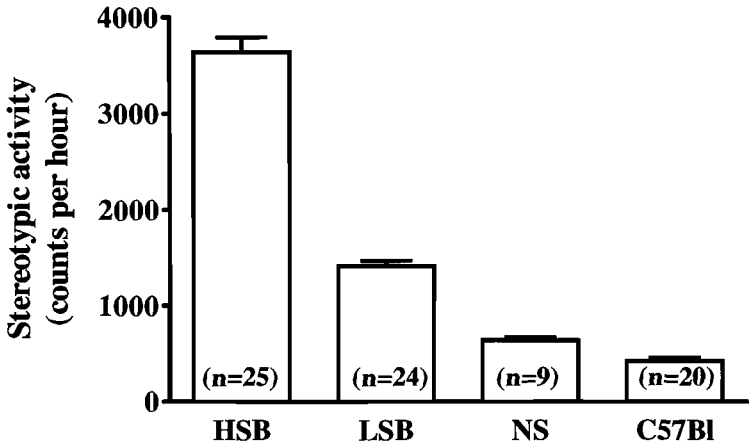


Figure 4.1 Basal stereotypic activities of deer mice and CB57Bl mice. Three distinct groups of deer mice were identified, namely high stereotypic (HSB), low stereotypic (LSB) and non-stereotypic (NS) animals. Included in the graph is the behavioural assessment of mice (C57Bl) that do not develop stereotypic behaviour under standard laboratory conditions. Data represent the average of three behavioural assessment sessions and is expressed as the mean ± SEM. The number of animals used (n value) is given in brackets within each bar.

The most common topographies of stereotypy in the colony of deer mice were repetitive jumping, patterned running, and backward somersaults, while C57Bl did not perform any stereotypic behaviour although did occasionally engage in vertical activity, i.e. to jump from the cage floor to the cage top. A representative example of these behaviours is depicted in table 1. Enough animals for the study to allow for necessary screening into specific categories of stereotypy intensity was based on a prior pilot study performed before commencement of the study. Overall, 194 litters were used to establish successful topographical screening of deer mice. Animals were assembled into groups according to their stereotypic score (see figure 4.1). Of the 58 deer mice screened, 25 mice were consequently classified as high stereotypic (43%), 24 as low stereotypic (41%) and 9 as non-stereotypic mice (16%). Of the 20 C57Bl mice monitored for the same behaviours, all presented with scores lower than 1000 cph, and were accordingly classified as non-stereotypic animals (figure 4.1). A representative example of the typical behaviours monitored in deer mice and C57Bl in this study are depicted in table 4.1. The three behavioural sessions spaced 7 days apart were used to allow the animals to recover from the stress of handling. Behavioural scores indicate that deer mice did not habituate across these sessions.

The DAAM system is able to measure a number of locomotor and stereotypic movements. For this study, we were interested in cage revolutions, defined by DAAM as the number of times an animal runs around in a circle, however, if the animal is rotating (e.g. around one leg) rather than travelling in a circle, this variable is not incremented. Two other parameters namely vertical activity, which represent repetitive jumping (i.e. the total number of beam interruptions that occur in the vertical sensor during a sample period), and vertical movement, which represent backward somersaults (i.e. just as vertical activity, but the animal must go below or above the vertical sensor for at least one second before the next movement can be registered) were also used. Since C57Bl occasionally engage in vertical activity, this parameter was taken into account when the final cph score of all mice used in the study were calculated. DAAM has in-built criteria for stereotypy count and stereotypy number, and is programmed so that if the animal breaks the same set of beams repeatedly, then the monitor considers the animal to be exhibiting stereotypy. This however typically happens during grooming, head bobbling,

Table 4.1 Behavioural activity data directly from Digiscan Animal Activity Monitor (DAAM)

Animal #	Classification	Drug treatment	Vertical activity	Vertical Movement	Cage Revolutions	DAAM Stereotypy count	Counts per hour (cph)
1	LSB		1194	228	51	1858	**1422**
2	NS		235	187	18	4109	**522**
3	HSB		1117	238	1296	6160	**2651**
4	NS		94	54	9	5452	**148**
5	NS		344	127	25	5667	**471**
6	HSB		1986	1565	154	6932	**3551**
		Fluoxetine	1802	494	183	5322	**2296**
7	HSB		2672	793	85	5696	**3465**
		Desipramine	2718	814	79	5702	**3532**
8	HSB		2870	805	45	4423	**3675**
		Saline	2895	977	65	4385	**3872**

nibbling on cage material etc. and could not be considered stereotypy. Thus, even when 'stereotypy' is performed according DAAM, it does not match the topographies engaged by deer mice. Therefore only vertical activity, vertical movement, and cage revolution data from the DAAM system were used corresponding to repetitive jumps, pattern running and back somersaults, respectively. These stereotypy's were confirmed visually during extensive pilot studies and indeed, extremely high correlations between visual and automated recordings of all recorded behaviour were found in the present study as well as previous seminal studies (Sanberg et al., 1987; Sanberg et al., 1983).

Representative data from the DAAM describing the key stereotypy parameters measured are presented in table 4.1. Cph scores were determined as follows: animal #1 has a vertical movement score of 228 per hour, a vertical activity score of 1194, giving a final cph score of 1422, and is designated LSB. Animal #2 has a vertical movement score of 187 per hour, a vertical activity score of 235, giving a final cph score of 522, and is designated NS. Animal #3 has a vertical activity score of 1117 per hour, and performs 1296 cage revolutions, giving a final cph score of 2651, and is designated HSB. An example of the typical topographies performed by deer mice include back somersaults (#6, vertical movement score of 1565), repetitive jumping (#1, vertical activity score of 1194), and pattern running (#3, cage revolution score of 1296). Animals #4 and #5 are C57Bl mice and, although they occasionally jump from the cage floor, they do not engage in repetitive jumping, pattern running or back somersaults. In deer mice, this triad of 'stereotypy' parameters were confirmed visually at the outset of the study, especially since stereotypy as defined by the DAAM system is not representative of the typical stereotypy performed by deer mice, but in fact behaviour such as grooming, head bobbing etc. Thus, using the above-mentioned triad of parameters across deer mice as well as C57Bl mice, we were able to confirm that deer mice display distinct behaviour that is stereotypic in nature and that are displayed to different degrees in a given population, designated HSB, LSB and NS (figure 4.1).

4.2.2 Predictive validation

4.2.2.1 Effect of 10 mg/kg SSRI/NRI on stereotypic behaviour of LSB mice

Pharmacological isomorphism is an important factor for assessing the validity of an animal model and hence the validation process involves testing the effects of relevant pharmacological agents. For this reason, empirical validity (pharmacological isomorphism) was evaluated through the differential response of the model to a drug of known efficacy in OCD, namely fluoxetine, and a drug known not to be effective in OCD, namely, desipramine. All mice used during pharmacological studies were naïve and treatment began one week after the last behavioural assessment session. LSB mice received 10 mg/kg of either fluoxetine or desipramine, while no-drug controls received an equivalent volume of vehicle. Administration of 10 mg/kg fluoxetine resulted in a significant decrease in spontaneous stereotypic behaviour (figure 4.2; $t(8) = 3.98$, $p = 0.005$). In contrast, desipramine ($t11) = - 0.89$, $p = 0.399$) and saline ($t(8) = - 1.26$, $p = 0.246$) did not significantly modify stereotypic behaviour. Table 4.1 provides a representative illustration of the effects of drug treatment (of animals #6-8), or lack thereof, on baseline stereotypic behaviours in HSB deer mice as recorded by the DAAM. Thus, fluoxetine reduced stereotypic behaviour, (i.e. vertical movement/back somersaulting of deer mouse #6), whereas stereotypic behaviours were unchanged by treatment with desipramine (deer mouse #7) or saline treatment (mouse #8).

4.2.2.2 Effect of 10 mg/kg SSRI/NRI on stereotypic behaviour of HSB mice

HSB mice received a 10 mg/kg dose of fluoxetine, desipramine, or vehicle. Neither 10mg/kg desipramine ($t(9) = - 2.04$, $p = 0.0.75$) nor vehicle ($t(14) = - 0.207$, $p = 0.838$) modified stereotypic behaviour in these animals (figure 4.3) although 10mg/kg fluoxetine ($t(9) = 2.431$, $p = 0.032$) significantly reduced stereotypic behaviour in the HSB group (figure 4.3).

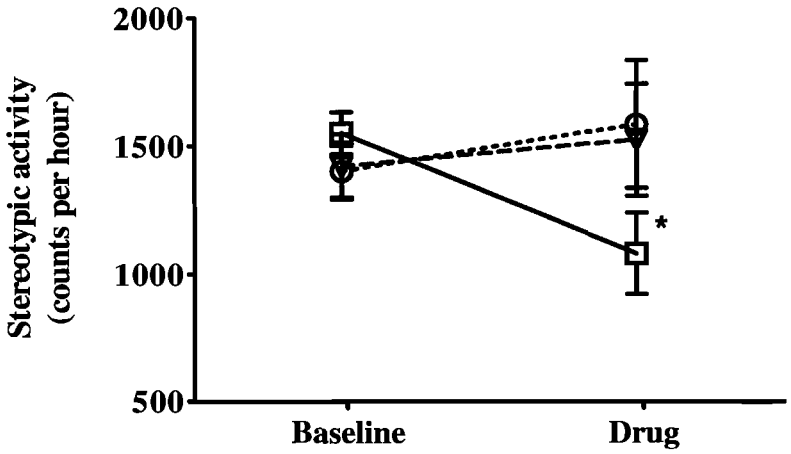


Figure 4.2 Effect of 10 mg/kg SSRI/NRI on stereotypic behaviour of LSB mice. Baseline stereotypic activity for each group is provided as well as the cph-score following chronic treatment with fluoxetine (solid line with open square, n = 9), desipramine (broken line with triangle, n = 12) or vehicle (dotted line with circle, n = 9). Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement for the drug altered score. Data is expressed as the mean ± SEM and * indicates $p < 0.05$ for fluoxetine or desipramine v saline treated basline.

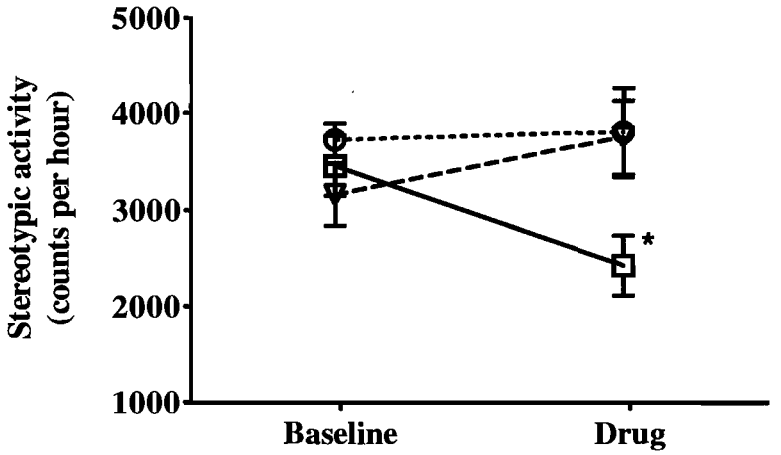


Figure 4.3 Effect of 10 mg/kg SSRI/NRI on stereotypic behaviour of HSB mice. Baseline stereotypic activity for each group is provided as well as the cph-score following chronic treatment with fluoxetine (solid line with open square, n = 10), desipramine (broken line with triangle, n = 10) or vehicle (dotted line with circle, n = 15). Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement for the drug altered score. Data is expressed as the mean ± SEM and * indicates $p < 0.05$ for fluoxetine or desipramine v saline treated basline.

4.2.2.3 Effect of 20 mg/kg SSRI/NRI on stereotypic behaviour of LSB mice

SRIs and SSRIs are, to date, the only effective pharmacological treatment of OCD. Since OCD patients often require much higher than standard antidepressant doses of SRI (Goodman et al., 1990; Goodman et al., 1991). The role of dosage of treatment in this putative animal model was studied. Mice were treated with a 'high' dose (20 mg/kg) of fluoxetine or desipramine. As shown in figure 4.4, treatment of LSB mice with 20 mg/kg fluoxetine resulted in a significant decrease in stereotypic behaviour ($t(17) = 3.20$, $p = 0.005$). Animals administered with 20 mg/kg desipramine ($t(17) = 1.30$, $p = 0.211$) and vehicle ($t(12) = - 0.33$, $p = 0.745$), however, did not respond to treatment.

4.2.2.4 Effect of 20 mg/kg SSRI/NRI on stereotypic behaviour of HSB mice

HSB mice also received a 'high' dose of fluoxetine, desipramine, or vehicle. Fluoxetine significantly reduced stereotypic behaviour in the HSB group (figure 4.5; $t(17) = 4.11$, $p = 0.0003$). Treatment with desipramine ($t(14) = - 0.365$, $p = 0.719$) or vehicle ($t(16) = 0.606$, $p = 0.551$) did not improve stereotypic behaviour over the 21 day period it was administered. In a separate analysis, no significant differences between high and low dose fluoxetine treatments were evident in both HSB and LSB animals, although of note was that the degree of scatter in standard deviation were less pronounced in the high dose group (table 4.2).

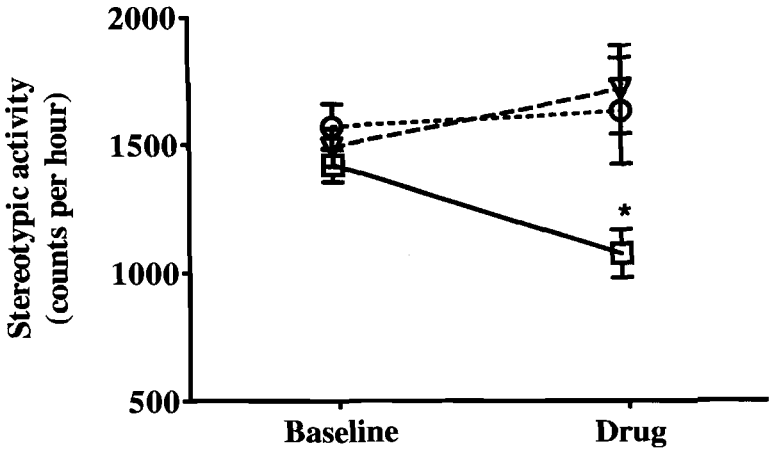


Figure 4.4 Effect of 20 mg/kg SSRI/NRI on stereotypic behaviour of LSB mice. Baseline stereotypic activity for each group is provided as well as the cph-score following chronic treatment with fluoxetine (solid line with open square, n = 18), desipramine (broken line with triangle, n = 18) or vehicle (dotted line with circle, n = 13). Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement for the drug altered score. Data is expressed as the mean ± SEM and * indicates $p < 0.05$ for fluoxetine or desipramine v saline treated basline.

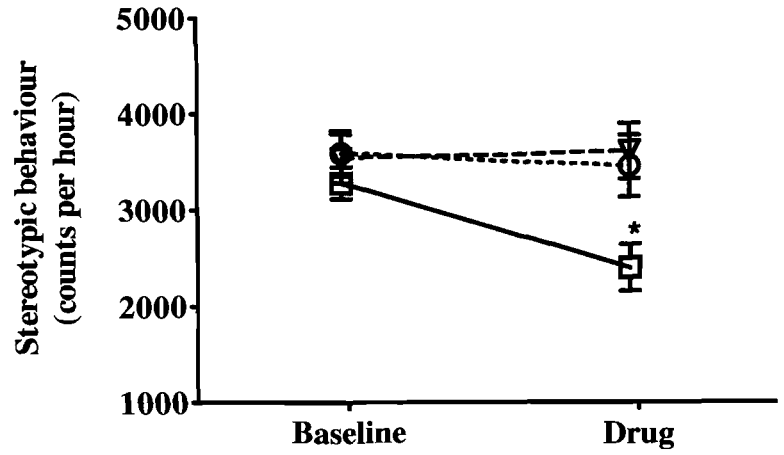


Figure 4.5 Effect of 20 mg/kg SSRI/NRI on stereotypic behaviour of HSB mice. Baseline stereotypic activity for each group is provided as well as the cph-score following chronic treatment with fluoxetine (solid line with open square, n = 18), desipramine (broken line with triangle, n = 15) or vehicle (dotted line with circle, n = 17). Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement for the drug altered score. Data is expressed as the mean ± SEM and * indicates $p < 0.05$ for fluoxetine or desipramine v saline treated basline.

Table 4.2 Standard deviation (SD) in stereotypic mice subsequent to drug treatment.

	10 mg/kg fluoxetine		20 mg/kg fluoxetine	
	Mean (cph)	**SD (cph)**	**Mean (cph)**	**SD (cph)**
LSB	1081	160	1078	94
HSB	2432	314	2407	240

4.2.3 Construct validation

The objective of this component of the study was to validate spontaneous stereotypic behaviour in the deer mouse as a putative animal model of OCD. With the increasing evidence for a causal role for dopamine and 5-HT in the neurobiology and treatment response in OCD (Goodman et al 1990; Goodman et al 1991; Denys et al., 2004), the study set out to evaluate the construct validity of the model. This was done by studying the behavioural response of deer mice to subacute (4 days) serotonergic (mCPP, 2 mg/kg) and dopaminergic (quinpirole, 5 mg/kg; SKF, 5 mg/kg) challenges. In addition, the ability of chronic fluoxetine or desipramine to prevent said changes was explored.

4.2.3.1 Subacute drug challenge of stereotypic deer mice

Prior to exploring the ability of fluoxetine and desipramine to attenuate behaviour, a study was performed to establish the degree and tendency (increase or decrease) of attenuation of subacute drugs in stereotypic deer mice. LSB and HSB mice was therefore subactutely (4 days) challenged with either mCPP, SKF or QNP. LSB mice treated with subacute mCPP ($t(9) = 2.54$, $p = 0.011$) as well as QNP ($t(9) = 2.86$, $p = 0.007$) demonstrated significantly reduced stereotypic activity compared to baseline (figure 4.6A). Treatment with D_1 agonist, SKF ($t(9) = 1.148$, $p = 0.135$) did not significantly reduce stereotypic behaviour, although a trend was noted. Treatment of LSB mice with saline ($t(8) = 3.19$, $p = 0.0048$) resulted in a significant decrease in stereotypic behaviour in LSB mice (figure 4.6A).

HSB mice treated with mCPP ($t(12) = 5.52$, $p = 0.0001$), SKF ($t(11) = 2.81$, $p = 0.006$), and QNP ($t(9) = 3.83$, $p = 0.0005$) resulted in a significant decrease in stereotypic behaviour in this group compared to baseline (figure 4.6B). It was furthermore noted that stereotypic behaviour was significantly reduced in HSB mice following treatment with saline ($t(9) = 3.04$, $p = 0.004$).

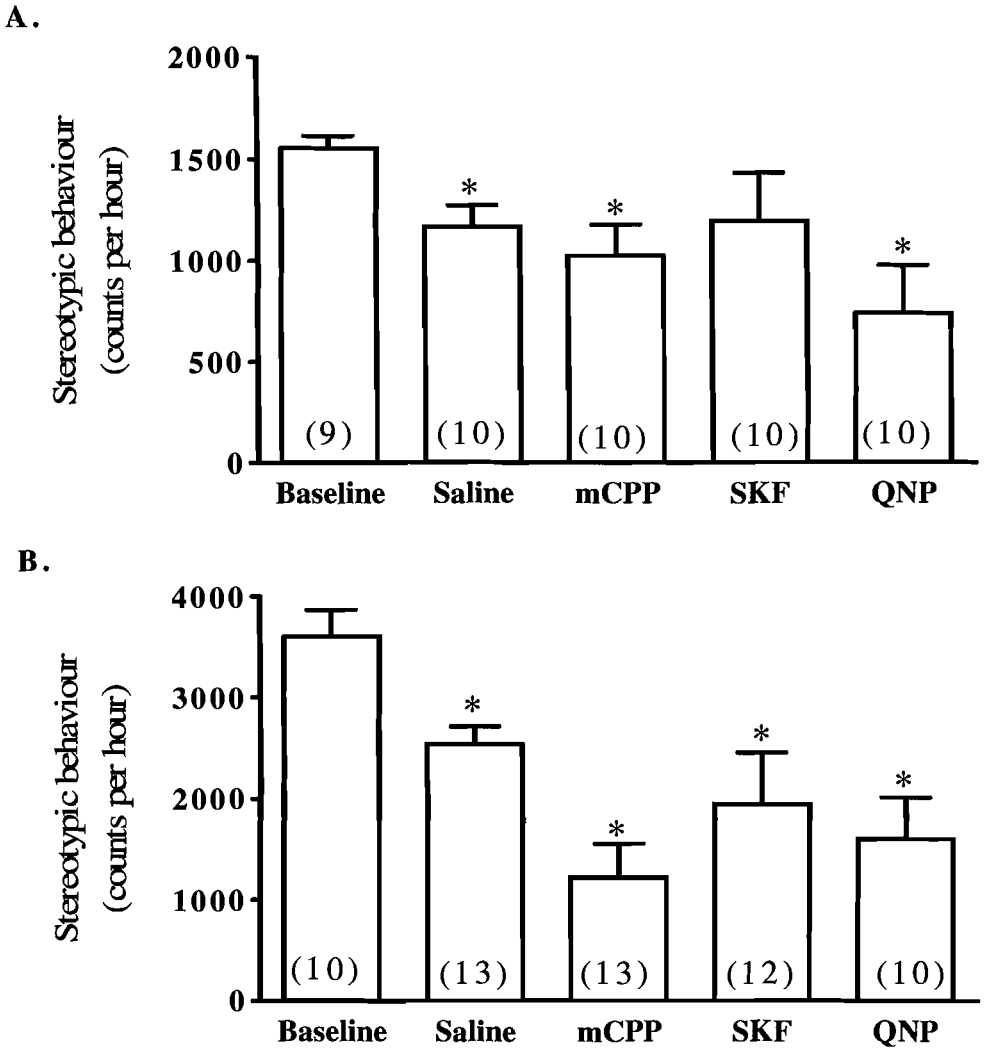


Figure 4.6 Subacute drug challenge of stereotypic deer mice. LSB mice (A) and HSB mice (B) were subacutely challenged with mCPP, SKF and QNP for a period of 4 days. Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement after drug treatment for the drug altered scores. Data is expressed as the mean ± SEM and the number of animals used (n value) is given in brackets within each bar. For subacute challenges, data was analyzed by ANOVA followed by Bonferonni test (e.g. drug challenge v baseline). Student t test was performed for baseline v saline data. In all instances * indicates $p < 0.05$.

4.2.3.2 Subacute mCPP challenge and effect of chronic NRI/SRI treatment in deer mice

LSB mice treated with subacute mCPP ($t(9) = 2.55$, $p = 0.041$) and those receiving chronic saline plus subacute mCPP ($t(10) = 4.14$, $p = 0.025$) both demonstrated significantly reduced stereotypic activity compared to baseline (figure 4.7A). Interestingly, chronic fluoxetine (20 mg/kg; $F = 1.89$, $p = 0.003$) but not desipramine (20mg/kg; $F = 2.91$, $p = 0.396$) pre-treatment prior to mCPP challenge significantly prevented the decrease in stereotypic behaviour evoked by mCPP in LSB mice (figure 4.7A).

Subacute mCPP challenge ($t(14) = 4.92$, $p = 0.014$) and mCPP plus subacute saline ($t(11) = 1.03$, $p = 0.027$) both indicated that mCPP induces significantly reduced stereotypic behaviour in HSB mice versus baseline (figure 4.7B). Additionally, neither fluoxetine (20mg/kg; $F = 1.21$, $p = 0.311$) nor desipramine (20mg/kg; $F = 2.30$, $p = 0.161$) pre-treatment prior to subacute mCPP challenge significantly altered mCPP-induced changes in stereotypic behaviour in HSB mice (figure 4.7B).

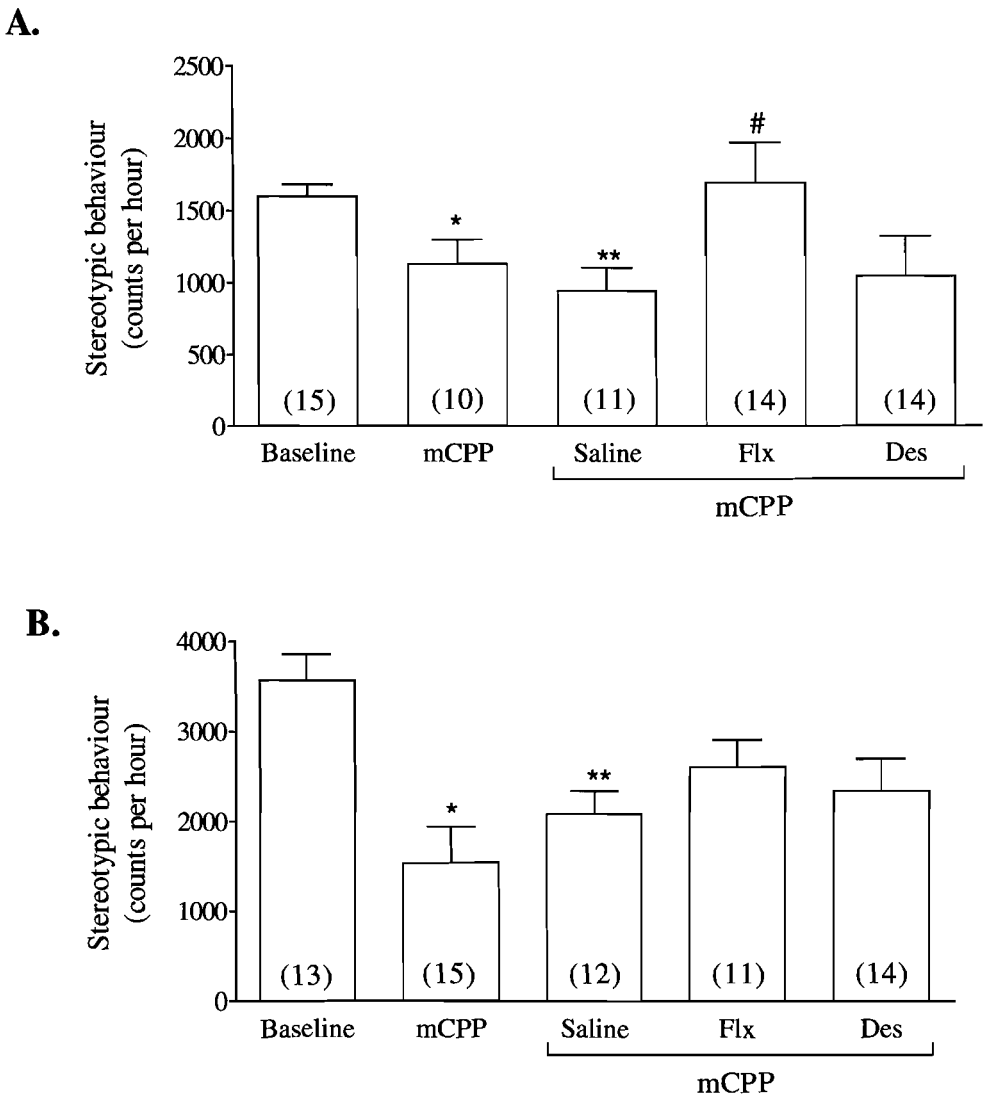


Figure 4.7 Effect of mCPP in stereotypic deer mice. The effect of mCPP challenge and pre-treatment plus challenge in LSB mice (A) and HSB mice (B) is shown. Empty bars express the stereotypic behaviour in deer mice that were untreated (baseline) or mCPP challenged. Filled bars indicate mCPP challenged mice following pre-treatment with saline, fluoxetine or desipramine. Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement after drug treatment for the drug altered scores. Data is expressed as the mean ± SEM and the number of animals represented (n value) is given in brackets within each bar. * indicates $p < 0.05$ (mCPP v baseline), ** indicates $p < 0.05$ (chronic saline plus mCPP v baseline). # indicates $p < 0.05$ (vehicle v fluoxetine). For subacute challenge following pretreatment, data was analyzed by ANOVA followed by Bonferonni test. Student t test was performed for baseline v saline or mCPP challenge.

4.2.3.3 Subacute QNP challenge and effect of chronic NRI/SRI treatment in deer mice

LSB mice receiving subacute QNP (D_2 agonist) treatment ($t(11) = 3.37$, $p = 0.006$) as well as receiving chronic saline plus subacute QNP ($t(17) = 4.75$, $p = 0.02$) both demonstrated a significantly attenuated stereotypic behaviour versus baseline (figure 4.8A). Furthermore, chronic pre-treatment with fluoxetine (20mg/kg; $F = 3.12$, $p = 0.04$) resulted in a significant reversal of QNP-induced behaviour, with desipramine (20mg/kg; $F = 8.41$, $p = 0.952$) not having marked effect in this regard.

In HSB mice subacute QNP administration ($t(14) = 3.54$, $p = 0.006$) and QNP plus subacute saline treatment ($t(14) = 2.37$, $p = 0.039$) indicated that QNP evokes a significant decrease in stereotypic behaviour (figure 4.8B). However, neither fluoxetine (20 mg/kg; $F = 6.23$, $p = 0.055$) nor desipramine pre-treatment (20 mg/kg; $F = 1.09$, $p =$ 241) prior to subacute QNP challenge modified QNP induced behaviours (figure 4.8B), although with fluoxetine a definite trend towards reversal was noted.

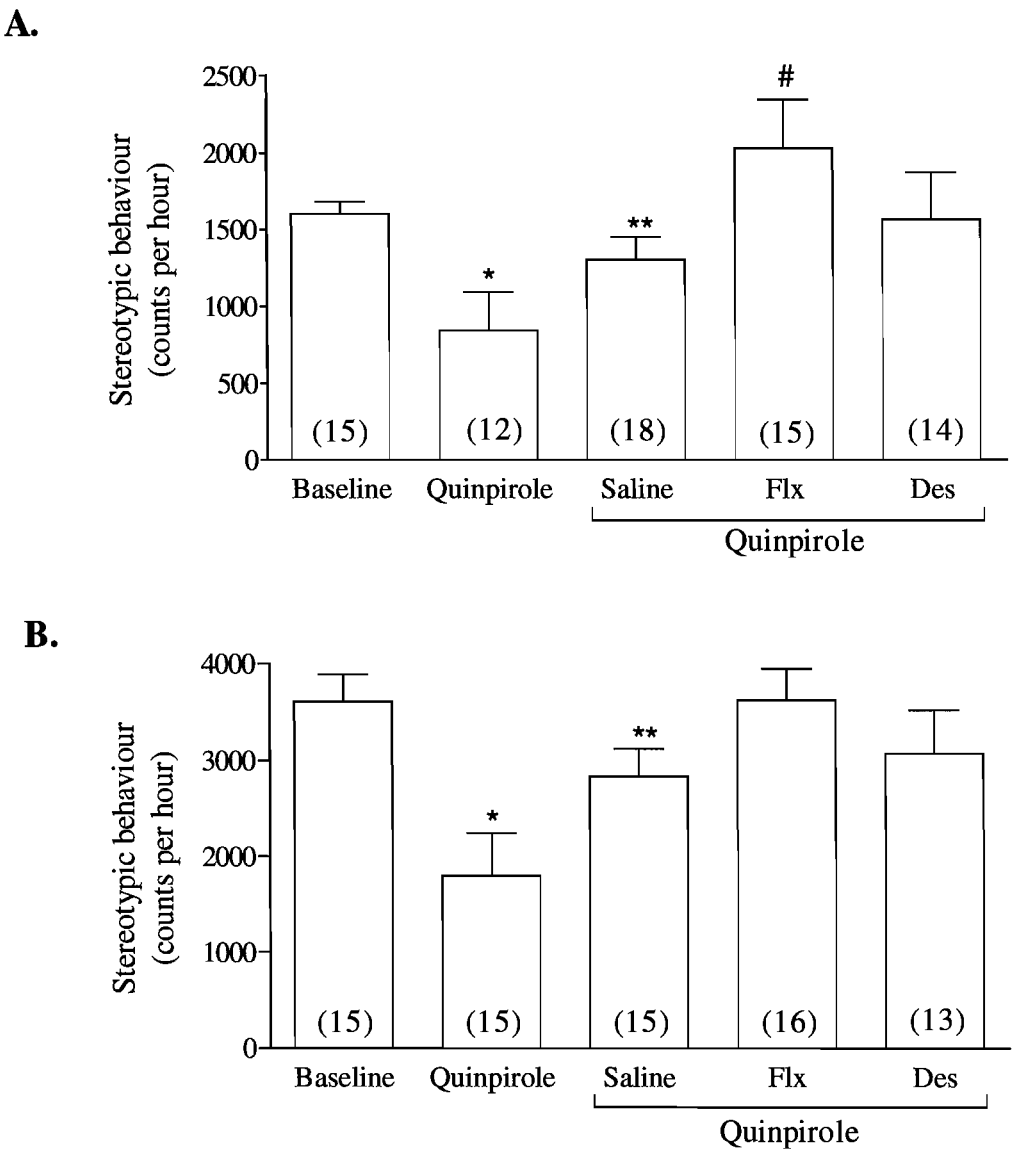


Figure 4.8 Effect QNP in stereotypic deer mice. The effect of QNP challenge and pre-treatment plus challenge in LSB mice (A) and HSB mice (B) is shown. Empty bars express the stereotypic behaviour in deer mice that were untreated (baseline) or QNP challenged. Filled bars indicate QNP challenged deer mice following pre-treatment with saline, fluoxetine or desipramine. Data represent the average of three behavioural assessment sessions for the baseline score and a once-off measurement after drug treatment for the drug altered score. Data is expressed as the mean ± SEM and the number of animals represented (n value) is given in brackets within each bar.* indicates $p < 0.05$ (QNP v baseline), ** indicates $p < 0.05$ (chronic saline plus QNP v baseline). # indicates $p < 0.05$ (vehicle v fluoxetine). For subacute challenge following pretreatment, data was analyzed by ANOVA followed by Bonferonni test. Student t test was performed for baseline v saline or QNP challenge.

4.3 Summary of results: validation of animal model

4.3.1 Behavioural assessment and face validity

Since stereotypic behaviour in deer mice becomes evident early in life, being most notable at about 20 days of age and becomes well developed by 30 days of age, the study was performed on adult mice older than two months (56 days). Animals of this age were chosen in order to reduce the degree of variability in the rate of development observed between animals. Comparing jumping or pattern running to backward somersaulting, typical topographies seen in deer mice, suggests that somersaulting develops later than jumping or running. This differential rate of development may relate to the animal's physical development with backward somersaulting requiring greater motor competence (Powell et al., 1999).

The most frequent compulsions noted in patients with OCD are checking, arranging, ordering, cleaning or washing, hoarding and counting (Rasmussen & Eisen, 1992). Deer mice engage in various compulsive stereotypic behaviours, including repetitive jumping, patterned running, and backward somersaults (Powell et al., 1999), that are strongly reminiscent of the aforementioned range of compulsive and stereotypic behaviours seen in OCD. Furthermore, while housing conditions remained constant throughout the study and in all groups, it was observed that deer mice develop the above stereotypic behaviours to different degrees, allowing the separation of animals into high and low stereotypic groups within the population. This would indicate individual susceptibility within groups that promotes the development of heightened stereotypic behaviour in some individuals but less so in others. While it would be over-speculation to compare this directly to human OCD there is some correlation in that first-degree relatives of OCD adult probands have been noted to more likely suffer from OCD or other anxiety disorder (Black et al., 1995).

A larger percentage of OCD patients have an early age of onset with symptoms usually developing before 18 years of age (Morer et al., 2006). Deer mice develop characteristic stereotypic behaviour at approximately 20 days of age (Powell et al., 1999), more or less when these mice are weaned. Thus, spontaneous stereotypic behaviour in deer mice has an early age of onset, similar to individuals who develop OCD early in life and indulge in stereotypic activities reminiscent of the range of compulsive behaviours seen in patients

with OCD. Furthermore, stereotyping deer mice are easier to agitate than non-stereotypers when handled or disturbed which adds to the suggestion that the performance of stereotypies could be related to human anxiety disorders (Schoenecker & Heller, 2003). Since deer mice develop stereotypic behaviours to different degrees, it allows a separation between stereotypic and non-stereotypic animals within the population. To broaden face validity of stereotyping deer mice, this distinctive behaviour in deer mice was compared to a species that do not develop spontaneous stereotypic behaviour under standard laboratory conditions, namely C57Bl mice.

It is important to note that according to the methods discussed in section 3.3, C57Bl mice are strictly classified as non-stereotypic mice (Cph < 1000) and did not engage in topographies such as patterned running and backward somersaulting. Topographies most frequently displayed by C57Bl mice include walking about in the cage, but without a clear pattern. C57Bl did not engage in stereotypic behaviour but did perform various locomotor behaviours such vertical activity, i.e. to jump from the cage floor to the cage cage in an exploratory fashion. These mice also did not engage in any backward somersaulting even thought they share a similar physical development with deer mice (Crawley et al., 1997).

Of the 58 deer mice screened, 25 mice were consequently classified as high stereotypic (43%), 24 as low stereotypic (41%), and 9 as non-stereotypic mice (16%). Of the 20 C57Bl mice monitored for the same behaviours, all presented with scores lower than 1000 cph, and were accordingly classified as non-stereotypic animals. The present study also supports previous findings (Powell et al., 1999) that gender does not affect stereotypic behaviour in the deer mice.

NS mice engaged in all topographies, albeit to a lesser extent than stereotypic animals (LSB and HSB mice). Deer mice thus present with a distinct array of stereotypic behaviours that was found to show diverse variation. These phenomena lend significant face validity to the model in that patients suffering from OCD experience different degrees of symptom severity and perform compulsions at different rates (American Psychiatric Association 2000). Face validity is however subjective, and therefore can only be considered as a secondary criterion. This, together with our behavioural studies, would

suggest that stereotypy in deer mice presents with a reliable behavioural parameter for pharmacological validation studies.

4.3.2 Pharmacological challenge with fluoxetine and desipramine

In the present animal study, low dose fluoxetine (10 mg/kg), but not desipramine, significantly reduced stereotypic behaviour compared to vehicle-treated animals in both LSB and HSB mice, confirming a selective response to fluoxetine treatment. Similarly, treatment of mice with high dose 20 mg/kg fluoxetine, but not 20mg/kg desipramine, resulted in a significant decrease in stereotypic behaviour. Response to the high dose (20 mg/kg) fluoxetine was not shown to be more effective in reducing stereotypic behaviour than low dose fluoxetine. In addition, since the higher dose of desipramine failed to decrease stereotypic behaviour, these studies further confirm the serotonin-dependence of this response to drug treatment, and the close analogy of deer mice-associated stereotypy with that of OCD.

Interestingly, the standard deviation of these data indicate that scatter of cph-data is less pronounced in animals receiving a high dose compared to low dose fluoxetine, suggesting that a higher dose may be more effective in reducing the stereotypic behaviour in these animals. Indeed, as has been the case in treating OCD, higher doses for a longer treatment period may have realised a better outcome (Baxter, 1999). Data thus suggests that the deer mouse model demonstrates noteworthy pharmacological isomorphism with OCD.

4.3.3 Serotonergic function in stereotypic behaviour

A second, but related objective of the present study was to investigate the regulatory contribution of 5-HT and dopamine pathways and their receptors to spontaneous stereotypic behaviour in the deer mice model. Treatment of deer mice with mCPP, a predominantly $5HT_{2A/C}$ receptor agonist (Barnes & Sharp, 1999), resulted in a significant decrease in stereotypic behaviour in both LSB and HSB mice. This response seems somewhat unexpected and the precise mechanism whereby this decrease in stereotypic behaviour in deer mice is evoked remains speculative since mCPP is a relatively broad-spectrum serotonergic receptor agonist, which complicates any prediction of how this drug will act (Barnes & Sharp 1999). Chronic pre-treatment of deer mice with 20 mg/kg

fluoxetine prior to mCPP challenge significantly reversed stereotypic behaviour in LSB mice as well as HSB mice, although the latter narrowly missed statistical significance. These data provide convincing evidence for the involvement of 5-HT (hyposerotonergia) in deer mice-associated stereotypy and that it can be attenuated by OCD-specific medication. This not only provides noteworthy construct validity to the model, but also supports predictive validity.

4.3.4 Dopaminergic function in stereotypic behaviour

QNP treatment of deer mice resulted in a significant decrease in stereotypic behaviour in both LSB and HSB mice. On the other hand, chronic pre-treatment of deer mice with 20 mg/kg fluoxetine prior to a subacute QNP challenge resulted in significant reversal (increase) in stereotypic behaviour in LSB mice. The same pattern was seen in HSB mice, although it did not reach statistical significance. Dopamine therefore plays an important role in deer mice-associated stereotypy, while its reversal by fluoxetine is supportive of clinical studies of dopamine involvement in OCD, thus again emphasizing construct and predictive validity. The behavioural and phenomenological data presented in this chapter will be discussed in more detail in chapter 7, but suffice to say that the deer mice model of spontaneous stereotypy presents with prominent face, construct, and predictive validity that will allow further investigations into the neurobiology and neurochemistry of OCD.

5. Results – Neurochemistry

5.1 Introduction and experimental design

Several neural systems have been implicated in the pathophysiology of OCD. Dysregulation of the serotonergic system has been suggested primarily on the basis of the effectiveness of SRIs in alleviating obsessions and compulsions in patients (section 2.4.3). Abnormalities of the dopaminergic system have also been implicated in the pathophysiology of OCD, based on the therapeutic benefits obtained with co-administration of SRIs and dopamine receptor blockers (section 2.6.1). Furthermore, the results of neuroimaging studies in OCD patients have consistently implicated the orbitofrontal cortex, the cingulate cortex and the basal ganglia, particularly the striatum, in the pathophysiology of obsessions and compulsions. These regions are interconnected and are densely innervated by dopaminergic and serotonergic terminals (section 2.4.1). The nature of the dysfunction of these regions and the relation between their malfunction and neurotransmitter disturbance postulated to be involved in OCD, is still unknown. Down-stream neural messengers known to be regulated by antidepressants, namely cAMP (Ozawa & Rasenick, 1991) and activity of PDE4 (Takahashi et al., 1999), were examined. More specifically, the role of cAMP and PDE4 in the prefrontal cortex and striatum were explored since these areas are thought to be involved in OCD pathophysiology (Baxter, 1999; Dubois et al., 1994; Kelly, 1999).

This study again followed the rationale described in chapter 4 in using fluoxetine and desipramine for 21 days to reverse the effects of subacute dopaminergic and serotonergic drugs, QNP and mCPP which were administered for 4 days immediately prior to sacrifice. Briefly, mice were treated chronically (21 days) with SRI, NRI or vehicle and immediately for the next 4 days challenged with said dopaminergic or serotonergic drugs. In the subacute mCPP and QNP-challenges preceded by chronic fluoxetine and desipramine administration, the latter two drugs were administered at high dose (20mg/kg). Following treatment, deer mice were sacrificed and the striatum and prefrontal cortex were rapidly dissected and frozen at -80°C for further analysis. The study was initiated with the determination of basal (untreated) cAMP levels and PDE4 activity. Stereotypic and non-stereotypic deer mice were also compared to a species that does not develop stereotypic behaviour under similar laboratory conditions, namely C57Bl mice. In subsequent experiments only stereotypic deer mice were used.

5.2 Basal cAMP levels and PDE4 activity in untreated mice

5.2.1 cAMP levels and PDE4 activity in prefrontal the cortex of untreated mice

In order to establish a point of reference for supplementary studies, cAMP levels and PDE4 activity of untreated stereotypic (LSB and HSB) as well as non-stereotypic (NS) deer mice were compared to that of C57Bl mice. It should be noted that mice were sacrificed 7 days after the last behavioural assessment session when cAMP and PDE4 activity were measured. This allowed animals to recover from the stress of handling and negates any influence these test may have on basal parameters. Basal (untreated) cAMP levels in the prefrontal cortex of LSB and HSB mice are significantly higher than their non-stereotypic deer mice and C57Bl counterparts (figure 5.1A). PDE4 activity in the prefrontal cortex of both LSB and HSB mice are significantly lower than non-stereotypic deer mice under basal conditions (figure 5.1B). However, untreated deer mice still presented with significantly higher PDE4 activity than C57Bl mice.

5.2.2 cAMP levels and PDE4 activity in the striatum of untreated mice

Striatal cAMP levels and PDE4 activity under basal condition were determined. Deer mice present with significantly higher basal cAMP levels than non-stereotypic C57Bl mice (figure 5.2A). Furthermore, striatal PDE4 activity is significantly higher in deer mice than C57Bl mice (figure 5.2B). Of interest was the stepwise diminution in PDE4 activity in NS, LSB, and HSB mice, although these were not significant.

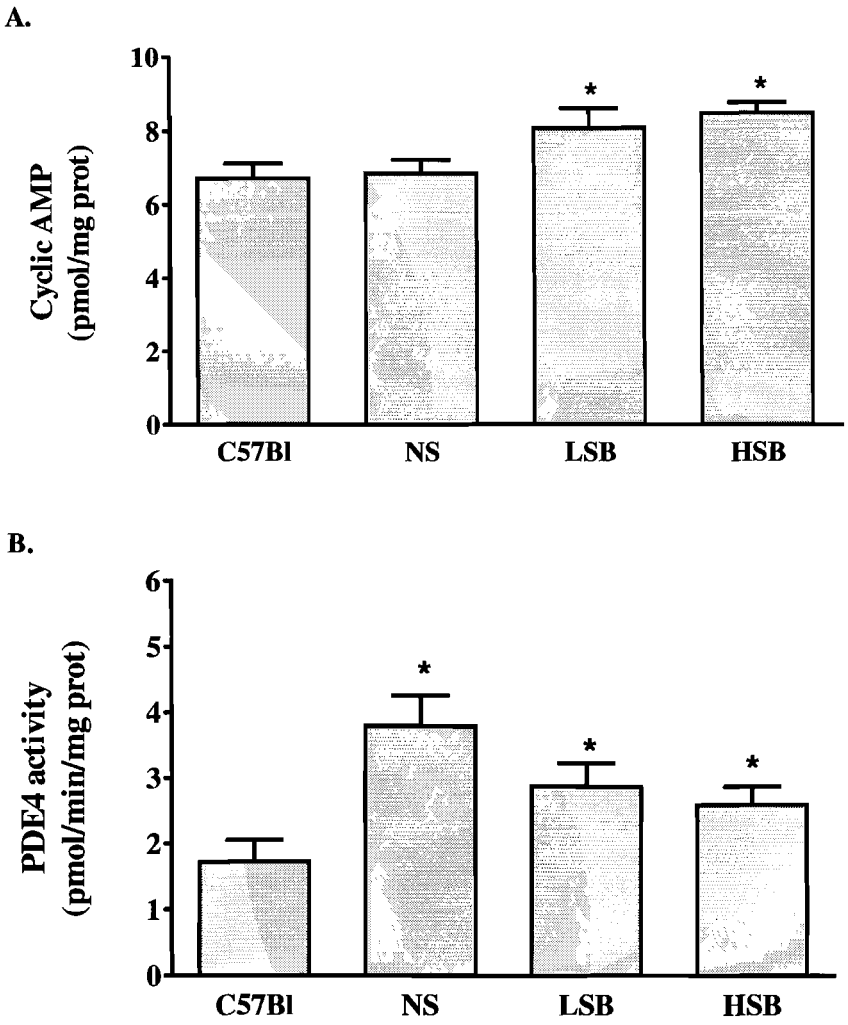


Figure 5.1 cAMP levels and PDE4 activity in the prefrontal cortex of untreated mice. Three groups were distinguished among deer mice namely high stereotypic (HSB, n = 19), low stereotypic (LSB, n = 18) and non-stereotypic (NS, n = 12) animals, and compared to C57Bl mice (n = 20). Subsequent to behavioural assessment mice were sacrificed and cAMP levels (A) and PDE4 enzyme activity (B) determined. Data is expressed as averages ± SEM and * indicates $p < 0.05$ for HBS, LSB or NS deer mice v C57Bl mice. Data was analysed by one-way ANOVA followed by Dunnett's test.

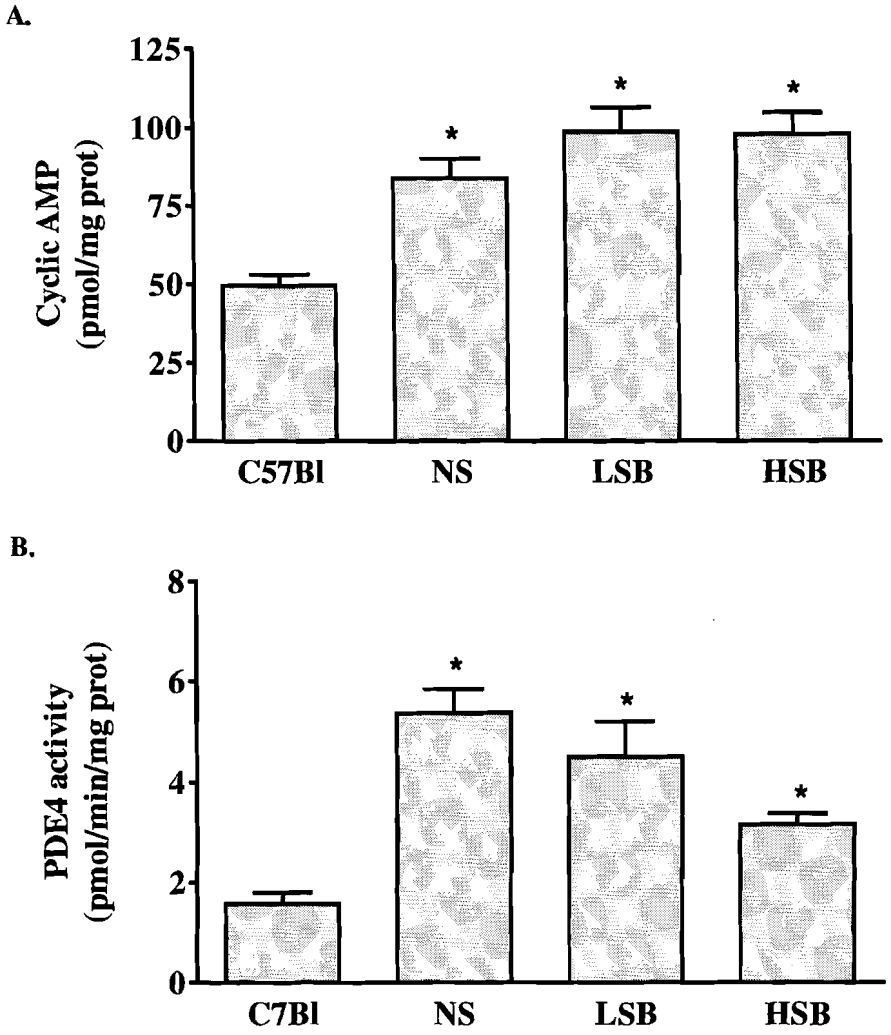


Figure 5.2 cAMP levels and PDE4 activity in the striatum of untreated mice.
Three groups of deer mice were distinguished namely high stereotypic (HSB, n = 19), low stereotypic (LSB, n = 18) and non-stereotypic (NS, n = 12) animals and compared to C57Bl mice (n = 20). Subsequent to behavioural assessment mice were sacrificed and cAMP levels (A) and PDE4 enzyme activity (B) determined. Data is expressed as averages ± SEM and * indicates $p < 0.05$ for HBS, LSB, or NS deer mice v C57Bl mice. Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.3 Effect of chronic drug treatment on cAMP levels and PDE4 activity in prefrontal cortex of deer mice

5.3.1 Effect of 10 mg/kg SRI/NRI in LSB mice

Treatment of LSB mice with 10 mg/kg desipramine resulted in a significant decrease in cAMP levels compared to control values (saline)(figure 5.3A). Fluoxetine treatment, however, did not alter cAMP levels. PDE4 enzyme activity did not change following desipramine or fluoxetine treatment of LSB mice (figure 5.3B).

5.3.2 Effect of 10 mg/kg SRI/NRI in HSB mice

Both fluoxetine and desipramine treatment resulted in significant decreases in cAMP levels in the prefrontal cortex of HSB mice (figure 5.4A). PDE4 activity however, was left unaltered by either desipramine or fluoxetine treatment (figure 5.4B).

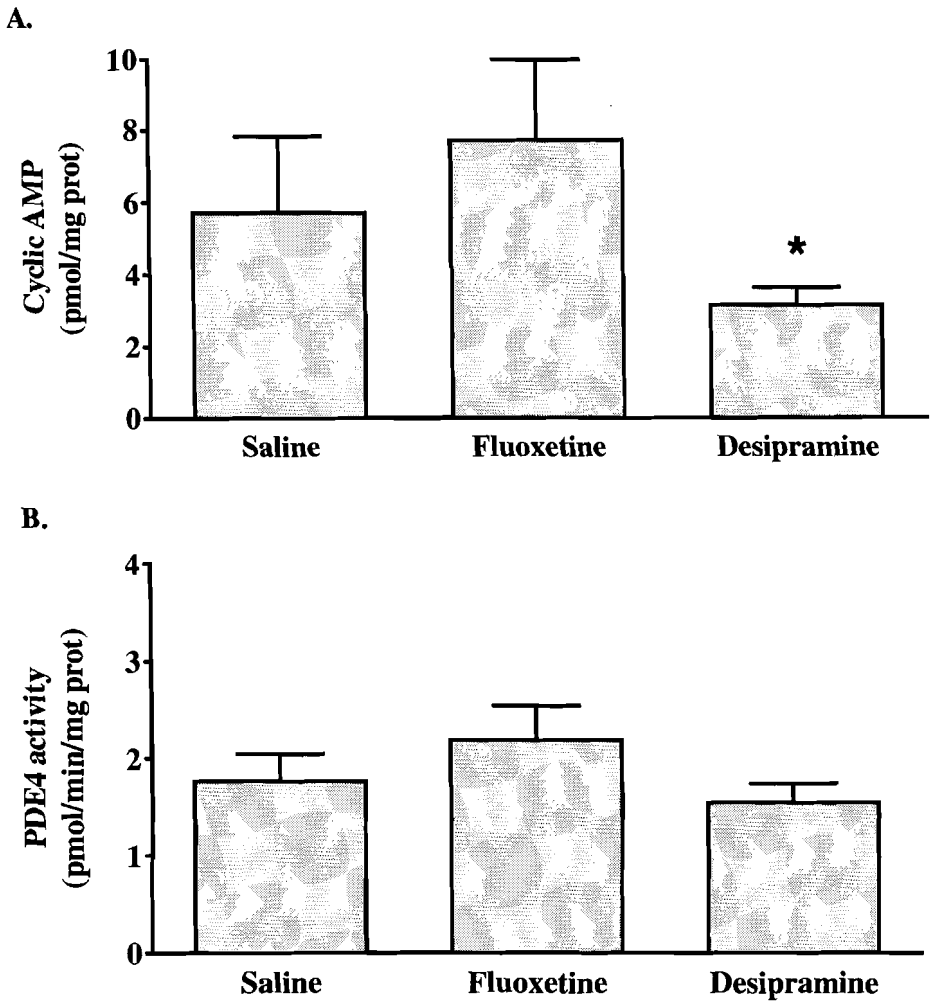


Figure 5.3 The effect of 10 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 9), desipramine (n = 8) or saline (n = 8). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and * indicates $p < 0.05$ for desipramine v saline. Data was analyzed one-way ANOVA followed by Dunnett's test was performed.

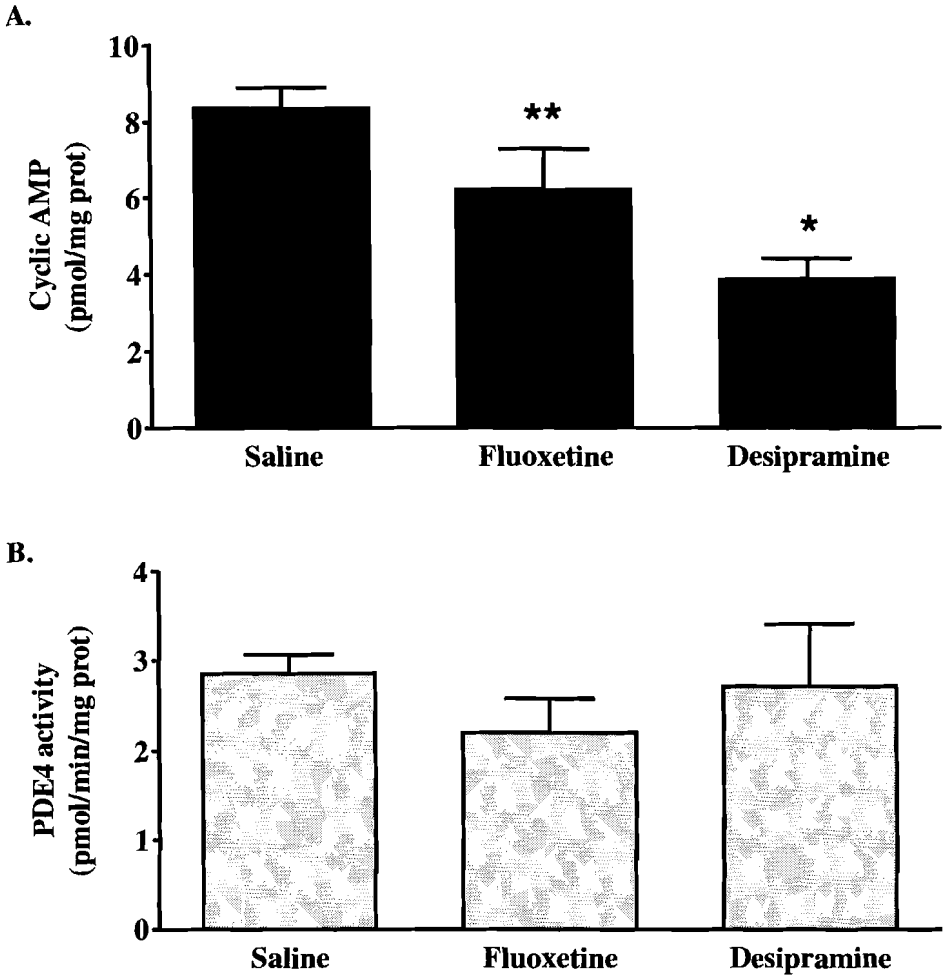


Figure 5.4 The effect of 10 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the prefrontal cortex of HSB mice. Mice were treated for 21 days fluoxetine (n = 10), desipramine (n = 10) or saline (n = 10). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v saline and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed one-way ANOVA followed by Dunnett's test.

5.3.3 Effect of 20 mg/kg SRI/NRI in LSB mice

cAMP levels were significantly reduced in the prefrontal cortex of LSB mice by treatment with either fluoxetine or desipramine (figure 5.5A). PDE4 activity however, was not significantly affected by similar treatment (figure 5.5B).

5.3.4 Effect of 20 mg/kg SRI/NRI in HSB mice

cAMP levels were significantly reduced in the prefrontal cortex of HSB mice following drug treatment with either fluoxetine or desipramine (figure 5.6A). Moreover, both fluoxetine and desipramine significantly reduced PDE4 activity in the prefrontal cortex of HSB mice compared to control values (figure 5.6B).

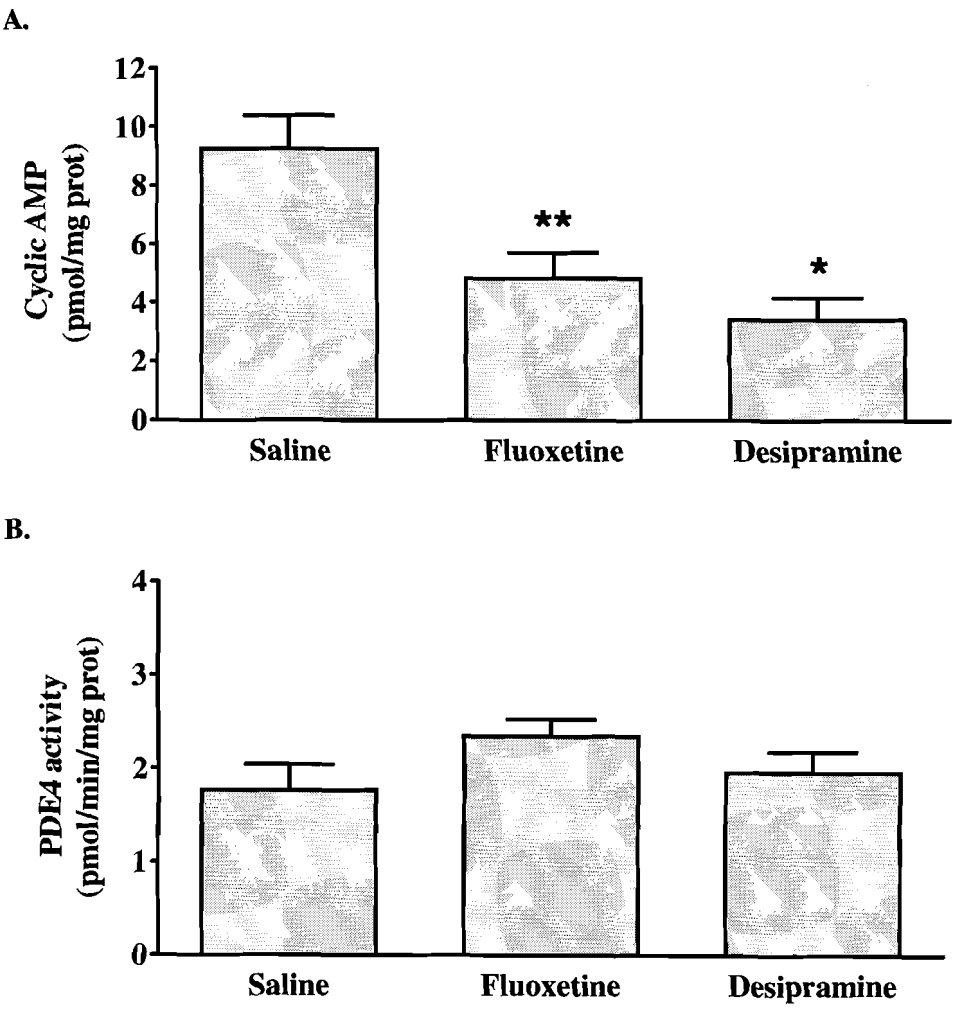


Figure 5.5 The effect of 20 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 8), desipramine (n = 9) or saline (n = 9). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v saline and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed one-way ANOVA followed by Dunnett's test was performed.

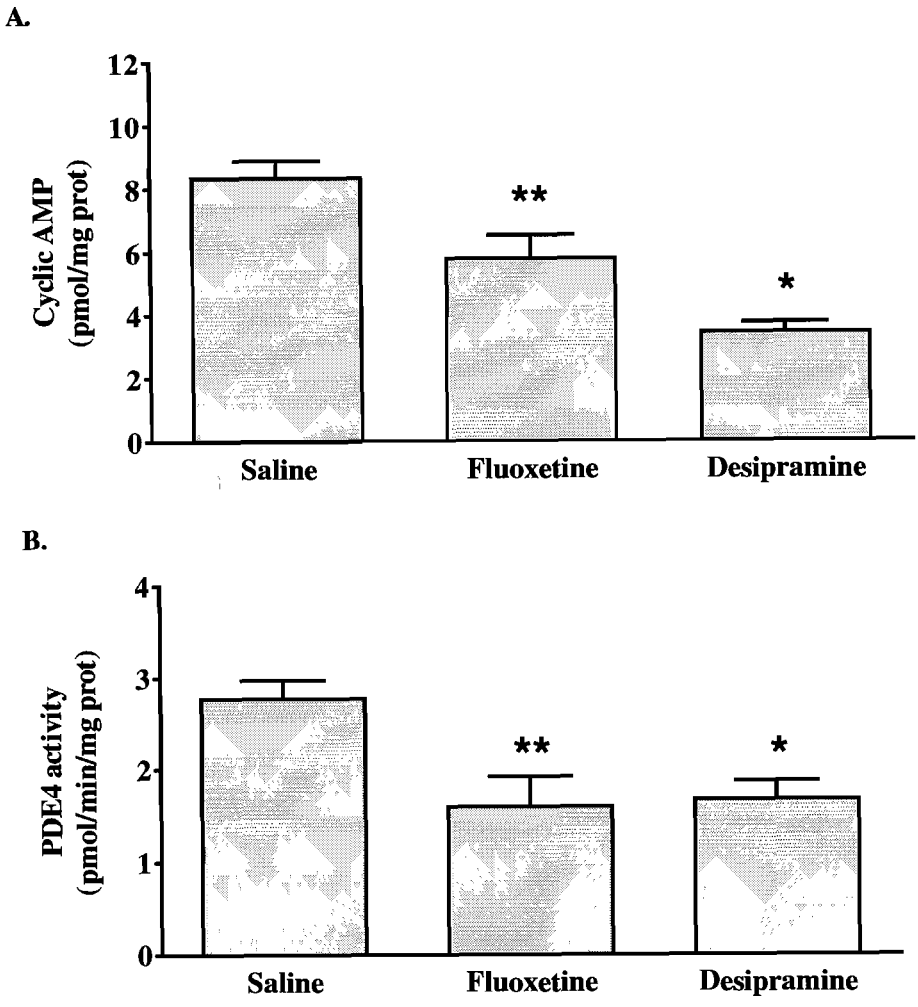


Figure 5.6 The effect of 20 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the prefrontal cortex of HSB mice. Mice were treated for 21 days with fluoxetine (n = 11), desipramine (n = 9) or saline (n = 10). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v vehicle and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed one-way ANOVA followed by Dunnett's test.

5.4 Effect of chronic drug treatment on cAMP levels and PDE4 activty in striatum of deer mice

5.4.1 Effect of 10 mg/kg SRI/NRI in LSB mice

Fluoxetine treatment did not change cAMP in the striatum of LSB mice, however, cAMP levels were significantly decreased by desipramine (figure 5.7A). Furthermore, PDE4 activity was also increased by both fluoxetine and desipramine treatment of LSB mice (figure 5.7B).

5.4.2 Effect of 10 mg/kg SRI/NRI in HSB mice

Treatment of HSB mice with desipramine resulted in a significant decrease in striatal cAMP levels (figure 5.8A), whereas treatment with fluoxetine failed to alter cAMP levels. PDE4 activity on the other hand was significanty decreased by treatment with both fluoxetine and desipramine (figure 5.8B).

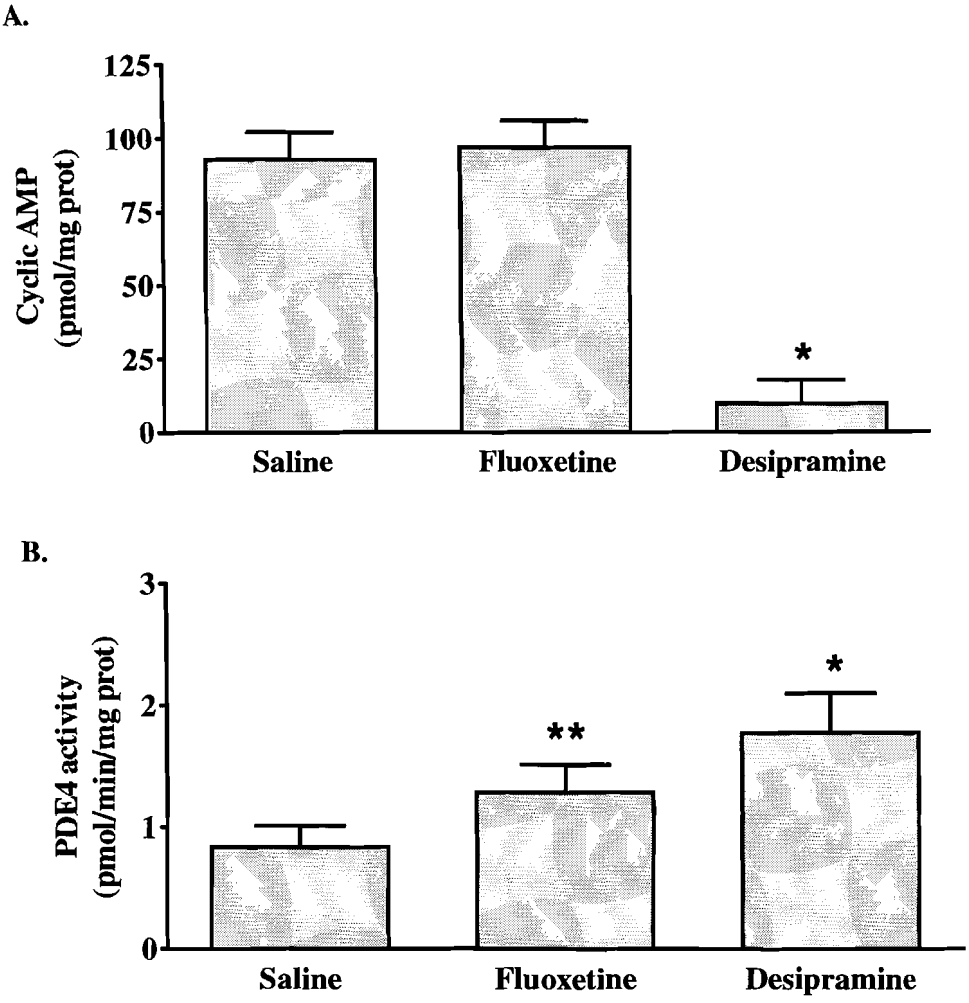


Figure 5.7 The effect of 10 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the striatum of LSB mice. Mice were treated for 21 days with fluoxetine (n = 9), desipramine (n = 10) or saline (n = 8). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v saline and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

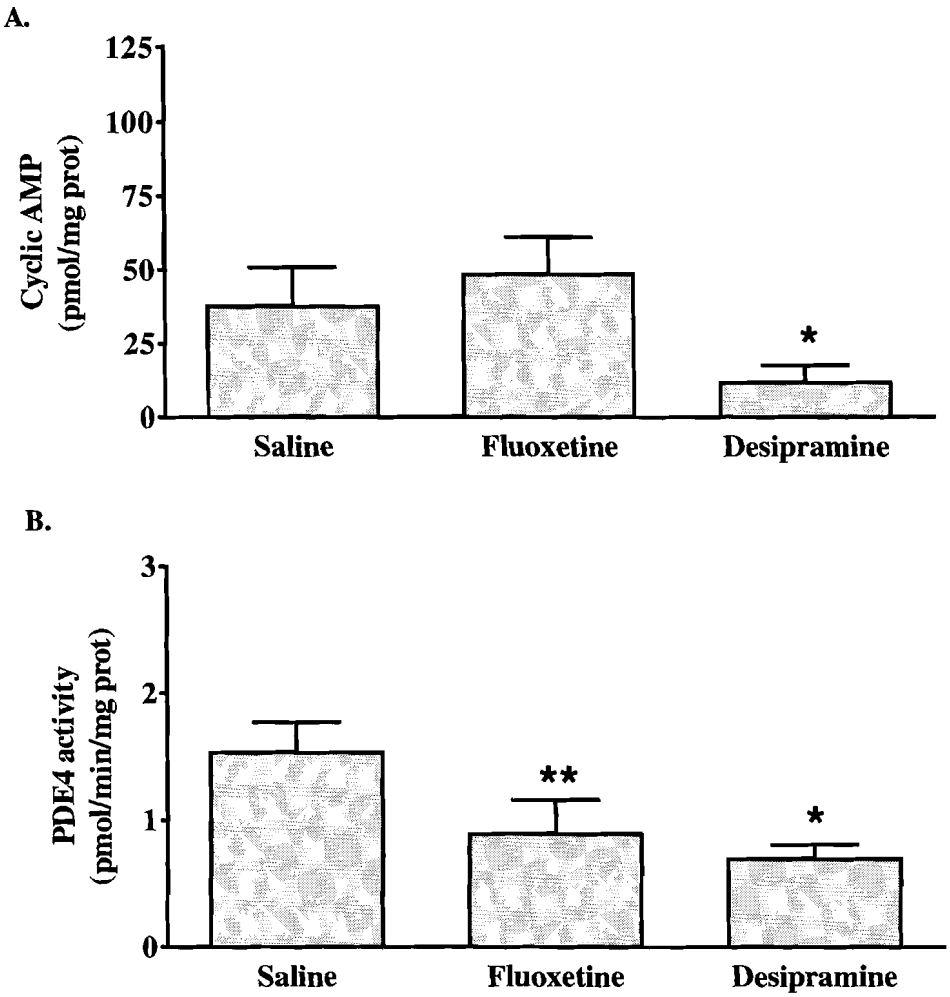


Figure 5.8 The effect of 10 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the striatum of HSB mice. Mice were treated for 21 days with fluoxetine (n = 11), desipramine (n = 11) or saline (n = 13). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v saline and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.4.3 Effect of 20 mg/kg SRI/NRI in LSB mice

Fluoxetine (20 mg/kg) significantly increased cAMP levels in the striatum of LSB mice compared to control values (figure 5.9A). Desipramine however, failed to alter cAMP levels. Neither drug treatment affected striatal PDE4 activity (figure 5.9B).

5.4.4 Effect of 20 mg/kg SRI/NRI in HSB mice

Treatment of HSB mice with desipramine resulted in a significant decrease in striatal cAMP levels (figure 5.10A), with fluoxetine not altering cAMP levels in a significant manner. Concerning PDE4 activity, the opposite applied, with desipramine treatment not altering striatal PDE4 activity significantly, and fluoxetine resulting in a significant decrease (figure 5.10B).

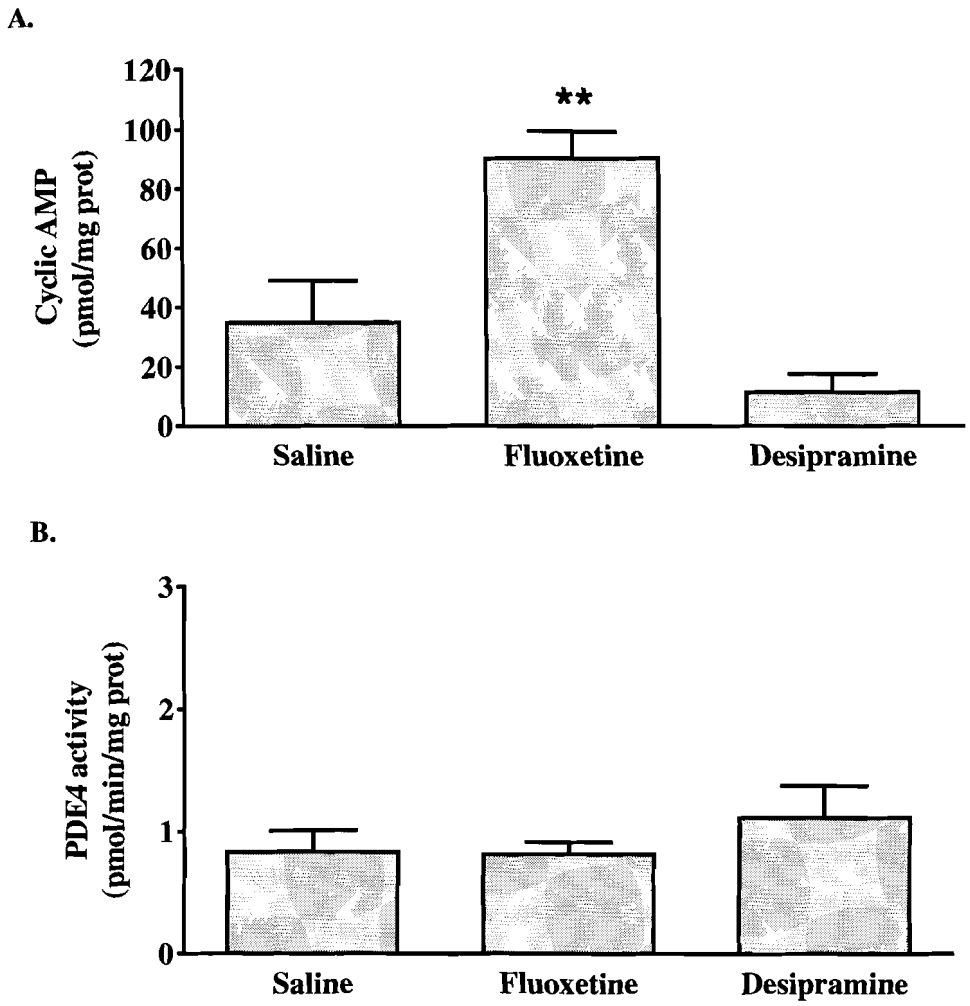
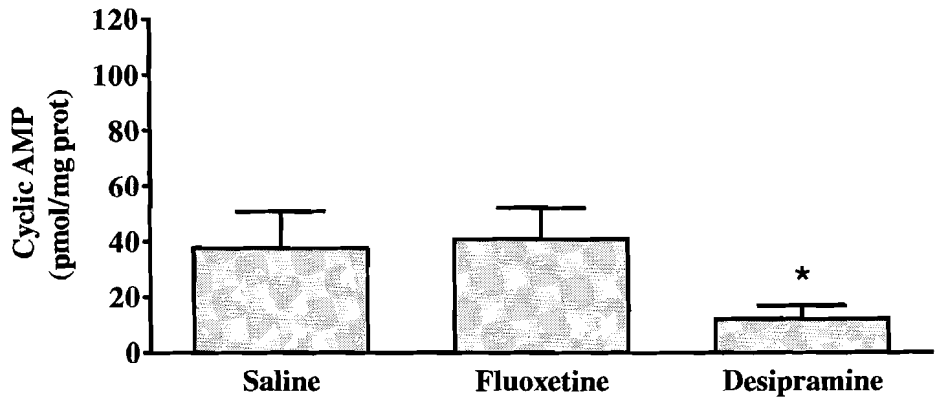


Figure 5.9 The effect of 20 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the striatum of LSB mice. Mice were treated for 21 days with fluoxetine (n = 9), desipramine (n = 8) or saline (n = 8). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and ** indicates $p < 0.05$ for fluoxetine v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

A.



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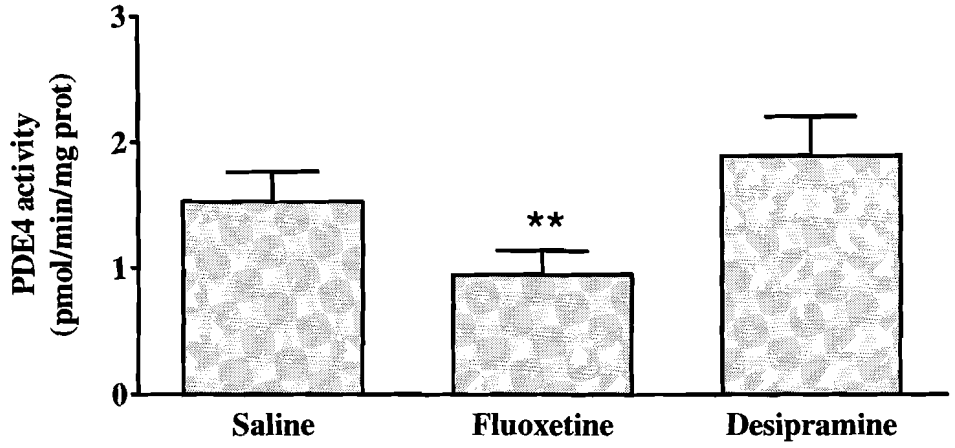

Figure 5.10 The effect of 20 mg/kg SRI/NRI on cAMP levels and PDE4 activity in the striatum of HSB mice. Mice were treated for 21 days with fluoxetine (n = 12), desipramine (n = 11) or saline (n = 14). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for desipramine v saline and ** indicates $p < 0.05$ for fluoxetine v saline. Dara was analyzed by one-way ANOVA followed by Dunnett's test.

5.5 Effect of subacute drug challenge on cAMP levels and PDE4 activity in prefrontal cortex of deer mice

This section of the results accounts for the affect of serotonergic and dopaminergic drugs on neurochemical parameters in stereotypic deer mice. Here, mCPP (a non-selective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist), QNP (a D_2 selective agonist) as well as SKF (a D_1 selective agonist) were the drugs of choice. mCPP were chosen because of the central role of 5-HT in OCD and deer mice, while both D_1 and D_2 agonist were included because both receptors form part of the CSTC circuit (section 2.4.2) and have been implicated in stereotypy in deer mice and in the pathophysiology of OCD (section 2.9.2.3).

5.5.1 Effect of subacute challenge in LSB mice

QNP significantly increased cAMP levels in the prefrontal cortex of LSB mice compared to control values (figure 5.11A). Neither mCPP nor SKF had any significant effect on prefrontal cortical cAMP levels. PDE4 activity in the prefrontal cortex of LSB mice was significantly increased by mCPP and QNP, whereas SKF did not have any significant effect on PDE4 enzyme activity (figure 5.11B).

5.5.2 Effect of subacute challenge in HSB mice

Treatment of HSB mice with mCPP and SKF resulted in a significant decrease in cAMP levels in the prefrontal cortex of HSB mice (figure 5.12A). QNP did not alter cAMP levels. mCPP and SKF reduced prefrontal cortical PDE4 activity in HSB mice (figure 5.12B). While QNP failed to have any significant effect on PDE4 activity.

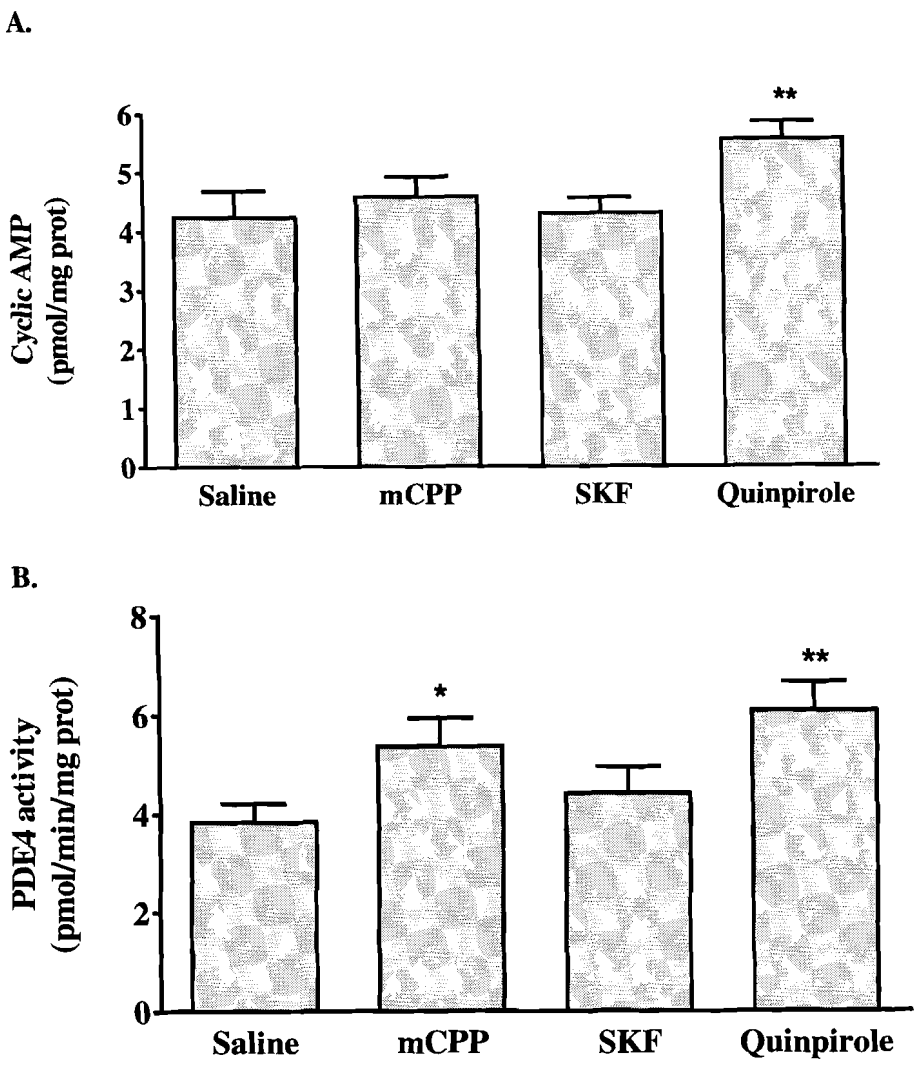

Figure 5.11 Effect of subacute challenge on cAMP levels and PDE4 activity in prefrontal cortex of LSB mice. Deer mice received subacute treatment with either mCPP (n = 8), SKF (n = 8) or QNP (n = 9) or saline (n = 7). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for mCPP v saline and ** indicates $p < 0.05$ for quinpirole v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

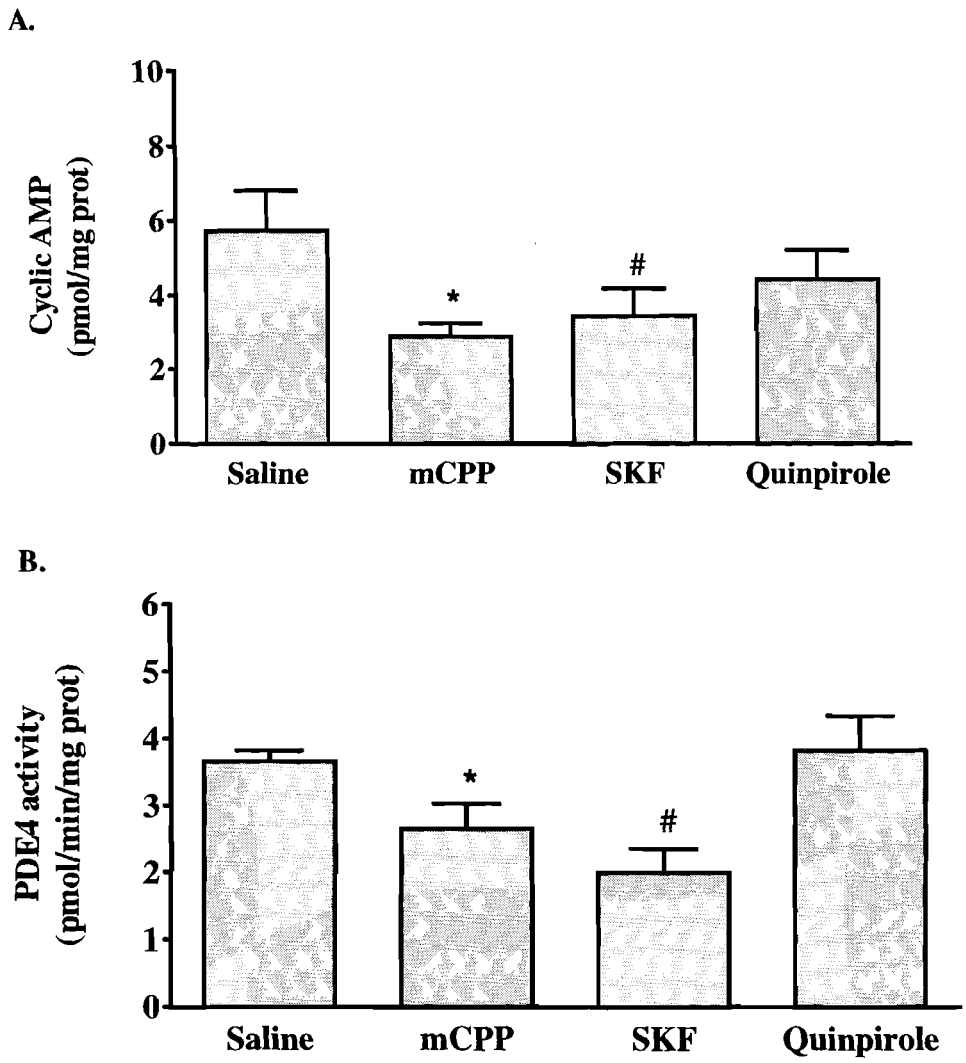

Figure 5.12 Effect of subacute challenge on cAMP levels and PDE4 activity in prefrontal cortex of HSB mice. Deer mice received subacute treatment with either mCPP (n = 10), SKF (n = 10) or QNP (n = 10) or saline (n = 8). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for mCPP v saline and # indicates $p < 0.05$ for SKF v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.6 Effect of subacute drug challenge on cAMP levels and PDE4 activity in striatum of deer mice

5.6.1 Effect of subacute challenge in LSB mice

Subacute treatment with mCPP, SKF and QNP resulted in a significant increase in striatal cAMP levels in LSB mice compared to controls (figure 5.13A). In contrast, only QNP reduced striatal PDE4 activity significantly in LSB mice (figure 5.13B).

5.6.2 Effect of subacute challenge in HSB mice

QNP significantly increased striatal cAMP levels in HSB mice compared to control values (figure 5.14A), while mCPP and SKF failed to alter cAMP levels. In addition, subacute challenge with QNP resulted in a significant increase in striatal PDE4 activity in HSB mice (figure 5.14B), while neither mCPP nor SKF significantly modified PDE4 activity.

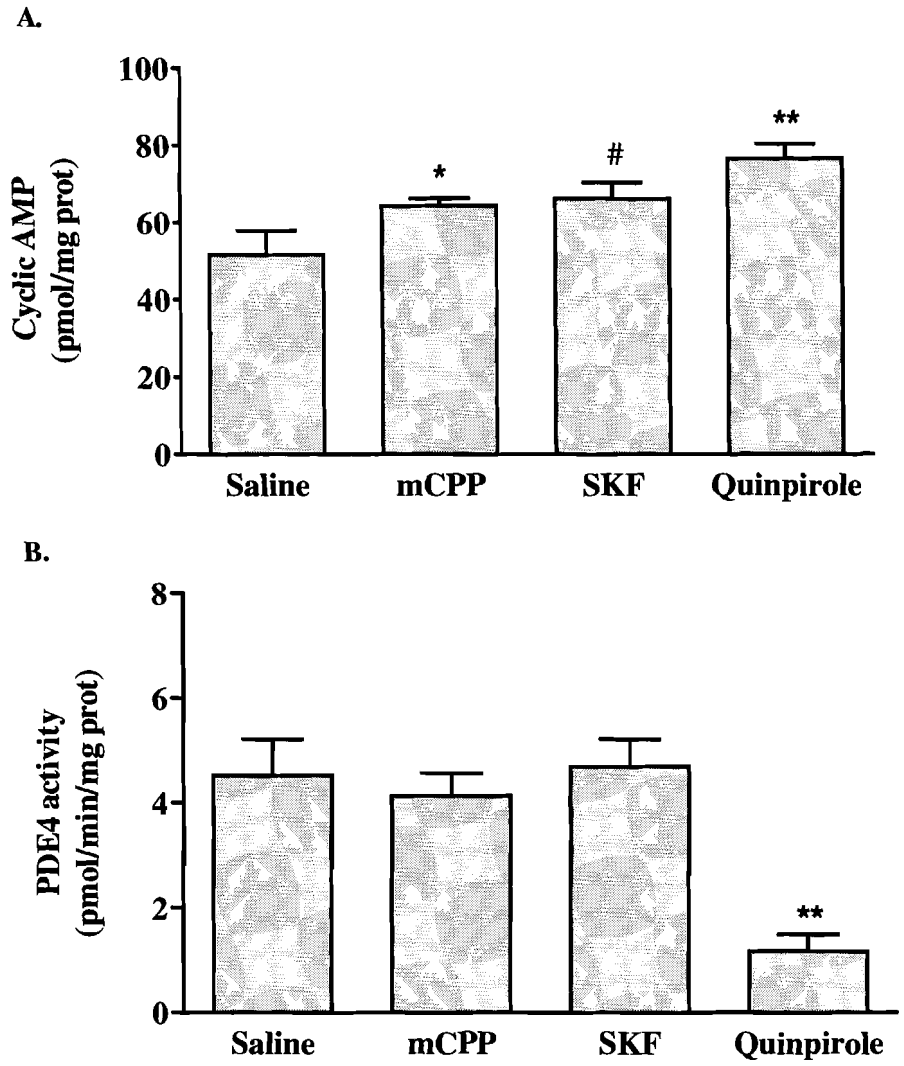

Figure 5.13 Effect of subacute challenge on cAMP levels and PDE4 activity in striatum of LSB mice. Deer mice received subacute treatment with either mCPP (n = 9), SKF (n = 8) or QNP (n = 9) or saline (n = 7). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM. * indicates $p < 0.05$ for mCPP v saline, # indicates $p < 0.05$ for SKF v saline and ** indicates $p < 0.05$ for quinpirole v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

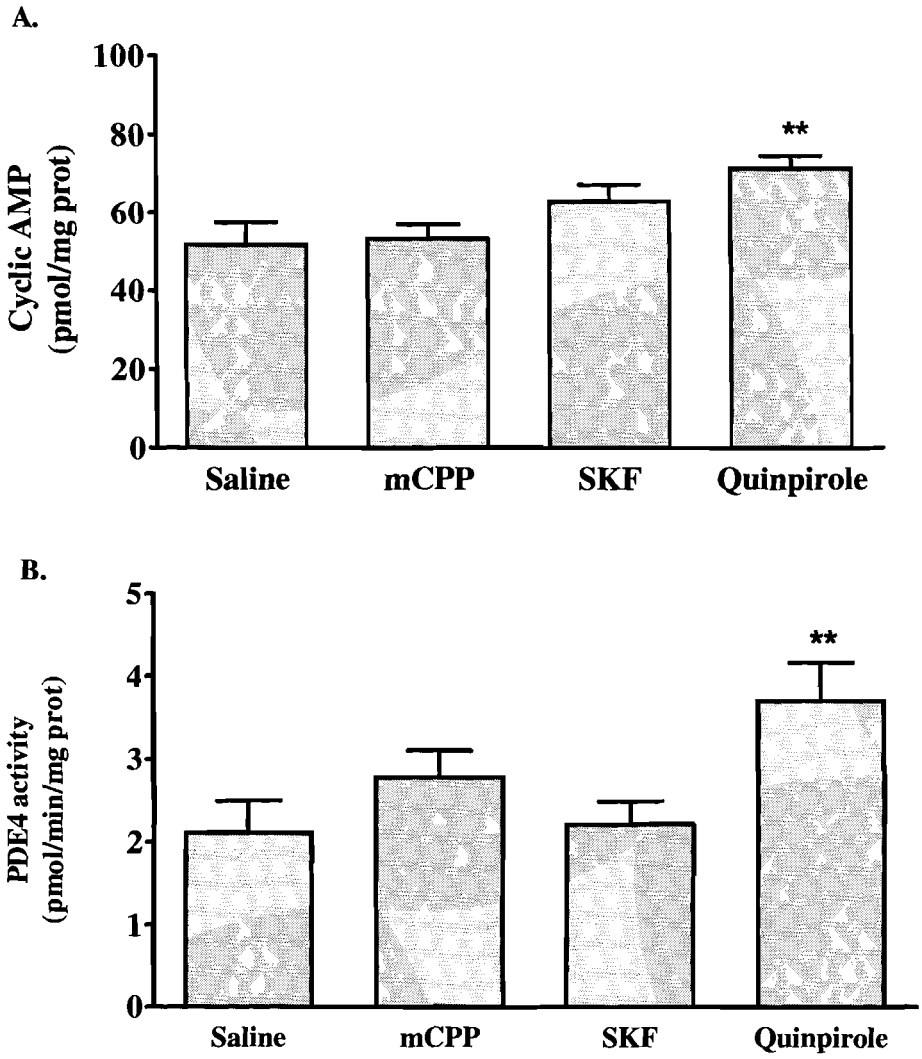

Figure 5.14 Effect of subacute challenge on cAMP levels and PDE4 activity in striatum of HSB mice. Deer mice received subacute treatment with either mCPP (n = 10), SKF (n = 10) or QNP (n = 10) or saline (n = 8). Following sacrifice and tissue collection, samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and ** indicates $p < 0.05$ for quinpirole v saline. Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.7 Effect of subacute drug challenge following chronic SRI/NRI pre-treatment on cAMP levels and PDE4 activity in prefrontal cortex of deer mice

This study followed the rationale described in chapter 4 in using fluoxetine and desipramine for 21 days to inhibit the effects of subacute dopaminergic and serotonergic drugs, mCPP and QNP. SKF was not included since results from behaviour data indicate a more prominent role for dopamine D_2 receptors, and not D_1 in deer mice. Briefly, mice were treated chronically (21 days) with SRI, NRI, or vehicle and immediately for the next 4 days challenged with one of the alone dopaminergic or serotonergic drugs. 24 hours after the last drug treatment, mice were sacrificed and striatal and prefrontal cortical cAMP and PDE4 enzyme activity determined.

5.7.1 Effect of mCPP challenge in LSB mice

LSB mice receiving subacute mCPP and those receiving chronic saline plus subacute mCPP both demonstrated significantly reduced prefrontal cortical cAMP compared to baseline (figure 5.15A). Interestingly, chronic fluoxetine but not desipramine pre-treatment prior to mCPP challenge significantly blocked the decrease in cAMP evoked by mCPP in LSB mice (figure 5.15A). Treatment of LSB mice with mCPP significantly increased prefrontal cortical PDE4 activity (figure 5.15B). Furthermore, chronic pre-treatment with fluoxetine resulted in a significant decrease in PDE4 activity versus mCPP plus saline, i.e. PDE4 activity was reduced compared to saline control values, with desipramine not having a marked effect in this regard (figure 5.15B).

5.7.2 Effect of mCPP challenge in HSB mice

HSB mice receiving subacute mCPP treatment as well as those receiving chronic saline plus subacute mCPP both demonstrated significantly attenuated prefrontal cortical cAMP levels versus baseline (figure 5.16A). In contrast to LSB mice, desipramine significantly increased cAMP levels in the prefrontal cortex of HSB mice compared to saline (figure 5.16A). Fluoxetine pre-treatment on the other hand, did not attenuate cAMP levels significantly in the prefrontal cortex compared to mCPP plus saline. Interestingly, neither mCPP challenge nor fluoxetine or desipramine pre-treatment prior to mCPP challenge had any significant effect on PDE4 in the prefrontal cortex of HSB mice (figure 5.16B).

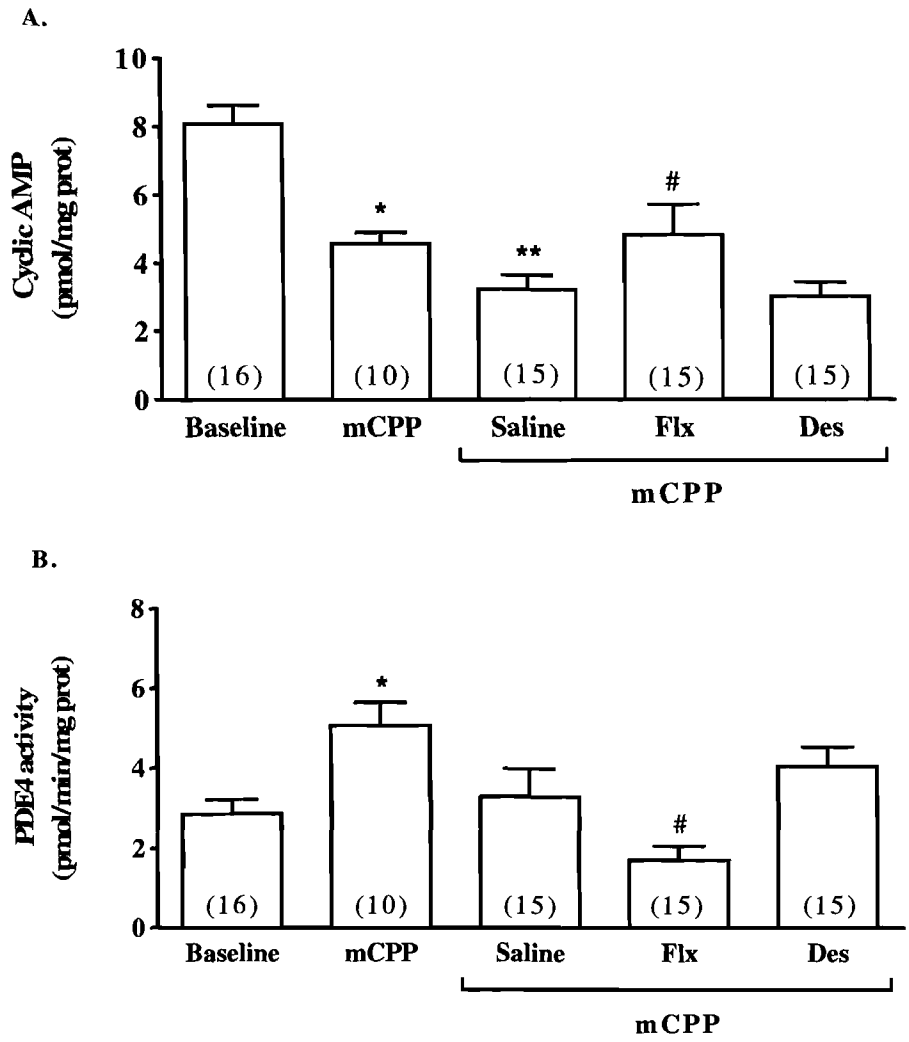

Figure 5.15 Effect of mCPP challenge on cAMP levels and PDE4 activity in the prefrontal cortex of LSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute mCPP (filled bars). For comparison, LSB mice that were untreated (baseline) or received mCPP challenge (empty bars) are shown. Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicates $p < 0.05$ (mCPP v baseline), and # indicates $p < 0.05$ (saline v fluoxetine) and ** indicates $p < 0.05$ (chronic saline plus mCPP challenge v baseline Data was analyzed by one-way ANOVA followed by Dunnett's test.

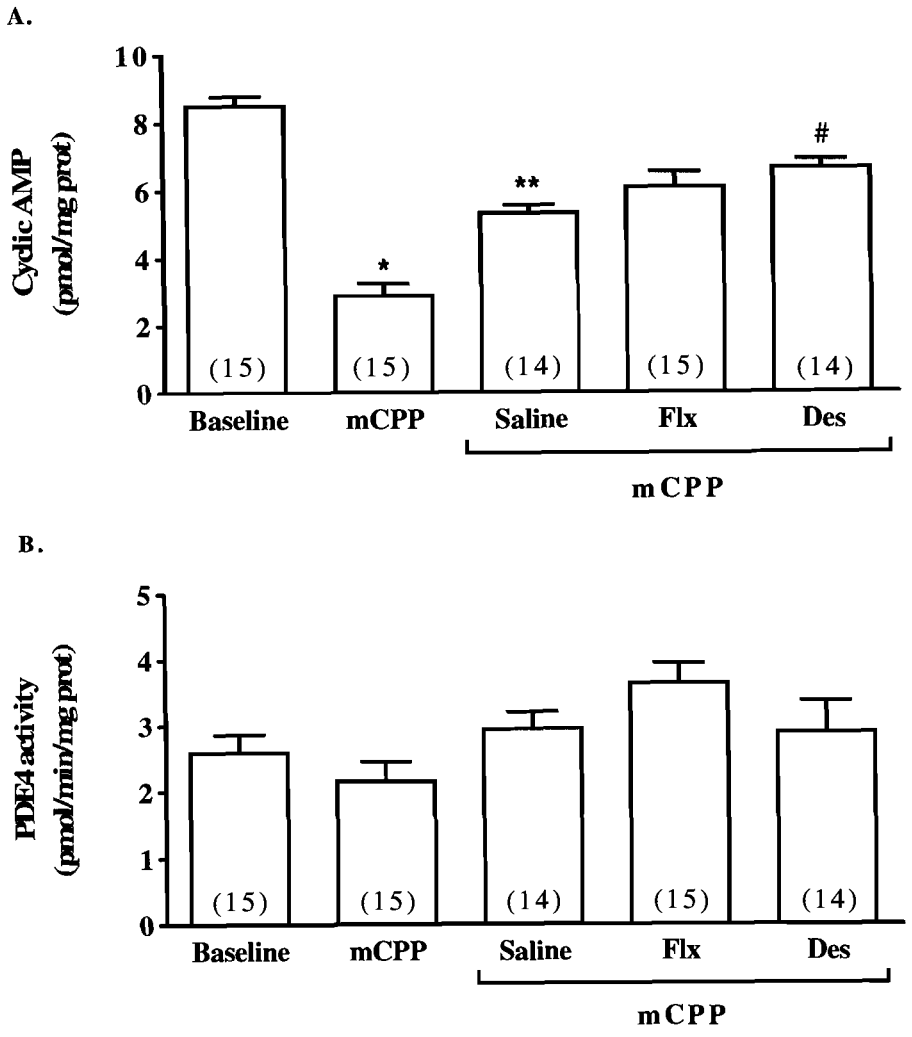

Figure 5.16 Effect of mCPP challenge on cAMP levels and PDE4 activity in the prefrontal cortex of HSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment which was followed by subacute treatment (filled bars). For comparison, HSB mice that were untreated (baseline) or mCPP challenge (empty bars) are shown. Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicates $p < 0.05$ (mCPP v baseline), ** indicates $p < 0.05$ (chronic saline plus mCPP challenge v baseline) and # indicates $p < 0.05$ (saline v desipramine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.7.3 Effect of quinpirole challenge in LSB mice

LSB mice receiving subacute QNP treatment as well as receiving chronic saline plus subacute QNP both demonstrated significantly reduced prefrontal cortical cAMP levels versus baseline (figure 5.17A). In addition, chronic fluoxetine but not desipramine pre-treatment prior to QNP challenge significantly prevented the decrease in cAMP evoked by QNP compared to saline pretreated controls (figure 5.17A). Subacute QNP challenge significantly increased PDE4 activity in the prefrontal cortex of LSB mice (figure 5.17B). Furthermore, chronic fluoxetine but not desipramine pre-treatment preceding QNP challenge increased PDE4 activity compared to saline pretreated controls (figure 5.17B), with desipramine not having any marked effect.

5.7.4 Effect of quinpirole challenge in HSB mice

Subacute QNP challenge in HSB mice resulted in a significant reduction in prefrontal cortical cAMP compared to untreated (baseline) animals (figure 5.18A). In addition, treatment of HSB mice receiving chronic saline plus QNP challenge resulted in a significant decrease in cAMP levels. Neither fluoxetine nor desipramine pre-treatment prior to QNP challenge had any significant effect on prefrontal cortical cAMP in HSB mice (figure 5.18A). Treatment of HSB mice with QNP (subacute challenge) significantly increased prefrontal cortical PDE4 activity (figure 5.18B). Chronic pre-treatment with fluoxetine as well as with desipramine resulted in a significant increase in PDE4 activity in the prefrontal cortex of HSB mice compared to QNP plus saline challenged animals (figure 5.18B).

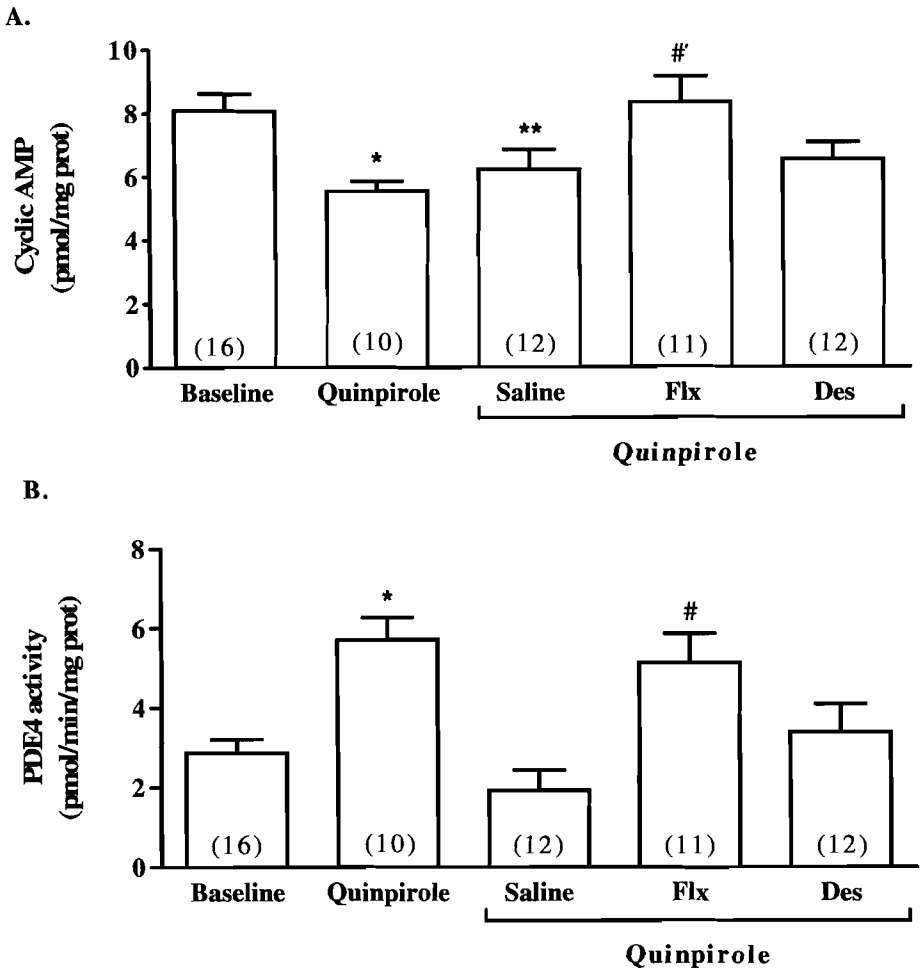

Figure 5.17 Effect of quinpirole challenge on cAMP levels and PDE4 activity in prefrontal cortex of LSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute quinpirole (filled bars). For comparison, LSB mice that were untreated (baseline) or quinpirole challenge (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicates $p < 0.05$ (quinpirole v baseline), ** indicates $p < 0.05$ (chronic saline plus quinpirole challenge v baseline), and # indicates $p < 0.05$ (saline v fluoxetine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

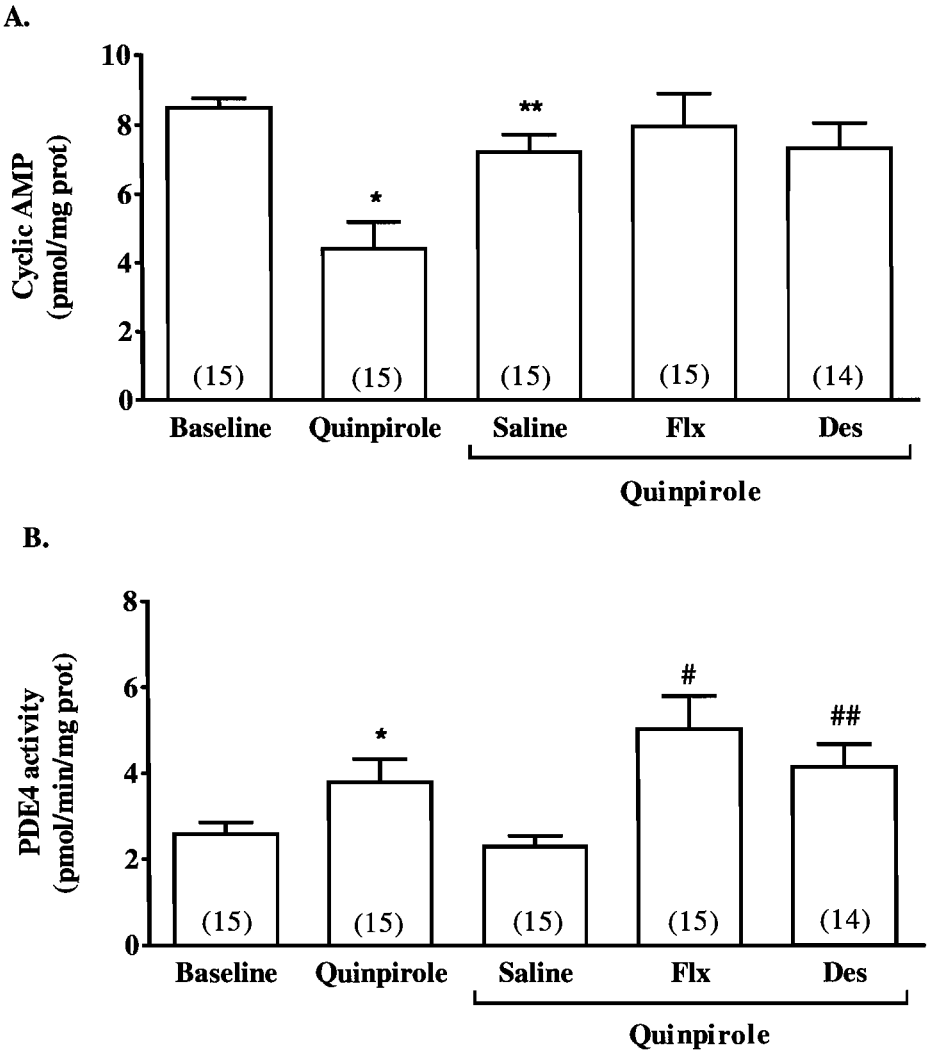

Figure 5.18 Effect of quinpirole challenge on cAMP levels and PDE4 activity in prefrontal cortex of HSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute quinpirole (filled bars). For comparison, HSB mice that were untreated (baseline) or quinpirole challenge (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicates $p < 0.05$ (quinpirole v baseline), ** indicates $p < 0.05$ (chronic saline plus quinpirole challenge v baseline), # indicates $p < 0.05$ (saline v fluoxetine), and ## indicates $p < 0.05$ (saline v desipramine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.8 Effect of subacute drug challenge following chronic SRI/NRI pre-treatment on cAMP levels and PDE4 activity in striatum of deer mice

5.8.1 Effect of mCPP challenge in LSB mice

LSB mice receiving subacute mCPP treatment as well as those receiving chronic saline plus subacute mCPP both demonstrated significantly attenuated striatal cAMP levels versus baseline (figure 5.19A). In addition, pre-treatment with fluoxetine or desipramine resulted in a decrease in cAMP levels in the striatum of LSB mice compared to saline plus mCPP. Subacute mCPP did not significantly affect PDE4 activity, but pre-treatment with fluoxetine or desipramine prior to mCPP challenge resulted in a significant decrease in PDE4 activity in LSB striatum compared to saline plus mCPP (figure 5.19B).

5.8.2 Effect of mCPP challenge in HSB mice

Subacute mCPP challenge of HSB mice as well as those receiving chronic saline plus subacute mCPP resulted in significantly reduced striatal cAMP levels versus baseline (figure 5.20A). Pre-treatment with fluoxetine or desipramine preceding mCPP challenge did not have a significant effect on cAMP levels in the striatum of HSB mice compared to saline plus mCPP. Striatal PDE4 activity however, was significantly increased compared to baseline following chronic saline plus mCPP challenge (figure 5.20B). PDE4 activity was, however, significantly reduced after fluoxetine or desipramine pre-treatment compared to saline plus mCPP (figure 5.20B).

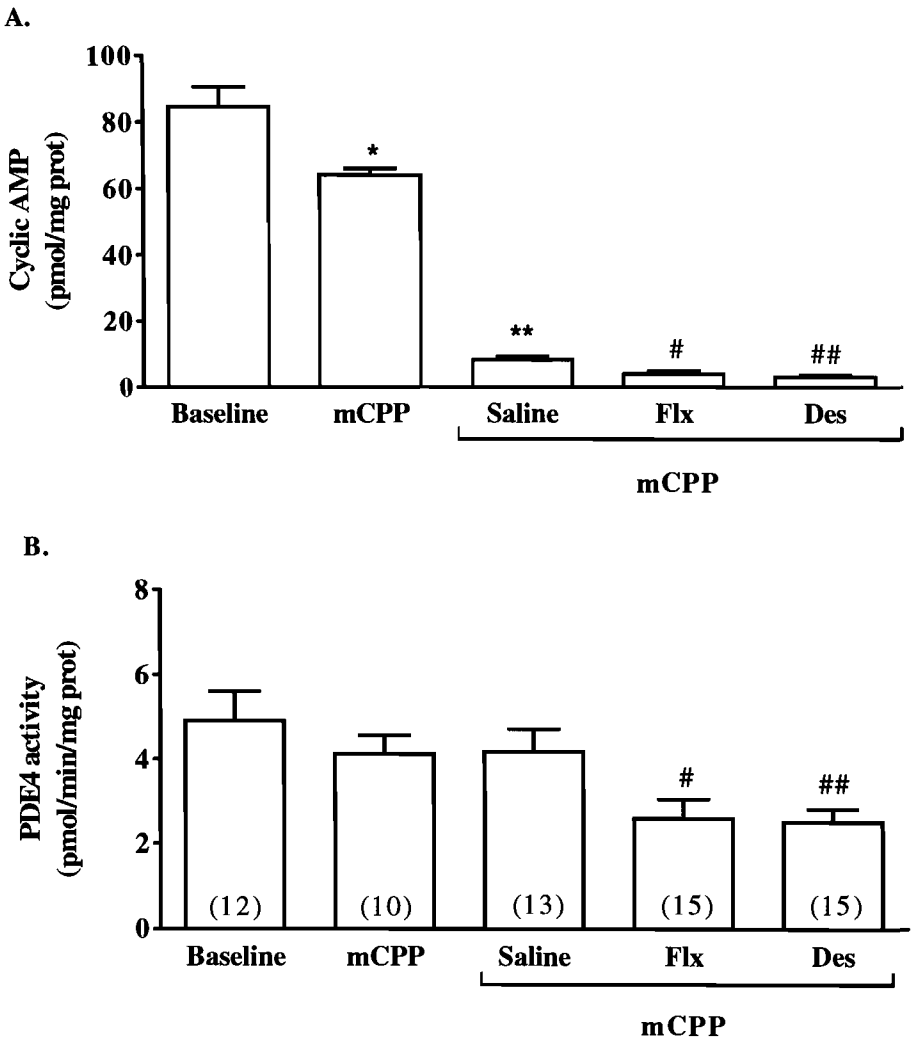

Figure 5.19 Effect of mCPP challenge on cAMP levels and PDE4 activity in striatum of LSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute mCPP (filled bars). For comparison, LSB mice that were untreated (baseline) or mCPP challenge (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. The same number of animals was used for cAMP and PDE4 activity determination. * indicates $p < 0.05$ (mCPP v baseline), ** indicates $p < 0.05$ (chronic saline plus mCPP challenge v baseline), # indicates $p < 0.05$ (saline v fluoxetine), and ## indicates $p < 0.05$ (saline v desipramine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

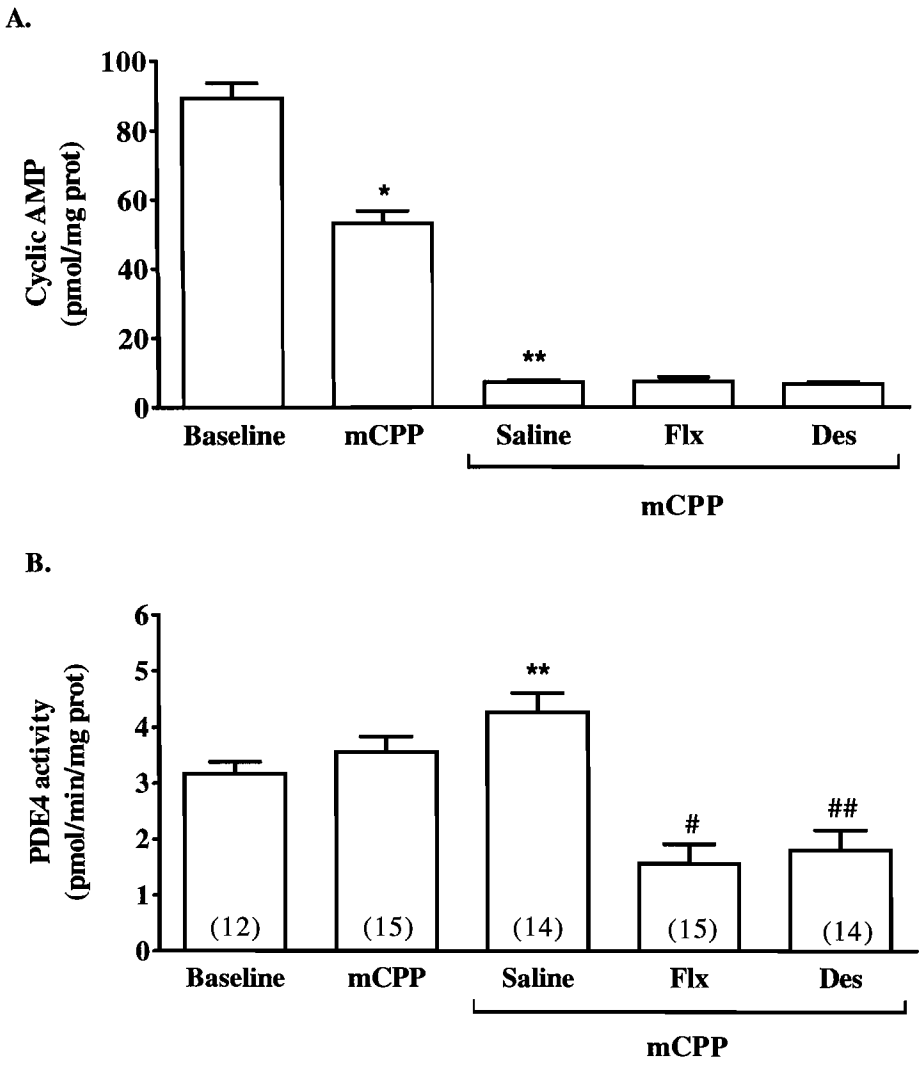

Figure 5.20 Effect of mCPP challenge on cAMP levels and PDE4 activity in striatum of HSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute mCPP (filled bars). For comparison, HSB mice that were untreated (baseline) or mCPP challenge (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. The same number of animals was used for cAMP and PDE4 activity determination. * indicate $p < 0.05$ (mCPP v baseline), ** indicates $p < 0.05$ (chronic saline plus mCPP challenge v baseline), # indicates $p < 0.05$ (saline v fluoxetine), and ## indicates $p < 0.05$ (vehichle v desipramine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.8.3 Effect of quinpirole challenge in LSB mice

QNP challenge and chronic saline pre-treatment prior to QNP challenge significantly reduced striatal cAMP levels in LSB mice (figure 5.21A). Moreover, fluoxetine pre-treatment as well as desipramine pre-treatment prior to QNP challenge resulted in reduced cAMP levels in LSB mice striatum compared to saline plus QNP (figure 5.21A). Subacute treatment of LSB mice with QNP resulted in a significant decrease in striatal PDE4 activity compared to untreated (baseline) mice (figure 5.21B). Similarly, chronic saline pre-treatment followed by QNP challenge also decreased PDE4 activity. Interestingly, both chronic fluoxetine and desipramine pre-treatment partially reversed the QNP induced reduction in PDE4 activity, so that PDE4 enzyme activity was significantly increased compared to saline plus QNP (figure 5.21B).

5.8.4 Effect of quinpirole challenge in HSB mice

Subacute QNP challenge of HSB mice as well as those receiving chronic saline plus subacute mCPP resulted in significantly reduced striatal cAMP levels compared to untreated (baseline) mice (figure 5.22A). Pre-treatment of HSB mice prior to QNP challenge with fluoxetine or desipramine did not have any significant effect on striatal cAMP levels (figure 5.22A). QNP challenge following chronic saline resulted in a significant decrease in PDE4 activity in the striatum of HSB mice (figure 5.22B). Furthermore, chronic pre-treatment with fluoxetine resulted in a significant increase PDE4 activity compared to saline plus QNP control, with desipramine not having a marked effect in this regard (figure 5.22B).

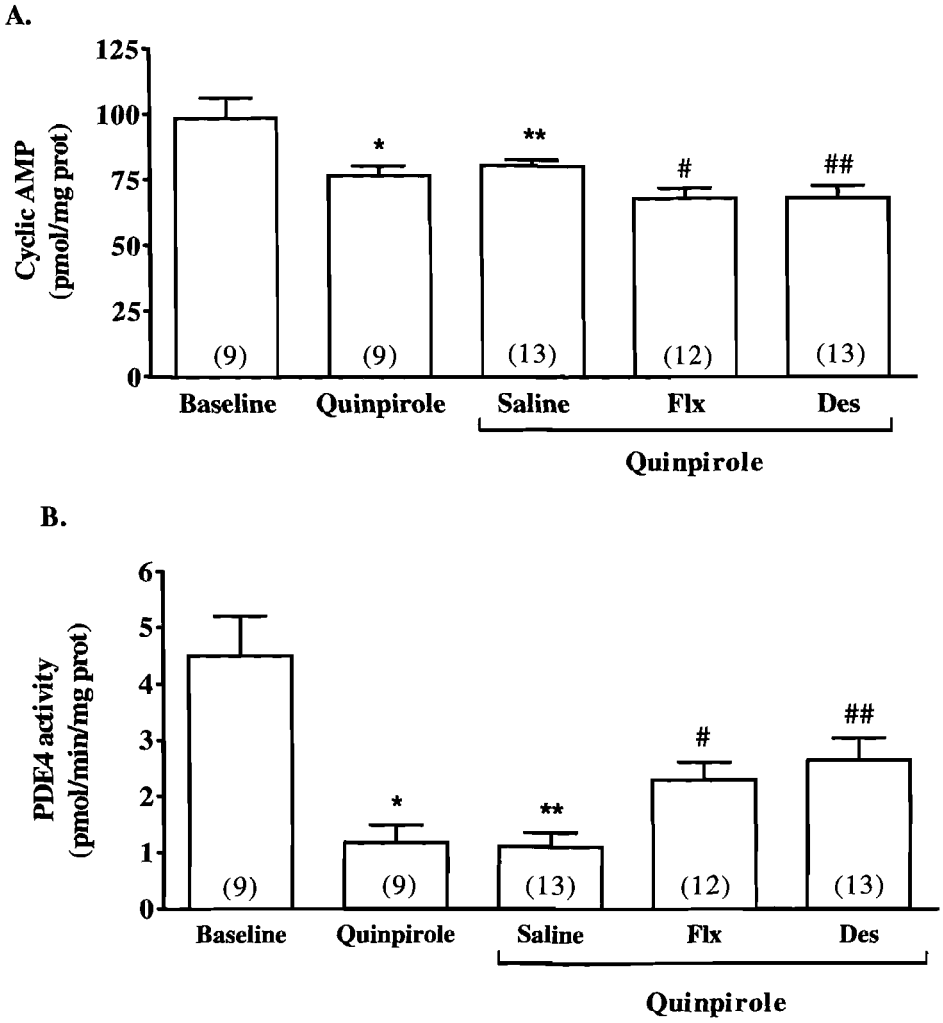

Figure 5.21 Effect of quinpirole challenge on cAMP levels and PDE4 activity in striatum of LSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute quinpirole (filled bars). For comparison, LSB mice that were untreated (baseline) or quinpirole challenged (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicate $p < 0.05$ (quinpirole v baseline), ** indicates $p < 0.05$ (chronic saline plus quinpirole challenge v baseline), # indicates $p < 0.05$ (vehichle v fluoxetine), and ## indicates $p < 0.05$ (vehichle v desipramine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

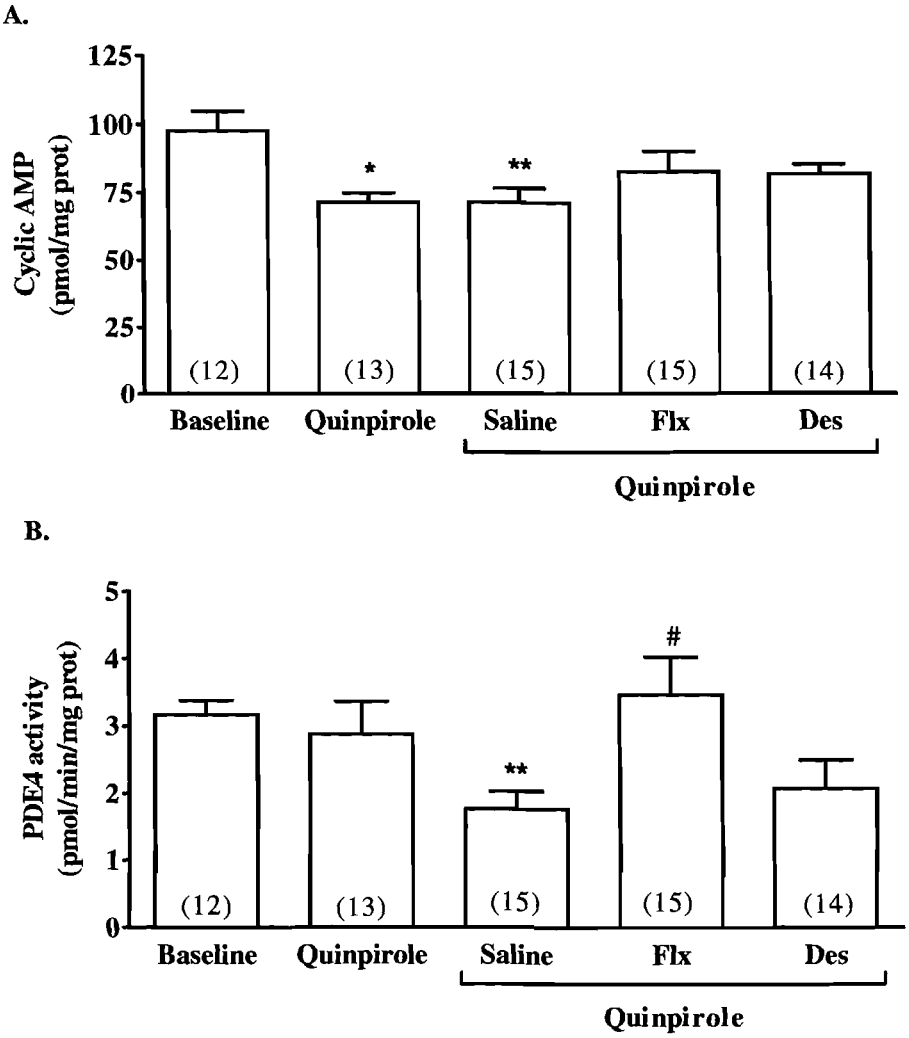

Figure 5.22 Effect of quinpirole challenge on cAMP levels and PDE4 activity in striatum of HSB mice following SRI/NRI pre-treatment. Deer mice received chronic SRI/NRI pre-treatment plus subacute quinpirole (filled bars). For comparison, HSB mice that were untreated (baseline) or quinpirole challenged (empty bars) are shown (Students t test). Subsequent to drug treatment, mice were sacrificed and dissected. Samples were analysed for cAMP levels (A) and PDE4 activity (B). Data is expressed as averages ± SEM and the number of animals used (n value) is provided in brackets within each bar. * indicates $p < 0.05$ (quinpirole v baseline), ** indicates $p < 0.05$ (chronic saline plus quinpirole challenge v baseline), and # indicates $p < 0.05$ (saline v fluoxetine). Data was analyzed by one-way ANOVA followed by Dunnett's test.

5.9 Summary of results: neurochemistry

Data presented in chapter 4 suggests that spontaneous stereotypic behaviour in deer mice respond in a similar fashion to pharmacological treatment in OCD patients namely that stereotypy is selectively reversed by fluoxetine and not desipramine (section 4.2.2). The precise mechanisms of action of antidepressant drugs remain unclear, however their effect on the cAMP pathway is important (Nibuya et al., 1996; Ye et al., 1997). Since this pathway appears to play a role in OCD (Perez et al., 2000) and antidepressant action (Ozawa & Rasenick, 1991; Takahashi et al., 1999), this part of the study focussed on cAMP levels as well as PDE4 activity in the prefrontal cortex and striatum of deer mice after antidepressant use and the effects of dopamine and 5-HT in regulating these two parameters.

As mentioned before (section 2.9.1 – 2.9.5), the binding of ligands such as neurotransmitters to their receptors located in the plasma membranes of cells, activates G-proteins that are coupled to adenylyl cyclase which converts ATP to cAMP. Binding of 5-HT to its receptors can either activate or inhibit adenylyl cyclase activity (section 2.9.1.1 – 2.9.1.7). This will result in either an increase or decrease in cAMP levels, depending on the type of receptor involved. PDE4 furthermore controls intracellular concentrations of cAMP by catalyzing its hydrolysis (section 2.9.8.2). PDE4 not only responds to cAMP levels, but results from recent studies indicate that this enzyme mediates some of the effects of antidepressants (Fleischhacker et al., 1992) and that these drugs primarily regulate PDE4 expression (Dlaboga et al., 2006).

A complete summary of the outcome following drug treatment as well as drug challenges is provided in table 5.1. A comprehensive discussion of these results is provided in chapter 7 where differential response to drug treatment is considered along with the possible role of neuroanatomy (striatal v prefrontal cortex) and pre-existing neurochemical abnormalities (LSB v HSB mice) in regulating cAMP levels and PDE4 activity. The next few paragraphs (5.9.1 – 5.9.5) exclusively highlight the effect of drug treatment on cAMP levels and PDE4 activity in the deer mice. Suffice to say that in comparing stereotypic deer mice to non-stereotypic C57Bl mice (figure 5.1 and 5.2) it is very clear that the two strains show remarkably different cAMP levels and PDE4 activity in the prefrontal cortex and striatum. Deer mice have significantly higher levels of cAMP

and PDE4 activity in the striatum, with a similar observation in the prefrontal cortex. These marked differences do suggest differences at the functional level in these brain regions, particularly locomotor function as has been described with stereotypy in section 4.2.2. It is of significant interest that cAMP and PDE4 activity are significantly altered in stereotypic (LSB and HSB) deer mice compared to non-stereotypic deer mice in both the striatum and prefrontal cortex. Interestingly, a reciprocal (inverse) relationship is seen in deer mice, with an increase in cAMP correlating with a decrease in PDE4 activity.

5.9.1 The effect of chronic fluoxetine on cAMP levels and PDE4 activity

Treatment of LSB and HSB mice with fluoxetine (20 mg/kg) resulted in a significant decrease in prefrontal cortex cAMP levels in both LSB and HSB mice (section 5.3 and 5.4 and summarized in table 5.1), with the 10 mg/kg dose less effective in the prefrontal cortex of LSB mice. In the striatum however, only a high dose (20 mg/kg) fluoxetine significantly increased cAMP levels. SRI treatment also significantly altered both striatal and prefrontal cortex PDE4 activity in deer mice. In this regard, fluoxetine decreased striatal PDE4 activity in HSB mice and up-regulated its activity in LSB mice (section 5.3 and 5.4).

In HSB mice, fluoxetine (10 mg/kg and 20 mg/kg) significantly reduced striatal PDE4 activity, whereas in LSB mice (10 mg/kg), a trend towards up-regulation was noted. From this it is clear that drug dosage and degree of stereotypy are important in regulating cAMP and PDE4 activity. However, a clearer picture regarding the role of different PDE4 isoforms and their regulation of cAMP levels following fluoxetine treatment is needed. In this regard, protein expression studies (see section 6.3 and 6.4) will provide valuable information. Suffice to say that current data suggest that the diverse but distinct actions of antidepressants on cAMP and PDE4 activity are also profoundly influenced by the degree of stereotypy.

5.9.2 The effect of chronic desipramine on cAMP levels and PDE4 activity

This section of results summarizes the neurochemical effects of desipramine on cAMP and PDE4 activity. Desipramine significantly reduces prefrontal as well as striatal cAMP in both LSB and HSB mice (table 5.1). Desipramine (10 mg/kg) furthermore significantly

increased PDE4 activity in the striatum of HSB mice (table 5.1), whereas the only significant effect of desipramine (10 mg/kg) in LSB mice was an increase in striatal PDE4. Data suggest that cAMP levels are significantly altered in both the striatum and prefrontal cortex of stereotypic deer mice. A comprehensive discussion regarding these data is provided in chapter 7.

5.9.3 The effect of subacute drug treatment on cAMP levels and PDE4 activity

Deer mice were exposed to subacute drug challenges to investigate the construct validity of the model, and to establish the effects of these challenges on cAMP levels and PDE4 activity in deer mice. Subacute challenge of deer mice with mCPP, SKF and QNP resulted in a significant increase in striatal cAMP levels in LSB mice but not HSB, with only QNP increasing cAMP in HSB mice. mCPP is a non-selective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist, although it is thought that this drug acts primarily through the 5-$HT_{2A/C}$ receptors (Ni & Miledi, 1997). Stimulation of 5-HT_{2A} would explain the increase in striatal cAMP levels, since this receptor have been shown to stimulate cAMP production (Berg et al., 1994). Interestingly, in the deer mice, the D_1 agonist, SKF, and the D_2/D_3 agonist, QNP, both stimulated adenylyl cyclase activity. Interestingly, prefrontal cAMP levels differed between LSB and HSB mice following subacute challenges. In LSB mice, subacute challenge with QNP resulted in an increase in cAMP levels, with mCPP and SKF not having a marked effect. In HSB mice however, both mCPP and SKF significantly reduced cAMP levels, with QNP not having any significant effect.

Treatment of LSB mice with mCPP and QNP significantly increased prefrontal PDE4 activity. SKF did not have any effect in this regard. In HSB mice however, mCPP and SKF decreased PDE4 activity, with QNP not having an effect (table 5.1). Striatal PDE4 activity were not influenced by subacute drug challenges with the exception of QNP. In LSB mice, QNP reduced enzyme activity whereas in HSB mice, the challenge resulted in an increase in PDE4 activity. This suggests that D_2 agonists act differently in the presence of already heightened stereotypy. As with fluoxetine and desipramine treatment, subacute challenges have differential effects on the cAMP-PDE4 system and the effects of drug treatment on PDE4 will better emerge following protein expression studies (see section 6.3 and 6.4).

Table 5.1 Effect of drug treatment on cAMP levels and PDE4 activity in stereotypic deer mice. (↑/↓ indicates non-significant increase or decrease in enzyme activity or second messenger levels; ⇑/⇓ indicates significant change; -- indicate no change; Str = striatum, PFC = prefrontal cortex)

Treatment regime	LSB mice				HSB mice			
	cAMP levels		PDE4 activity		cAMP levels		PDE4 activity	
	Str	PFC	Str	PFC	Str	PFC	Str	PFC
10 mg/kg chronic fluoxetine	--	↑	⇑	↑	↑	⇓	⇓	↓
20 mg/kg chronic fluoxetine	⇑	⇓	--	↑	--	⇓	⇓	⇓
10 mg/kg chronic desipramine	⇓	⇓	⇑	↓	⇓	⇓	⇑	--
20 mg/kg chronic desipramine	↓	⇓	↑	--	⇓	⇓	↑	⇓
mCPP challenge	⇑	↑	↓	⇑	--	⇓	↑	⇓
SKF challenge	⇑	--	--	↑	↑	⇓	--	⇓
QNP challenge	⇑	⇑	⇓	⇑	⇑	↓	⇑	--
fluoxetine pre-treatment + mCPP	⇓	⇑	⇓	⇓	--	↑	⇓	↑
desipramine pre-treatment + mCPP	⇓	--	⇓	--	--	⇑	⇓	--
saline pre-treatment + mCPP	⇓	⇓	↓	--	⇓	⇓	⇑	--
fluoxetine pre-treatment + QNP	⇓	⇑	⇑	⇑	↑	↑	⇑	⇑
desipramine pre-treatment + QNP	⇓	--	⇑	↑	↑	--	↑	⇑
saline pre-treatment + QNP	⇓	⇓	⇓	↓	⇓	⇓	⇓	--

5.9.4 The effect of chronic antidepressant pre-treatment plus mCPP challenge on cAMP levels and PDE4 activity

Chronic pre-treatment with fluoxetine plus mCPP challenge in LSB resulted in a decrease in striatal cAMP levels compared to saline plus mCPP. Pre-treatment followed by desipramine challenge similarly resulted in a decrease in striatal cAMP levels compared to saline plus mCPP. The effect of pre-treatment with either fluoxetine or desipramine may also be masked, since pre-treatment with saline also resulted in adenylyl cyclase inhibition (decrease in cAMP levels). This suggests a possible influence of injection stress and/or handling stress on the animal. In contrast to striatal levels, fluoxetine pre-treatment plus mCPP in LSB increased prefrontal cortex cAMP compared to saline suggesting partial reversal. Importantly, this trend was noted in HSB mice, but it did not reach significance. Desipramine failed to reverse mCPP-induced decreases in cAMP in the prefrontal cortex of LSB mice, but did significantly reverse cAMP after mCPP in HSB mice.

Striatal and prefrontal cAMP levels were significantly decreased following chronic saline plus mCPP compared to mCPP in both LSB and HSB mice. From this it is clear that injection stress may have pronounced effects on cAMP levels, possibly via activation of the hypothalamic-pituitary-adrenal (HPA) axis and modulation of 5-HT receptors (Leonard, 2005) or more specifically the stimulation of 5-HT_1 receptors. Nevertheless, it is clear that cAMP is reduced in the prefrontal cortex and striatum.

Focussing on striatal PDE4 enzyme activity, chronic fluoxetine reversed the effects of mCPP on PDE4 in LSB and HSB mice, with chronic desipramine similarly reversing mCPP-induced effects on PDE4 activity in stereotypic (LSB and HSB) deer mice (table 5.1). In contrast, only fluoxetine pre-treatment decreased mCPP-induced effects on PDE4 in the prefrontal cortex of LSB and HSB mice. From this it is evident that PDE4, and to a lesser extent cAMP, is differentially regulated in the striatum compared to the prefrontal cortex. Data also highlight the fact that the degree of pre-existing stereotypy may furthermore influence antidepressant action.

5.9.5 The effect of chronic antidepressant pre-treatment plus quinpirole challenge on cAMP levels and PDE4 activity

Chronic pre-treatment with fluoxetine or desipramine resulted in a decrease in striatal cAMP in LSB mice but not HSB mice. Chronic fluoxetine plus QNP resulted in an increase in prefrontal cortex cAMP levels in LSB, but not HSB mice, while desipramine plus QNP did not have any effect on cAMP in stereotypic mice. In LSB and HSB mice, chronic saline plus QNP decreased striatal and prefrontal cortex cAMP significantly. Chronic fluoxetine plus QNP reversed (increased) QNP-altered PDE4 activity in both the prefrontal cortex and striatum of LSB and HSB deer mice, while desipramine only reversed QNP effects in the striatum of LSB mice. Chronic saline plus QNP resulted in a decrease in striatal PDE4 activity whereas the same treatment did not have any effect on prefrontal cortex PDE4 activity. Again, the central role of stress (injection stress) in this model is apparent and additional information (section 6.3 and 6.4) will be necessary to explain current results. Yet again, this suggests that D_2 agonists act differently in the presence of already heightened stereotypy.

As a final point, data from this section of the study directly implicates the serotonergic and dopaminergic systems in the cAMP-PDE4 cascade of stereotypic deer mice. Chronic drug treatment and challenge studies highlight the fact that the cAMP-PDE4 system in deer mice seems to be controlled or at the very least, substantially regulated by the degree of pre-existing stereotypic behaviour. It is also apparent that two regions implicated in the pathophysiology of OCD, namely the prefrontal cortex and the striatum, is differentially regulated in stereotypic deer mice and that antidepressants have diverse affects on the cAMP-PDE4 system in these regions.

6. Results – Protein expression and phosphorylation

6.1 Introduction and experimental design

The prominent neural second messenger cAMP is known to be up-regulated by antidepressants (Nibuya et al., 1996). It is furthermore strictly regulated by amongst other the phosphodiesterase family of enzymes (section 2.9.8.2). In this section, expression of a number of PDE4 subtypes (PDE 4A, PDE 4B and PDE 4D) were investigated to compliment and to extend PDE4 activity data described in chapter 5. The PDE 4C subtype is expressed sparsely, if at all, in brains of mice (Cherry & Davis, 1999) and was not investigated. The study furthermore examined phosphorylation of Darpp-32 at two key amino acid residues namely at Thr34 and Thr75.

As described in section 2.9.6, Darpp-32, is a key regulator of dopaminergic and glutamatergic signalling in the striatum and to a lesser extent the prefrontal cortex. The crucial link with OCD is that the nigrostriatal dopamine system plays a central role in the regulation of movements, and the mesolimbicortical system mediates the cognitive and rewarding effects of dopamine (Kalivas, 2002; Wise, 2004). Exploration of Darpp-32 regulation (phosphorylation) in the deer mice may contribute to our understanding of stereotypy and this in turn may facilitate our understanding of the role of dopamine in the stereotypic aspects of OCD.

Briefly, deer mice were treated with either SRI or NRI for 21 days where after mice were sacrificed and the striatum and prefrontal cortex dissected and frozen at -80°C for further analysis. The study was initiated with determination of PDE4 expression and Darpp-32 phosphorylation by western blotting. Stereotypic and non-stereotypic deer mice were compared to a species that does not develop stereotypic behaviour under standard laboratory conditions, namely C57Bl mice. In subsequent drug treatment experiments, only stereotypic deer mice were used.

It is important to mention that the polyclonal antibodies that were used during the study identified more than one isoform for each PDE4 subtype (table 3.2, section 3.7.1). Due to the difficulty resolving proteins during SDS-PAGE, a number of isoforms separated closely on immunoblots and is seen as a single band. For example, PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) is seen as a single band even though they are two separate

proteins. Following western blotting, densitometric analysis was performed separately for each isoform and the data displayed in graphs. A representative blot for each PDE4 isoform (and phosphorylated Darpp-32 protein) and a single data table is provided when results are described initially. This is also the case following drug treatment, however, in order to facilitate coherence and avoid unnecessary replication, certain data is reported in table format only.

6.2 Protein expression in untreated mice

6.2.1 Protein expression and phosphorylation in the prefrontal cortex of deer mice and C57Bl mice

Immunoblot analysis showed that expression of PDE 4A5 (109 kDA) and PDE 4A8 (106 kDa) was significantly decreased in the prefrontal cortex of deer mice (figure 6.1A) compared to C57Bl mice, but did not differ with the degree of stereotypy in these animals. Furthermore, immunoblot analysis showed that PDE 4A1 (66 kDa) expression was significantly decreased in the prefrontal cortex of HSB mice compared to C57Bl mice (figure 6.1B) but were expressed to a similar degree in C57Bl, NS, and LSB mice. The band intensity and p value for each of the individual PDE 4A isoforms are provided in tables 6.1 and 6.2

The immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was significantly increased in the prefrontal cortex of NS and LSB mice compared to C57Bl mice (figure 6.2A), with that of HSB not being significant. In contrast, PDE 4B4 (66 kDa) expression was significantly decreased in all groups of deer mice compared to C57Bl mice (figure 6.2B). However, the degree of expression of this PDE4 isoform did not differ with respect to the three deer mice groups. The band intensity and p value for each of the individual PDE 4B isoforms are provided in tables 6.3 and 6.4.

The immunoblot analysis showed that expression of PDE 4D4 (105 kDa) was significantly increased in all three deer mice groups compared to C57Bl mice (figure 6.3A). Interstingly, PDE 4D1 (68 kDa) was significantly decreased in all three deer mice groups compared to C57Bl mice (figure 6.3B). The band intensity and p value for each of the individual PDE 4D isoforms are provided in tables 6.5 and 6.6.

Immunoblot analysis did not detect any Darpp-32 phosphorylation on residue Thr34 in deer mice or C57Bl mice (figure 6.4A). Immunoblotting did however indicate a significant increase in Darpp-32 phosphorylation on Thr75 in stereotypic deer mice (HSB and LSB) compared to C57Bl mice (figure 6.4B), but not in NS deer mice. The relative band intensity and p value for phosphorylation of Darpp-32 on Thr75 is provided in table 6.7.

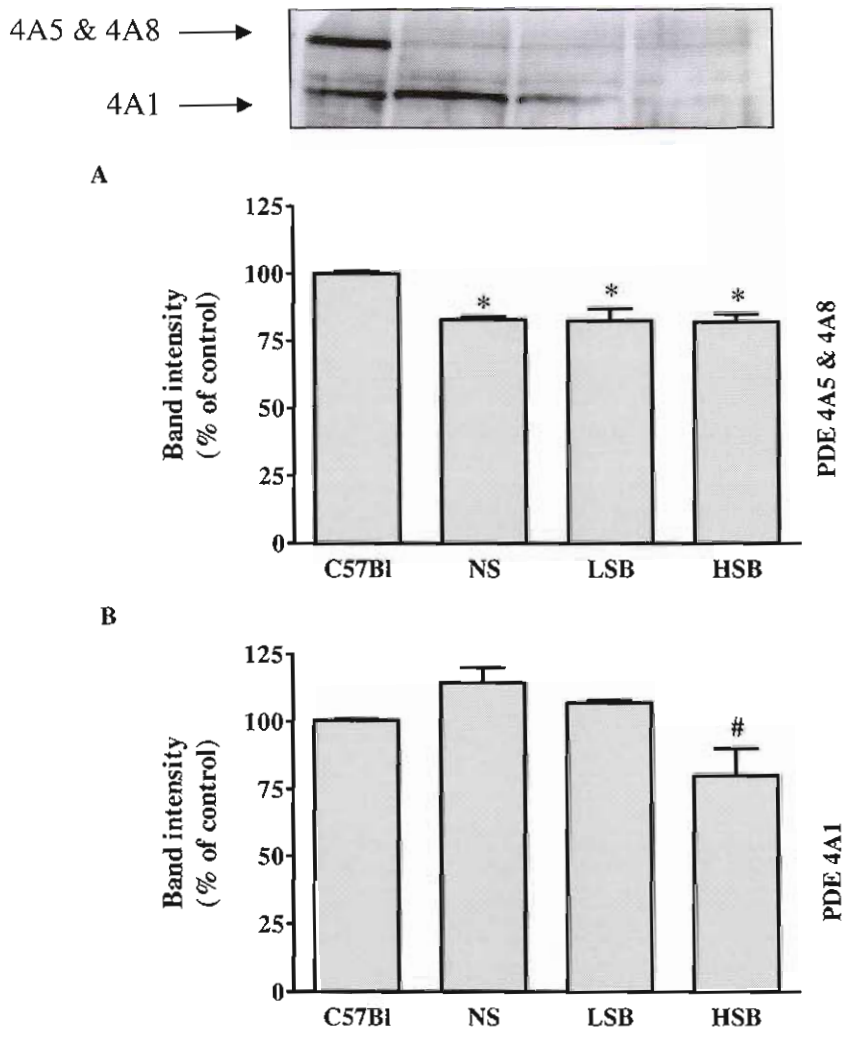

Figure 6.1 Protein expression of PDE 4A isforms in prefrontal cortex of untreated C57Bl and deer mice. Groups of mice were designated high stereotypic (HSB, n = 10), low stereotypic (LSB, n = 10) and non-stereotypic (NS, n = 8) deer mice and compared to C57Bl mice (n = 10). Subsequent to behavioural assessment, C57BL and deer mice were sacrificed and expression levels of PDE 4A determined. Shown is a representative immunoblot and the relative band intensities of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) (A) and PDE 4A1 (66 kDa) isoforms (B). Each point is the mean ± SEM and * denotes $p < 0.01$ compared to control (C57Bl) and # denotes $p < 0.05$ compared to C57Bl. Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

Table 6.1 **Expression of PDE 4A5 & 8 isoforms (109 & 106 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	83 ± 1	$p < 0.01$
LSB	83 ± 4.5	$p < 0.01$
HSB	82 ± 3	$p < 0.01$

Table 6.2 **Expression of PDE 4A1 isoform (66 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	115 ± 5.5	$p > 0.05$
LSB	107 ± 1	$p > 0.05$
HSB	80 ± 1	$p < 0.05$

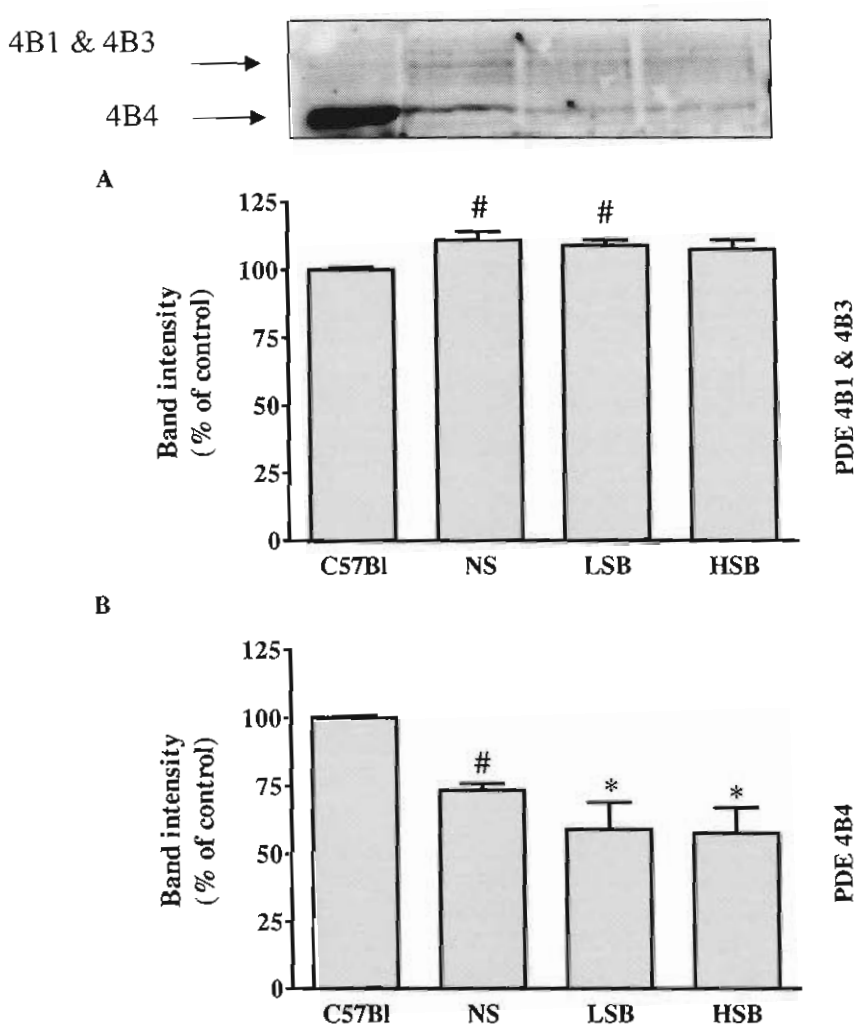

Figure 6.2 Protein expression of PDE 4B isforms in prefrontal cortex of untreated C57Bl and deer mice. Groups of mice were designated high stereotypic (HSB, n = 10), low stereotypic (LSB, n = 9) and non-stereotypic (NS, n = 8) deer mice and compared to C57Bl mice (n = 10). Subsequent to behavioural assessment, C57Bl and deer mice were sacrificed and expression levels of PDE 4B determined. Shown is a representative immunoblot and the relative band intensities of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) (A) and PDE 4B4 (66 kDa) isoforms (B). Each point is the mean ± SEM and * denotes $p < 0.01$ compared to control (C57BL) and # denotes $p < 0.05$ compared to C57Bl. Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

Table 6.3 **Expression of PDE 4B1 & 3 isoforms (107 & 100 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	111 ± 3	$p < 0.05$
LSB	109 ± 2	$p < 0.05$
HSB	108 ± 4.5	$p > 0.05$

Table 6.4 **Expression of PDE 4B4 isoform (66 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	74 ± 2.5	$p < 0.05$
LSB	59 ± 10	$p < 0.05$
HSB	57 ± 9	$p < 0.01$

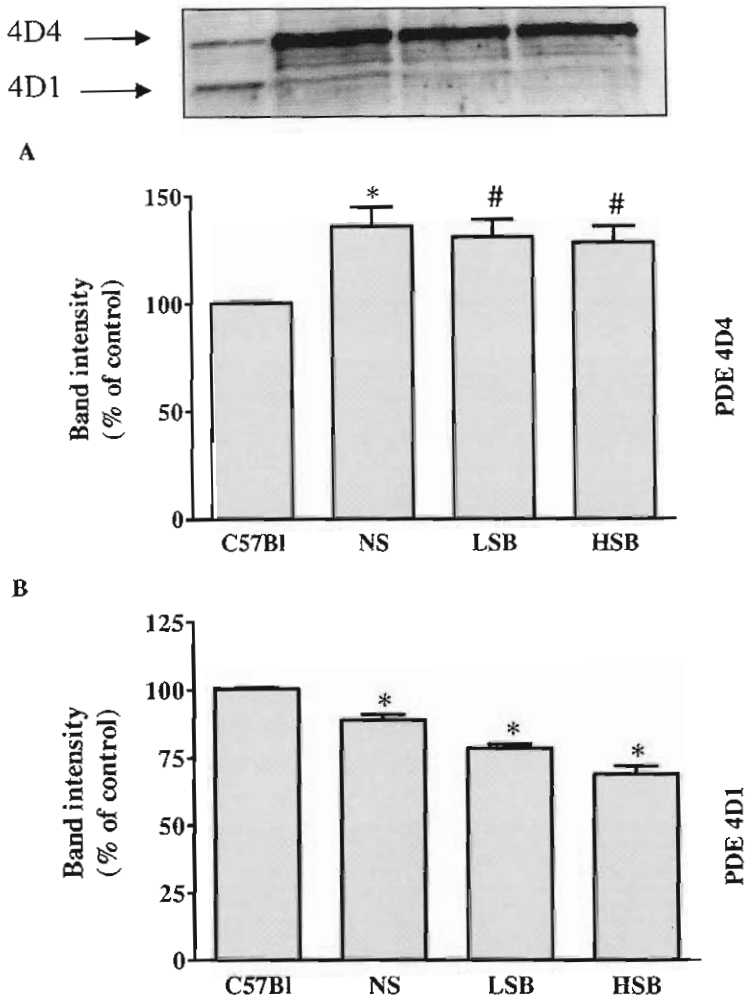

Figure 6.3 Protein expression of PDE 4D isforms in prefrontal cortex of untreated C57Bl and deer mice. Groups of mice were designated high stereotypic (HSB, n = 10), low stereotypic (LSB, n = 10) and non-stereotypic (NS, n = 10) deer mice and compared to C57Bl mice (n = 10). Subsequent to behavioural assessment, C57Bl and deer mice were sacrificed and expression levels of PDE 4D determined. Shown is a representative immunoblot and the relative band intensities of PDE 4D4 (105 kDa) (A) and PDE 4D1 (68 kDa) isoforms (B). Each point is the mean ± SEM and * denotes $p < 0.01$ compared to C57Bl and # denotes $p < 0.05$ compared to C57Bl. Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

Table 6.5 **Expression of PDE 4D4 isoform (105 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	136 ± 9	$p < 0.01$
LSB	131 ± 8	$p < 0.05$
HSB	128 ± 8	$p < 0.05$

Table 6.6 **Expression of PDE 4D1 isoform (68 kDa) in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	89 ± 2	$p < 0.01$
LSB	78 ± 1.5	$p < 0.01$
HSB	69 ± 3	$p < 0.01$

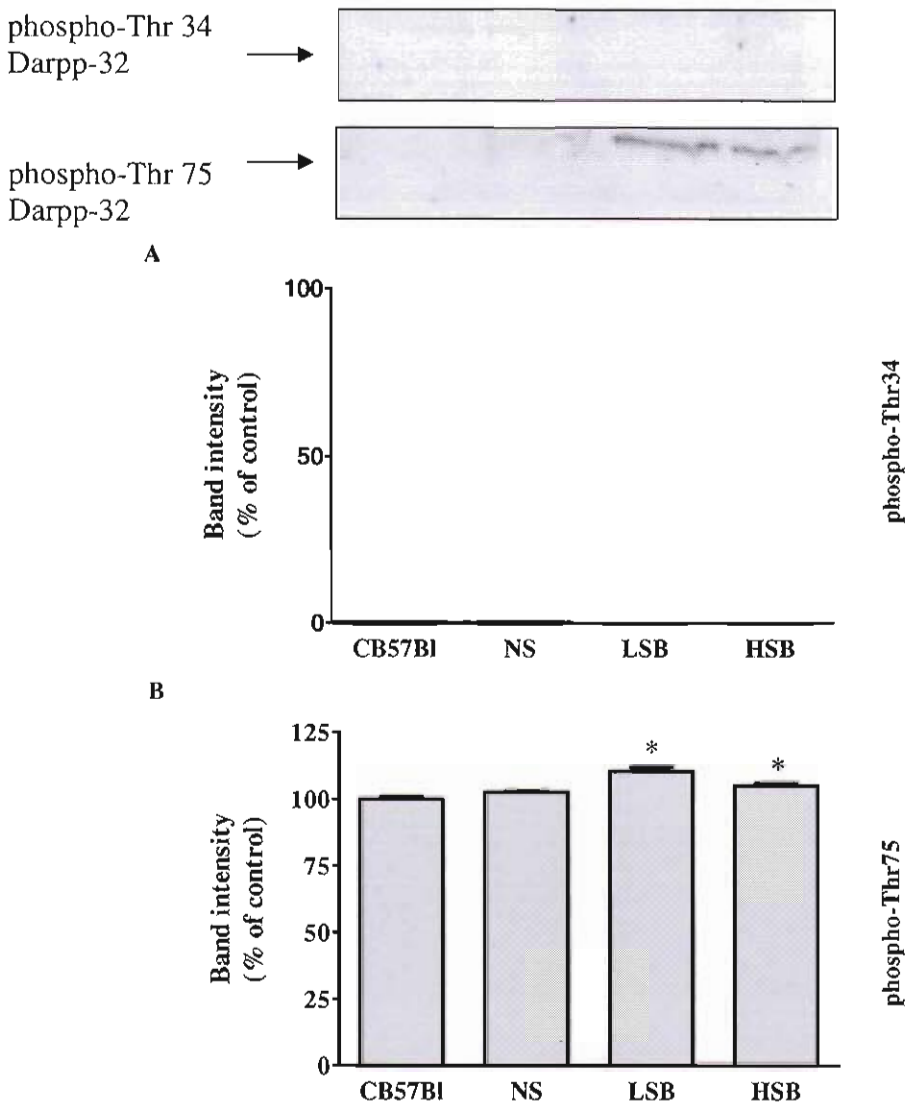

Figure 6.4 Phosphorylation of Darpp-32 in prefrontal cortex of untreated C57Bl and deer mice. Groups of mice were designated high stereotypic (HSB, n = 9), low stereotypic (LSB, n = 10) and non-stereotypic (NS, n = 10) deer mice and compared to C57Bl mice (n = 10). Subsequent to behavioural assessment, C57Bl and deer mice were sacrificed and expression levels of Darpp-32 phosphorylation determined. Shown is a representative immunoblot and the relative band intensities of Darpp-32 when phosphorylated on residue Thr34 (A) and on residue Thr75 (B). Each point is the mean ± SEM and * denotes $p < 0.01$ compared to C57Bl. Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

Table 6.7 **Level of Darpp-32 phosphorylation on residue Thr75 in the prefrontal cortex of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	102 ± 0.5	$p > 0.05$
LSB	110 ± 1.5	$p < 0.01$
HSB	105 ± 1	$p < 0.01$

6.2.2 Protein expression and phosphorylation in the striatum of deer mice and C57Bl mice

To avoid unnecessary repetition and since a representative immunoblot of each of the PDE4 isoforms and phosphorylated Darpp-32 proteins have been shown (figure 6.1 – 6.4), striatal protein expression data of PDE4 isoforms and Darpp-32 phosphorylation of untreated C57Bl and deer mice is given in table format. Immunoblot analysis showed that expression of PDE 4A5 (109 kDA) and PDE 4A8 (106 kDa) was significantly decreased in the striatum of all untreated deer mice compared to C57Bl mice. The relative band intensities and p values for PDE 4A5 and PDE 4A8 are provided in table 6.8. Immunoblot analysis moreover showed that PDE 4A1 (66 kDa) expression was significantly decreased in the striatum of HSB mice compared to C57Bl mice and that PDE 4A1 expression was slightly, although not significantly, decreased in LSB and NS deer mice. The relative band intensity of PDE 4A1 is provided in table 6.9.

The immunoblot analysis showed that expression of striatal PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) did not differ significantly between deer mice and C57Bl mice. A summary of the relative band intensities is provided in table 6.10. Striatal PDE 4B4 (66 kDa) expression was significantly decreased in all groups of deer mice compared to C57Bl mice, with a 40% decrease noted in HSB mice (table 6.11).

Expression of striatal PDE 4D4 (105 kDa) was significantly increased in all deer mice groups compared to C57Bl mice (table 6.12). However, striatal PDE 4D1 (68 kDa) expression was significantly decreased in LSB and HSB mice compared to C57Bl mice (table 6.13).

In contrast to prefrontal cortex samples, striatal phosphorylation of Darpp-32 on residue Thr34 was detected by immunoblotting. In fact, a significant increase in Darrp-32 phosphorylation on Thr34 was noted in HSB mice compared to C57Bl mice (table 6.14). Phosphorylation of Darpp-32 on Thr75 was significantly increased in LSB and HSB mice compared to C57Bl mice (table 6.15).

Table 6.8 **Expression of PDE 4A5 & 8 isoforms (109 & 106 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	80 ± 1.5	$p < 0.05$
LSB	81 ± 1.5	$p < 0.05$
HSB	69 ± 9	$p < 0.01$

Table 6.9 **Expression of PDE 4A1 isoform (66 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	89 ± 5.5	$p > 0.05$
LSB	82 ± 10	$p > 0.05$
HSB	77 ± 4	$p < 0.05$

Table 6.10 **Expression of PDE 4B1 & 3 isoforms (107 & 100 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	109 ± 2	$p > 0.05$
LSB	107 ± 6.5	$p > 0.05$
HSB	107 ± 4.5	$p > 0.05$

Table 6.11 **Expression of PDE 4B4 isoform (66 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	67 ± 3	$p < 0.01$
LSB	62 ± 1.5	$p < 0.01$
HSB	60 ± 1.5	$p < 0.01$

Table 6.12 **Expression of PDE 4D4 isoform (105 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	137 ± 11	$p < 0.05$
LSB	134 ± 5.5	$p < 0.05$
HSB	134 ± 10	$p < 0.05$

Table 6.13 **Expression of PDE 4D1 isoform (68 kDa) in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 0.5	-
NS	95 ± 4	$p > 0.05$
LSB	86 ± 2	$p < 0.05$
HSB	84 ± 5	$p < 0.01$

Table 6.14 **Levels of Darpp-32 phosphorylation on residue Thr34 in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	103 ± 1	$p > 0.05$
LSB	108 ± 3	$p > 0.05$
HSB	112 ± 4	$p < 0.05$

Table 6.15 **Levels of Darpp-32 phosphorylation on residue Thr75 in the striatum of untreated mice**

Stereotypy group	Band intensity (% control ± SEM)	p value
C57Bl	100 ± 1	-
NS	102 ± 1.5	$p > 0.05$
LSB	105 ± 0.5	$p < 0.01$
HSB	106 ± 0.5	$p < 0.01$

6.3 Effect of chronic drug treatment on protein expression and phosphorylation in the prefrontal cortex of deer mice

6.3.1 Effect of 10 mg/kg SRI/NRI in LSB mice

Immunoblot analysis showed that expression of PDE 4A5 (109 kDA) and PDE 4A8 (106 kDa) was not significantly altered in the prefrontal cortex of LSB mice (figure 6.5A) following chronic treatment with 10 mg/kg fluoxetine or desipramine. Immunoblot analysis furthermore showed that PDE 4A1 (66 kDa) expression was not significantly changed in the prefrontal cortex of LSB mice (figure 6.5B). The relative band intensities for the various PDE4 isoforms, expressed as percentage of control, are provided table 6.16.

The immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was significantly decreased in the prefrontal cortex of LSB mice following chronic desipramine treatment compared to control (figure 6.6A). Interestingly, PDE 4B4 (66 kDa) expression was significantly decreased in LSB mice following both fluoxetine and desipramine treatment (figure 6.6B and table 6.16).

The immunoblot analysis showed that expression of PDE 4D4 (105 kDa) was significantly decreased in the prefrontal cortex of LSB mice following chronic treatment with both fluoxetine and desipramine compared to saline control (figure 6.7A). Immunoblot analysis furthermore showed that fluoxetine, but not desipramine significantly decreased PDE 4D1 expression in prefrontal cortex of LSB mice (figure 6.7B and table 6.16).

Phosphorylation of Darpp-32 on Thr34 was not significantly altered by either chronic fluoxetine or desipramine treatment in the prefrontal cortex of LSB mice (figure 6.8A). Interestingly, chronic fluoxetine treatment, but not desipramine resulted in a significant increase in Darpp-32 phosphorylation on Thr75 compared to saline control (figure 6.8B and table 6.16).

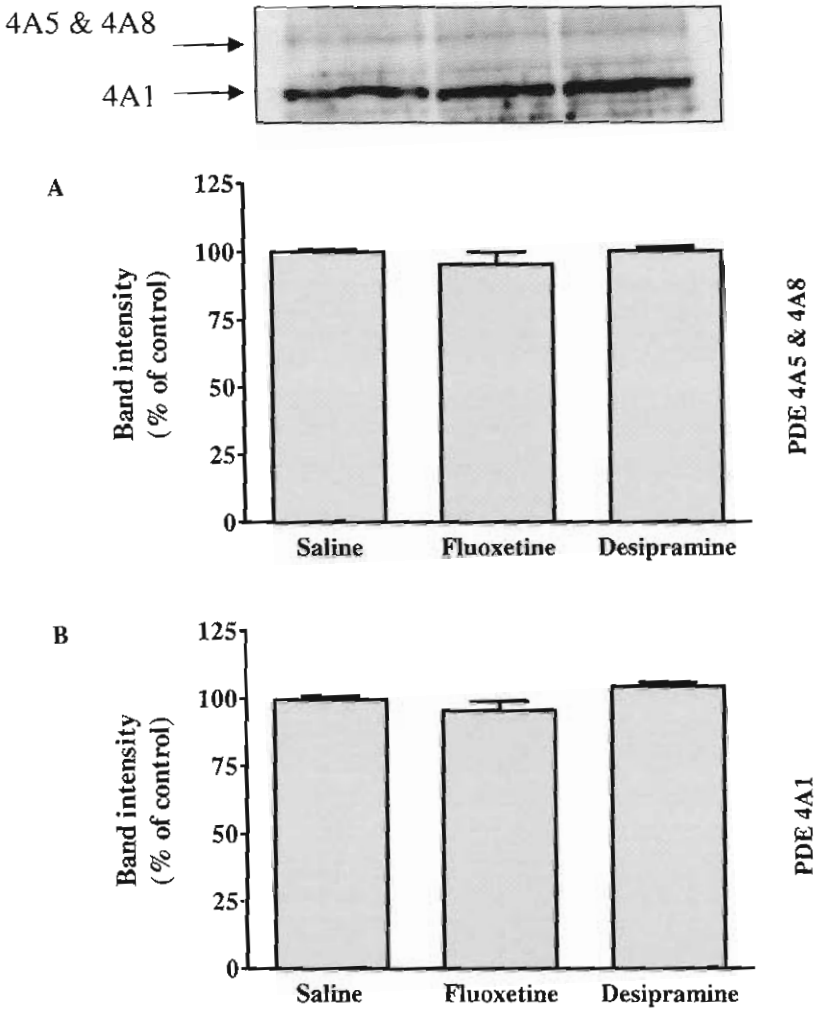

Figure 6.5 Effects of 10 mg/kg fluoxetine and desipramine on PDE 4A expression in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 11), desipramine (n = 10) or saline (n = 10). Following sacrifice and tissue collection, samples were analysed for protein expression. Shown is a representative immunoblot and the relative band intensities of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) (A) and PDE 4A1 (66 kDa) isoforms (B). Each point is the mean ± SEM with experiments carried out in duplicate. Data was analyzed by one-way ANOVA followed by Tukey's test.

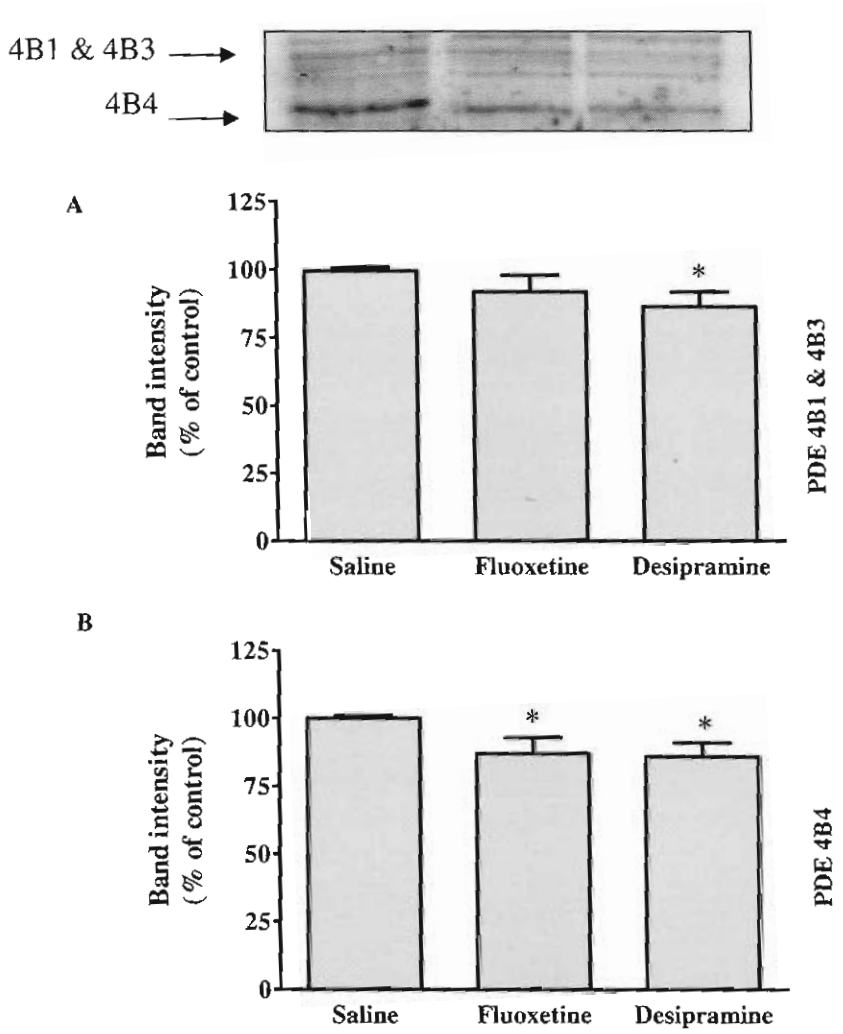

Figure 6.6 Effects of 10 mg/kg fluoxetine and desipramine on PDE 4B expression in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 10), desipramine (n = 10) or saline (n = 10). Following sacrifice and tissue collection, samples were analysed for protein expression. Shown is a representative immunoblot and the relative band intensities of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) (A) and PDE 4B4 (66 kDa) isoforms (B). Each point is the mean ± SEM and * denotes $p < 0.05$ compared to control (saline). Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

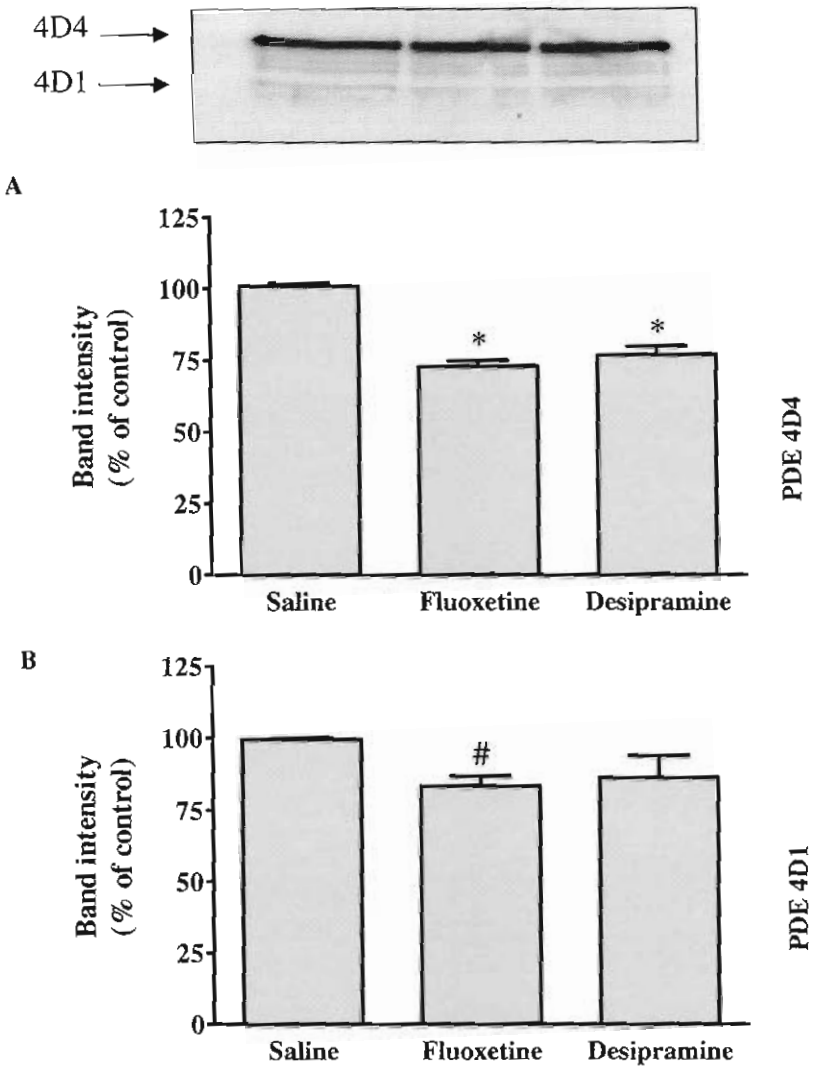

Figure 6.7 Effects of 10 mg/kg fluoxetine and desipramine on PDE 4D expression in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 10), desipramine (n = 10) or saline (n = 9). Following sacrifice and tissue collection, samples were analysed for protein expression. Shown is a representative immunoblot and the relative band intensities of PDE 4D4 (105 kDa) (A) and PDE 4D1 (68 kDa) isoforms (B). Each point is the mean ± SEM and * denotes $p < 0.01$ compared to control (saline) and # denotes $p < 0.05$ compared to control. Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

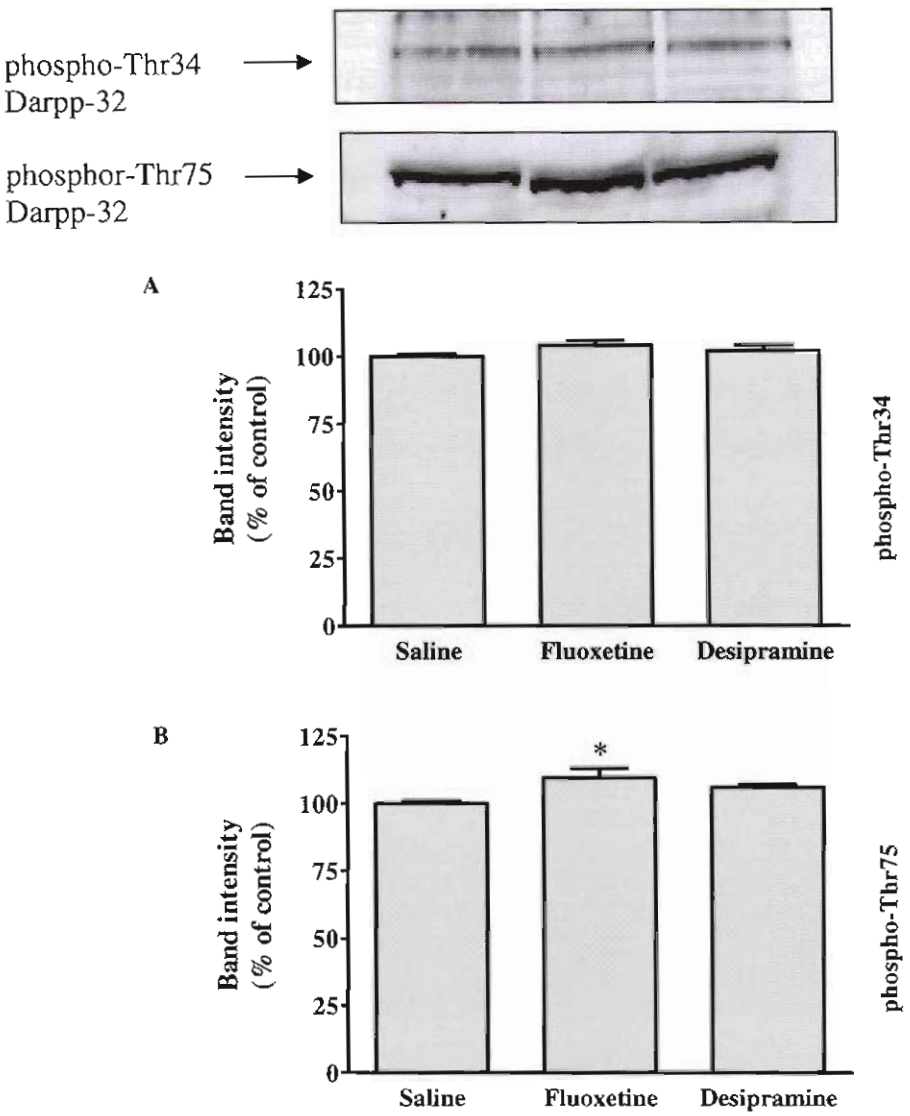

Figure 6.8 Effects of 10 mg/kg fluoxetine and desipramine on Darpp-32 phosphorylation in the prefrontal cortex of LSB mice. Mice were treated for 21 days with fluoxetine (n = 10), desipramine (n = 10) or saline (n = 10). Following sacrifice and tissue collection, samples were analysed for protein expression. Shown is a representative immunoblot of Darpp-32 when phosphorylated on residue Thr34 (A) and on residue Thr75 (B). Each point is the mean ± SEM and * denotes $p < 0.05$ compared to control (saline). Experiments were carried out in duplicate and data was analyzed by one-way ANOVA followed by Tukey's test.

6.3.2 Effect of 10 mg/kg SRI/NRI in the prefrontal cortex of HSB mice

Since a representative blot for each of the PDE4 isoforms has been shown, subsequent protein expression data is shown in table format. Immunoblot analysis indicated that expression of PDE 4A5 (109 kDA) and PDE 4A8 (106 kDa) were not significantly altered in the prefrontal cortex of HSB mice following chronic treatment. The relative band intensities for PDE 4A5 and PDE 4A8 are shown in table 6.16. Immunoblot analysis furthermore showed that PDE 4A1 (66 kDa) expression in HSB mice was not significantly changed following drug treatment (table 6.16).

The immunoblot analysis showed that expression of prefrontal cortex PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) in HSB mice was not significantly altered by chronic SRI/NRI treatment. Chronic desipramine did however significantly decrease PDE 4B4 (66 kDa) expression by 7% in the prefrontal cortex of HSB mice compared to saline control (table 6.16).

Immunoblot analysis showed that expression of PDE 4D4 (105 kDa) was significantly decreased in the prefrontal cortex of HSB mice following treatment with both fluoxetine and desipramine compared to control (table 6.16). Fluoxetine was shown to decrease PDE 4D4 expression by 19%, and desipramine reduced expression by 14%. In addition, PDE 4D1 (68 kDa) expression was significantly decreased in the prefrontal cortex of HSB mice following fluoxetine (45%) and desipramine (47%) treatment (table 6.16).

Phosphorylation of Darpp-32 on Thr34 was significantly reduced only by chronic fluoxetine treatment (7%) in the prefrontal cortex of HSB mice (table 6.16), while no significant change in Darpp-32 phosphorylation were noted on Thr34 or Thr75 following either drug treatment (table 6.16).

Table 6.16 Effect of 10 mg/kg fluoxetine or desipramine treatment in the prefrontal cortex of stereotypic deer mice.

PDE4 isoform & Darpp-32 residue	LSB mice		HSB mice	
	Fluoxetine	**Desipramine**	**Fluoxetine**	**Desipramine**
PDE 4A5 & 4A8	96 ± 4.5[a]	101 ± 1.5	96 ± 7	105 ± 1
PDE 4A1	96 ± 3	105 ± 1.5	100 ± 4	103 ± 0.5
PDE 4B1 & 4B3	92 ± 5	87 ± 6*	98 ± 0.5	102 ± 6
PDE 4B4	87 ± 6*	86 ± 5*	96 ± 3	93 ± 1*
PDE 4D4	73 ± 2**	77 ± 3**	81 ± 7*	86 ± 5*
PDE 4D1	83 ± 3*	87 ± 8	55 ± 3**	53 ± 2**
Thr34-Darpp-32	104 ± 2	102 ± 2	93 ± 1**	97 ± 1
Thr75-Darpp-32	110 ± 3*	106 ± 1	105 ± 0.5	104 ± 1

[a] Band intensitiy is expressed as % of control ± SEM.

* indicates $p < 0.05$ and ** indicates $p < 0.01$

6.3.3 Effect of 20 mg/kg SRI/NRI in prefrontal cortex of LSB mice

The immunoblot analysis showed that expression of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) in the prefrontal cortex of LSB mice was significantly decreased by 20 mg/kg fluoxetine treatment (10%) compared to control saline, but not by desipramine. A summary of the relative band intensities is provided in table 6.17. In contrast, PDE 4A1 (66 kDa) was not significantly changed by either fluoxetine or desipramine treatment (table 6.17).

Immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was not significantly changed in the prefrontal cortex of LSB mice following either fluoxetine or desipramine treatment (table 6.17). In addition, PDE 4B4 (66 kDa) expression was not significantly modified in the prefrontal cortex of LSB mice following either fluoxetine or desipramine treatment (table 6.17).

20 mg/kg Fluoxetine (15%), but not desipramine significantly decreased expression of PDE 4D1 (68 kDa) in the prefrontal cortex of LSB mice (table 6.17). However, chronic treatment with 20 mg/kg of either fluoxetine or desipramine did not alter expression of PDE 4D4 (105 kDa) in LSB mice (table 6.17).

Phosphorylation of Darpp-32 on Thr34 was significantly reduced by chronic desipramine treatment (10%) in the prefrontal cortex of LSB mice compared to saline control (table 6.17), but not by fluoxetine. In contrast, 20 mg/kg fluoxetine (9%) significantly increased phosphorylation of Darpp-32 on Thr75 (table 6.17) whereas desipramine treatment did not have any significant effect on Thr75 phosphorylation.

Table 6.17 Effect of 20 mg/kg fluoxetine and desipramine treatment in the prefrontal cortex of stereotypic deer mice.

PDE4 isoform & Darpp-32 residue	LSB mice		HSB mice	
	Fluoxetine	**Desipramine**	**Fluoxetine**	**Desipramine**
PDE 4A5 & 4A8	90 ± 0.5[*a]	102 ± 5	111 ± 5.5	110 ± 7
PDE 4A1	84 ± 8	91 ± 3	97 ± 1.5	109 ± 1.5[*]
PDE 4B1 & 4B3	87 ± 8	90 ± 6	104 ± 3	102 ± 0.5
PDE 4B4	89 ± 6	90 ± 6	88 ± 3[*]	92 ± 2
PDE 4D4	91 ± 6	93 ± 5	103 ± 3	109 ± 2[*]
PDE 4D1	85 ± 4[**]	95 ± 2.5	114 ± 2[**]	111 ± 2[**]
Thr34-Darpp-32	105 ± 2	90 ± 1.5[**]	95 ± 1	95 ± 2.5
Thr75-Darpp-32	109 ± 1.5[**]	97 ± 2	101 ± 1	96 ± 1

[a] Band intensitiy is expressed as % of control ± SEM.

[*] indicates $p < 0.05$ and [**] indicates $p < 0.01$

6.3.4 Effect of 20 mg/kg SRI/NRI in the prefrontal cortex of HSB mice

Treatment of HSB mice with either 20 mg/kg fluoxetine or desipramine did not have any effect on the expression of prefrontal cortex PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) (table 6.17). Chronic desipramine did however significantly increase expression of PDE 4A1 (66 kDa) in the prefrontal cortex of HSB mice by 9% (table 6.17).

Immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was not significantly changed in the prefrontal cortex of HSB mice following either fluoxetine or desipramine treatment (table 6.17). However, PDE 4B4 (66 kDa) expression was significantly decreased in the prefrontal cortex of HSB mice following fluoxetine treatment (table 6.17). In this instance, fluoxetine decreased expression by 12% compared to controls.

Desipramine, but not fluoxetine significantly increased expression of PDE 4D4 (105 kDa) in the prefrontal cortex of HSB mice by 9% compared to saline control (table 6.17). In addition, both 20 mg/kg fluoxetine and desipramine treatment significantly altered expression of PDE 4D1 (68 kDa) in HSB mice (table 6.45). Fluoxetine (14%) and desipramine (11%) both resulted in an increase in protein expression.

Treatment of HSB mice with 20 mg/kg SRI/NRI did not significantly alter the level of phosphorylation of Darpp-32 on either Thr34 or Thr75 (table 6.17).

6.4 Effect of chronic drug treatment on protein expression and phosphorylation in the striatum of deer mice

6.4.1 Effect of 10 mg/kg SRI/NRI in the striatum of LSB mice

Immunoblot analysis showed that expression of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) was not significantly changed in the striatum of LSB mice following either fluoxetine or desipramine treatment. A summary of the relative band intensities is provided in table 6.18. Similarly, PDE 4A1 (66 kDa) expression was unaltered in the striatum of LSB mice following chronic drug treatment (table 6.18). Although not statistically significant, a trend toward significance (increase in PDE 4A expression) was noted.

Treatment of LSB mice with 10 mg/kg fluoxetine or 10 mg/kg desipramine failed to change striatal expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) (table 6.18). Expression of PDE 4B4 (66 kDa) was also unaltered in the striatum of LSB mice following either drug treatment. It should be noted that a trend toward significance (decrease in PDE 4B4 expression) was observed after chronic fluoxetine treatment (table 6.18)

Strialtal PDE 4D4 (105 kDa) expression was significantly reduced following fluoxetine treatment (8%; table 6.18), but not by desipramine. In contrast, expression of PDE 4D1 (68 kDa) was unaltered in the striatum of LSB mice following either treatment (table 6.18).

Phosphorylation of Daprr-32 on Thr34 in LSB mice was significantly increased following both fluoxetine and desipramine treatment (table 6.18). Fluoxetine (8%) and desipramine (22%) both resulted in an increase in Darpp-32 phosphorylation on Thr34. On the contrary, neither drug treatment altered Darpp-32 phosphorylation on Thr75 (table 6.18).

Table 6.18 Effect of 10 mg/kg fluoxetine and desipramine treatment in the striatum of stereotypic deer mice.

PDE4 isoform & Darpp-32 residue	LSB mice		HSB mice	
	Fluoxetine	**Desipramine**	**Fluoxetine**	**Desipramine**
PDE 4A5 & 4A8	106 ± 0.5[a]	106 ± 0.5	107 ± 6	106 ± 1
PDE 4A1	101 ± 1.5	106 ± 4	92 ± 7	79 ± 1.5**
PDE 4B1 & 4B3	99 ± 5	100 ± 7	103 ± 2	90 ± 5
PDE 4B4	92 ± 6	99 ± 2.5	105 ± 2	89 ± 7
PDE 4D4	92 ± 1*	105 ± 3	97 ± 6	100 ± 5
PDE 4D1	100 ± 6	102 ± 4	92 ± 6	89 ± 5.5
Thr34-Darpp-32	108 ± 2*	122 ± 3**	85 ± 1.5**	80 ± 2**
Thr75-Darpp-32	100 ± 1	95 ± 0.5	104 ± 2	108 ± 2*

[a] Band intensitiy is expressed as % of control ± SEM.

* indicates $p < 0.05$ and ** indicates $p < 0.01$

6.4.2 Effect of 10 mg/kg SRI/NRI in the striatum of HSB mice

Treatment of HSB mice with either 10 mg/kg fluoxetine or 10 mg/kg desipramine did not change striatal expression of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) (table 6.18). Although not statistically significant, a trend toward significance (increase in PDE 4A5 and 4A8 expression) was noted following drug treatment. Interestingly, treatment of HSB mice with 10 mg/kg desipramine, but not with fluoxetine, resulted in a significant decrease (21%) in PDE 4A1 (66 kDa) expression (table 6.18).

Immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was unaltered in the striatum of HSB mice following either drug treatment (table 6.18). PDE 4B4 (66 kDa) expression was also unaltered in the striatum of HSB mice following either chronic drug treatment (table 6.18). Although not statistically significant, desipramine tended to decrease PDE 4B isoform expression.

Strialtal PDE 4D4 (105 kDa) expression was unaltered following either fluoxetine or desipramine treatment (table 6.18). In addition, treatment of HSB mice did not modify PDE 4D1 (68 kDa) expression (table 6.18), although desipramine tended to decrease PDE 4D1 expression by 10%.

Phosphorylation of Darpp-32 on Thr34 in the striatum of HSB mice was significantly altered following both fluoxetine and desipramine treatment (table 6.18). Fluoxetine (15%) and desipramine (20%) both resulted in a decrease in Darpp-32 phosphorylation on Thr34. Furthermore, desipramine, but not fluoxetine, significantly increased phosphorylation of Darpp-32 on Thr75 by 8% (table 6.18).

6.4.3 Effect of 20 mg/kg SRI/NRI in the striatum of LSB mice

The immunoblot analysis showed that expression of PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) in the striatum of LSB mice were significantly increased by both 20 mg/kg fluoxetine and desipramine treatment compared to control saline. A summary of the relative band intensities is provided in table 6.19. Fluoxetine increased PDE 4A5 and 4A8 by 24%, whereas desipramine increased expression by 32%. In addition, PDE 4A1 (66 kDa) was significantly increased by both fluoxetine (20%) and desipramine (26%) treatment.

Striatal PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) expression was unaltered in LSB mice following both drug treatments (table 6.19). In contrast, treatment of LSB mice with fluoxetine (28%) or desipramine (12%) both significantly decreased PDE 4B4 (66 kDa) expression (table 6.19).

Strialtal PDE 4D4 (105 kDa) expression was unchanged following either fluoxetine or desipramine treatment (table 6.19). In addition, neither drug treatment to LSB mice modified PDE 4D1 (68 kDa) expression. It was noted however, that desipramine caused a non-significant reduction of 10% in PDE 4D1 expression (table 6.19).

Treatment of LSB mice with either 20 mg/kg fluoxetine or desipramine did not significantly alter the level of phosphorylation of Darpp-32 on either Thr34 or Thr75 in the striatum of deer mice (table 6.19). It was noted however that both drug treatments tended to increase phosphorylation of Darpp-32 on both Thr residues.

Table 6.19 Effect of 20 mg/kg fluoxetine and desipramine treatment in the striatum of stereotypic deer mice.

PDE4 isoform & Darpp-32 residue	LSB mice		HSB mice	
	Fluoxetine	**Desipramine**	**Fluoxetine**	**Desipramine**
PDE 4A5 & 4A8	$124 \pm 5^{*a}$	$132 \pm 7^{**}$	$107 \pm 1^{*}$	$109 \pm 3^{*}$
PDE 4A1	$120 \pm 6.5^{*}$	$126 \pm 4^{**}$	97 ± 1	98 ± 0.5
PDE 4B1 & 4B3	105 ± 6	100 ± 8	105 ± 2.5	103 ± 7
PDE 4B4	$72 \pm 7^{**}$	$88 \pm 3^{**}$	102 ± 3	103 ± 1
PDE 4D4	105 ± 3	95 ± 8	104 ± 2	97 ± 6
PDE 4D1	96 ± 2	90 ± 9	$77 \pm 5^{**}$	$49 \pm 4^{**}$
Thr34-Darpp-32	104 ± 4	110 ± 7	98 ± 0.5	97 ± 1
Thr75-Darpp-32	106 ± 5	105 ± 3.5	103 ± 1.5	100 ± 2

[a] Band intensitiy is expressed as % of control ± SEM.

* indicates $p < 0.05$ and ** indicates $p < 0.01$

6.4.4 Effect of 20 mg/kg SRI/NRI in the striatum of HSB mice

Treatment of HSB mice with both fluoxetine (7%) or desipramine (9%) resulted in a significant increase in striatal PDE 4A5 (109 kDa) and PDE 4A8 (106 kDa) expression (table 6.19). PDE 4A1 (66 kDa) expression was unaltered in the striatum of HSB mice following either drug treatment (table 6.19).

Immunoblot analysis showed that expression of PDE 4B1 (107 kDa) and PDE 4B3 (100 kDa) was unaltered in the striatum of HSB mice following either fluoxetine or desipramine treatment (table 6.19). PDE 4B4 (66 kDa) expression was also unaltered in the striatum of HSB mice following either drug treatment (table 6.19).

Striatal PDE 4D4 (105 kDa) expression was unchanged following either fluoxetine or desipramine treatment (table 6.19). However, both fluoxetine (23%) and desipramine (51%) treatment of HSB modified striatal PDE 4D1 (68 kDa) expression.

Treatment of HSB mice with 20 mg/kg fluoxetine or desipramine did not significantly alter the striatal level of phosphorylation of Darpp-32 on either Thr34 or Thr75 amino acid residue (table 6.19).

6.5 Summary of results: PDE4 protein expression and Darpp-32 phosphorylation

6.5.1 Basal protein expression

Results in this chapter focus exclusively on PDE4 protein expression and phosphorylation levels of Darpp-32 in both chronically treated deer mice (LSB and HSB) as well as untreated C57Bl and deer mice. PDE4 is the predominant mediator of hydrolysis of cAMP formed by stimulation of monoaminergic receptors that are involved in the mediation of the effects of antidepressant drugs (Ye & O'Donnell, 2000). Elevating intracellular cAMP, via inhibition of PDE4, produces anxiogenic effects in animal models (Griebel et al., 1991; O'Donnell, 1993; Zhang et al., 2002). Recent neuroanatomical analysis shows that PDE4 is highly expressed in brain regions of mouse (Cherry & Davis, 1999) and rat (Iwahashi et al., 1996) associated with reward and affect. However, differing importance of PDE4 subtypes in the mediation of antidepressant effects has been suggested in mice (O'Donnell and Zhang, 2004) and rats (Takahashi et al., 1999).

In this study, stereotypic and non-stereotypic deer mice were compared to a strain that has been confirmed in this study not to develop stereotypic behaviour under similar laboratory conditions, namely C57Bl mice. A summary of PDE4 protein expression and Darpp-32 phosphorylation in untreated prefrontal cortex (table 6.20) and striatum (table 6.21) is provided. Expression of the PDE 4A5 and PDE 4A8 isoforms were significantly lower in untreated LSB and HSB deer mice than C57Bl mice in both the prefrontal cortex (section 6.2.1) as well as striatum (section 6.2.2). In addition, PDE 4A1 expression was significantly lower in HSB mice compared to C57Bl mice (section 6.2).

Expression of the PDE 4B1 and PDE 4B3 isoforms were significantly elevated in the prefrontal cortex of untreated LSB and HSB deer mice compared to C57Bl (section 6.2). The elevated levels of PDE 4B1 and B3 were mirrored in the striatum, although values did not research statistical significance. Examination of PDE 4B4 expression revealed a significantly lower presence of this particular PDE isoform in LSB and HSB deer mice compared to C57Bl in both the prefrontal cortex and striatum.

Untreated LSB and HSB deer mice expressed a significantly higher amount PDE 4D4 than C57Bl mice in both the prefrontal cortex and striatum (section 6.2.1 and 6.2.2). In addition, deer mice (LSB and HSB) expressed less of PDE 4D1 in both the prefrontal

cortex and striatum compared to C57Bl mice. Darpp-32 phosphorylation on Thr34 was not detected in the prefrontal cortex of either LSB or HSB deer mice or C57Bl but a significant increase in Thr34 phosphorylation was noted in the striatum of untreated HSB mice compared to C57Bl mice. Untreated LSB and HSB deer mice furthermore both expressed significantly more of Thr75 phosphorylated Darpp-32 than C57Bl mice in both the prefrontal cortex and striatum.

Table 6.20 Level of PDE4 expression and Darpp-32 phosphorylation in the prefrontal cortex of untreated deer mice compared to C57Bl mice. ↑ indicate significant increase, ↓ indicate significant decrease, and -- indicate no change in PDE4 protein expression or Darpp-32 phosphorylation levels.

PDE4 isoform & Darpp-32 residue	Protein expression of deer mice compared to C57Bl		
	NS	LSB	HSB
PDE 4A5 & 4A8	↓	↓	↓
PDE 4A1	--	--	↓
PDE 4B1 & 4B3	↑	↑	--
PDE 4B4	↓	↓	↓
PDE 4D4	↑	↑	↑
PDE 4D1	↓	↓	↓
phosphor-Thr34 Darpp-32	not detected	not detected	not detected
phosphor-Thr75 Darpp-32	--	↑	↑

Table 6.21 Level of PDE4 expression and Darpp-32 phosphorylation in the striatum of untreated deer mice compared to C57Bl mice. ↑ indicate significant increase, ↓ indicate significant decrease and -- indicate no change in PDE4 protein expression or Darpp-32 phosphorylation levels.

PDE4 isoform & Darpp-32 residue	Protein expression of deer mice compared to C57Bl		
	NS	LSB	HSB
PDE 4A5 & 4A8	↓	↓	↓
PDE 4A1	--	--	↓
PDE 4B1 & 4B3	--	--	--
PDE 4B4	↓	↓	↓
PDE 4D4	↑	↑	↑
PDE 4D1	--	↓	↓
phosphor-Thr34 Darpp-32	--	--	↑
phosphor-Thr75 Darpp-32	--	↑	↑

6.5.2 The effect of SRI/NRI treatment on PDE4 expression and Darpp-32 phosphorylation in LSB mice

This section of the study was performed to investigate the effects of chronic fluoxetine or desipramine treatment on deer mice associated PDE4 expression and Darpp-32 phosphorylation. A summary of the effects of drug treatment on PDE4 protein expression and Darpp-32 phosphorylation in the prefrontal cortex and striatum of LSB and HSB mice is provided in tables 6.22 and 6.23, respectively.

Treatment of LSB mice with 10 mg/kg fluoxetine significantly decreased striatal PDE 4D4 (section 6.4.1), whereas 20 mg/kg fluoxetine did not have any effect on this particular isoform. The high dose fluoxetine did however significantly decrease striatal PDE 4B4 and surprisingly increased all PDE 4A isoforms in this brain region (section 6.4.3). Fluoxetine (10 mg/kg) significantly decreased PDE 4B4, 4D4, and 4D1 in the prefrontal cortex of LSB mice (section 6.3.1). A high dose fluoxetine (20 mg/kg) on the other hand resulted in decreased expression of PDE 4A5, 4A8 and 4D1 isoforms in the prefrontal contex of LSB mice (section 6.3.3). In a recent study performed on male ICR mice repeatedly treated with a very low dose (5 mg/kg) fluoxetine, no change in cerebral cortex PDE 4B4 and a significant increase in PDE 4A5 was noted (Dlaboga et al., 2006). These discrepancies could be explained by the diffent anatomical areas investigated, the higher dose used in the present study, and the unrelated mouse strains used.

Chronic treatment of LSB mice with 10 mg/kg desipramine did not have any significant effect on striatal PDE4 expression (section 6.4.1). Desipramine (20 mg/kg) did however, significantly increase expression of all striatal PDE 4A isoforms (section 6.4.3) and simultaneously decreased PDE 4B4. Desipramine (10 mg/kg) treatment also resulted in an increase in striatal Darpp-32 phosphorylation on Thr34 in LSB mice (section 6.4.1). Fluoxetine (10 and 20 mg/kg) furthermore significantly increased Darpp-32 phosphorylation on Thr75 in the prefrontal cortex of LSB mice. Interestingly, 10 mg/kg fluoxetine significantly increased phosphorylation of Darpp-32 on Thr34 in the striatum of LSB mice. In a recent study however, Svenningson et al (2002b) found that in C57Bl mice Darpp-32 phosphorylation was not affected by chronic fluoxetine treatment.

Table 6.22 Effect of drug treatment on PDE4 expression and Darpp-32 phosphorylation in LSB mice. ⇑/⇓ indicate significant increase/decrease in expression or phosphorylation; ↑/↓ indicate non-significant trend noted; -- indicate no change. Strm = striatum; PFC = prefrontal cortex.

PDE4 isoforms & Darpp-32 residues	LSB mice							
	10 mg/kg fluoxetine		10 mg/kg desipramine		20 mg/kg fluoxetine		20 mg/kg desipramine	
	Strm	PFC	Strm	PFC	Strm	PFC	Strm	PFC
PDE 4A5 & 4A8	↑	↓	↑	--	⇑	⇓	⇑	--
PDE 4A1	--	↓	--	--	⇑	↓	⇑	↓
PDE 4B1 & 4B3	--	↓	--	⇓	--	↓	--	↓
PDE 4B4	↓	⇓	--	⇓	⇓	↓	⇓	↓
PDE 4D4	⇓	⇓	--	⇓	--	↓	--	↓
PDE 4D1	--	⇓	--	↓	↓	⇓	↓	↓
phospho-Thr34 Darpp-32	⇑	--	⇑	--	--	--	--	⇓
phospho-Thr75 Darpp-32	--	⇑	--	↑	--	⇑	--	--

Table 6.23 Effect of drug treatment on PDE4 expression and Darpp-32 phosphorylation in HSB mice. ⇑/⇓ indicate significant increase/decrease in expression or phosphorylation; ↑/↓ indicate non-significant trend noted; -- indicate no change. Strm = striatum; PFC = prefrontal cortex.

PDE4 isoforms & Darpp-32 residues	HSB mice							
	10 mg/kg fluoxetine		10 mg/kg desipramine		20 mg/kg fluoxetine		20 mg/kg desipramine	
	Strtm	PFC	Strm	PFC	Strm	PFC	Strm	PFC
PDE 4A5 & 8	--	--	--	--	↑	↑	↑	↑
PDE 4A1	↓	--	⇓	--	--	--	--	⇑
PDE 4B1 & 3	--	--	↓	--	--	--	--	--
PDE 4B4	--	--	↓	↓	--	⇓	--	↓
PDE 4D4	--	⇓	--	⇓	--	--	--	⇑
PDE 4D1	↓	⇓	↓	⇓	⇓	⇑	⇓	⇑
phospho-Thr34 Darpp-32	⇓	↓	⇓	--	--	--	--	--
phospho-Thr75 Darpp-32	↑	↑	⇑	↑	--	--	--	--

Desipramine (10mg/kg) significantly decreased expression of PDE 4B1, 4B3, 4B4, and 4D4 in the prefrontal cortex of LSB mice (section 6.3.1). In contrast, only PDE 4D1 expression was significantly decreased by 20 mg/kg desipramine in the prefrontal cortex of LSB mice (6.3.3). In the recent study by Dlaboga and coworkers (2006), treatment with 10 mg/kg desipramine resulted in an increase in cerebral cortex PDE 4D3 and 4A5 expression. It should also be noted that the mice used in said study (Dlaboga et al., 2006) do not engage in stereotypic behaviour under standard laboratory conditions.

6.5.3 The effect of SRI/NRI treatment on PDE4 expression and Darpp-32 phosphorylation in HSB mice

Treatment of HSB mice with 10 mg/kg fluoxetine did not have any significant effect on striatal PDE4 expression (section 6.4.2). Fluoxetine (20 mg/kg) however, did significantly decrease striatal PDE 4D1 expression in HSB mice. Low dose fluoxetine (10 mg/kg) significantly decreased PDE 4D4 and 4D1 expression in HSB prefrontal cortex (section 6.3.2). In contrast, treatment of HSB mice with 20 mg/kg fluoxetine resulted in an increase in prefrontal cortex PDE 4D1 (section 6.3.4). The high dose of fluoxetine furthermore resulted in a decrease in PDE 4B4 expresion in the prefrontal cortex of HSB mice.Treatment of HSB mice with 10 mg/kg desipramine significantly reduced striatal PDE 4A2 expression (section 6.4.2), whereas 20 mg/kg desipramine significantly reduced striatal PDE 4D1 (section 6.4.4). Chronic treatment of HSB mice with a low dose desipramine significantly reduced expression of all the PDE 4D isoforms in the prefrontal cortex. 20 mg/kg Desipramine conversely had the opposite effect, with the drug increasing expression of all the PDE 4D isoforms (section 6.3.4). The high dose desipramine furthermore resulted in an increase in prefrontal cortex PDE 4A1.

Chronic treatment of HSB mice with a high dose of either fluoxetine or desipramine did not have any effect on Darpp-32 phosphorylation (section 6.3.4 and 6.4.4). Interestingly, 10 mg/kg of either fluoxetine or desipramine resulted in a significant decrease in striatal Darpp-32 phosphorylation on Thr34. Treatment of HSB mice with 10 mg/kg desipramine furthermore resulted in considerable increase in Darpp-32 on residue Thr75. A comprehensive discussion of the effects of antidepressant treatment on PDE4 expression and Darpp-32 phosphorylation in deer mice is provided in chapter 7.

7. General discussion

7.1 Introduction

The aim of the initial part of the study was to develop a behavioural animal model of OCD. Patients afflicted with OCD experience intrusive, disturbing, repetitive thoughts (obsessions) and the uncontrollable urge to repeatedly enact stereotypic behaviours or rituals (compulsions), thereby reducing the psychic anxiety produced by the obsessional process (American Psychiatric association, 2000). More specifically, this study aimed to validate spontaneous naturalistic stereotypic behaviour in deer mice as an animal model of stereotypic behaviour seen in OCD. The model should therefore demonstrate sensitivity towards pharmacological treatment known to be effective in OCD patients, as well as insensitivity towards other classes of drugs that have been shown to be ineffective in OCD but effective in other conditions such as depression.

In the context of neurobiological research, in which the aim of an animal model is to promote our understanding of the modelled condition by elucidating its neurobiological mechanisms, it is widely agreed that a common physiological basis of the model and the modelled condition in humans contributes greatly to the model's validity. A critical component is the demonstration of a similar response to treatment, because the latter suggests similarity in the neurotransmitter systems involved. This makes pharmacological isomorphism an important factor in assessing the validity of an animal model, and indeed, the validation process of most animal models of psychopathology involves testing the effects of relevant pharmacological treatment.

This chapter will first set about highlighting the distinctive behavioural differences between the deer mice (*Peromyscus maniculatus bairdii*) and a species that does not develop spontaneous stereotypic behaviour under laboratory conditions namely C57Bl mice, and in so doing bring to the fore the potential value of the deer mice as an animal model of OCD. Disparity in stereotypic behaviour and the effect of SRI versus NRI treatment on these behavioural parameters will be discussed to emphasize face and predictive validity of the model. Further, serotonergic and dopaminergic challengers and the determination of cortical (prefrontal cortex) and striatal cAMP, PDE4 activity as well as PDE4 expression, will attempt to establish construct validity of the model.

7.2 Disparity between untreated deer mice and untreated C57Bl mice

7.2.1 Behavioural differences

A cornerstone of the study was to separate two strains of mice, C57Bl and deer mice, according to the degree of spontaneous, naturalistic stereotypy, and thereafter to demonstrate that stereotypic animals can further be separated according to degree of stereotypy, thereby broadening its face validity for OCD. According to earlier studies (Powell et al., 1999), three distinct groups of deer mice can be distinguished, namely HSB, LSB and non-stereotypic (NS) mice (section 4.2.1). Using the aforementioned criteria, C57Bl mice were found to have markedly lower stereotypy scores compared to LSB and HSB mice, indicating that stereotypy occurs spontaneously in *Peromyscus maniculatus bairdii* and is highly characteristic and easily measurable.

Deer mice, particularly HSB mice, are thus compelled to engage in stereotypic behaviour. Building on the summary provided in section 4.3.1, this study has established that stereotypy rate varies between individual animals, a phenomenon that lends face validity to the model in that patient's suffering from OCD experience different degrees of symptom severity and perform compulsions and stereotypic behaviours at different rates (American Psychiatric Association, 2000). Face validity is however subjective, and therefore can only be considered as a secondary criterion.

7.2.2 cAMP levels

Three groups were distinguished among deer mice, namely LSB, HSB and NS mice. cAMP levels in both the prefrontal cortex (section 5.2.1) and the striatum (5.2.2) were significantly increased in LSB and HSB mice compared to NS mice under basal conditions. Furthermore, NS mice presented with significantly higher striatal cAMP levels compared to C57Bl mice under basal conditions (section 5.2.3). This finding indicates that *Peromyscus maniculatus bairdii* can be distinguished from a non-stereotyping mouse strain at the molecular level. This has potential relevance for construct validity of the model since OCD has been associated with disturbances in the cAMP-PKA cascade (Perez et al., 2000), while SRI's are known to modulate this signalling system via action on 5-HT_{1A} (Barnes & Sharp, 1999; Bergqvist et al., 1999) and postsynaptic 5-HT_2 receptors (El Mansari & Blier, 2006).

Results presented (section 5.2) indicate that the degree of stereotypy could be linked to increased prefrontal cortex and striatal cAMP present under basal conditions. In this regard prefrontal cortex and striatal basal levels of cAMP in HSB mice are higher than that in NS and C57Bl mice. This is an important observation since cAMP stimulates PKA (section 2.9.7) and the latter has been shown to activate 5-HT reuptake (Blakely et al., 1998). Recent data have also demonstrated that the 5-HT transporter presents with at least three phosphorylation sites for the primary target of cAMP, namely PKA (Blakely et al., 1998).

An increase in cAMP, and therefore PKA, would result in increased 5-HT reuptake, and reduced synaptic levels of 5-HT. This could be of value since the main target of SRIs, particularly in OCD, is the inhibition of 5-HT transporter (Lesch et al., 1996; Mundo et al., 2002), where they act to increase available 5-HT in the synapse. Furthermore, it is plausible that the increase in cAMP in deer mice is a compensatory response to an as yet unknown dysfunction in the cAMP system. Nonetheless, this sequence of events provides substantial construct validity for the model.

7.2.3 PDE4 enzyme activity

The principle biological mediator of lowered levels of cAMP, as described above, is PDE, and in the CNS the PDE4 family (Beavo, 1995) primarily orchestrates this. This study demonstrates that PDE4 activity is significantly lower in the prefrontal cortex and striatum of stereotypic deer mice compared to NS mice (section 5.2.1 and 5.2.2) under basal conditions, which explains the increase in cAMP in LSB and HSB mice in these brain regions described earlier. Low stereotypy in NS deer mice may be linked to raised PDE4 activity, which was found to be significantly increased compared to that in C57Bl mice (section 5.2.3).

PDE4 activity data was found to closely compliment cAMP data of untreated deer mice, in that increased enzyme activity accompanied decreased cAMP levels. That HSB mice have lower basal PDE4 activity compared to LSB and NS mice indicates an inverse relationship between degree of stereotypy and PDE4 enzyme activity in deer mice. Changes in PDE4 activity may however be in response to cAMP levels, which in turn may be in response to up-stream dysfunction, e.g. receptor dysfunction. In order to

confirm a decrease in PDE4 activity, western blotting studies for PDE expression were undertaken on prefrontal cortex and striatal tissue extracts of the various groups of mice. This would also assist in shedding more light on the particular PDE isoform (s) involved in this response.

7.2.4 PDE4 protein expression

Expression of PDE4 isoforms do not always correspond to PDE4 activity such that an increase in enzyme activity is not necessarily accompanied by an increase in expression of PDE4 proteins (Houslay, 1998; Houslay & Adams, 2003). Nonetheless, results show significantly lower expression of prefrontal cortex PDE 4A5, 4A8, 4B4, and 4D1 in deer mice compared to C57Bl mice under basal conditions (section 6.2.1 and table 6.20). In contrast, PDE 4B1, 4B3, and 4D4 expression is significantly higher in deer mice than C57Bl mice. Since cAMP levels were consistently raised in HSB and in order of decreasing magnitude in LSB, NS, and C57Bl mice, this indicates that some isoforms, in particular PDE 4A5, 4A8, 4B4, and 4D1 have greater cAMP hydrolyzing activities than others, such as PDE 4B1, 4B3, and 4D4, and which represent the dominantly active isoforms in the prefrontal cortex and striatum of deer mice.

Given the increased basal expression of several PDE4 isoforms (PDE 4B1, 4B3 and 4D4) in deer mice, coupled with increased cAMP levels in the prefrontal cortex of stereotypic deer mice compared to C57Bl mice, it is plausible that these PDE4s are 'slow' hydrolyzers or may have a lower affinity for cAMP and therefore will hydrolyze cAMP at a reduced rate. As discussed in section 2.9.8.2, PDE4 isoforms can be divided into three major categories: long, short, and super-short (Houslay, 2001). Recent functional studies (MacKenzie et al. 2000) have established that upstream conserved regions 1 and 2 (UCR1 and UCR2, section 2.9.8.2.1) regulate PDE4 catalytic activity. PDE 4B1, 4B3, and 4D4 isoforms are so-called long-form PDE4s (O'Donnell & Zhang, 2004). The presence of UCR1/2 regulatory regions in these long-form enzymes indicates that catalytic activity, and hence affinity for cAMP, is differentially regulated compared to some of the short- (PDE 4D1) or super-short (PDE 4A1) forms.

A similar pattern of protein expression is seen within striatal PDE4 with isoforms either being expressed in lower (PDE 4A5, 4A8, 4B4) or higher (PDE 4D4) quantities, however,

a few exceptions in striatal protein expression patterns were noted. For example, basal PDE 4D1 expression is selectively reduced in LSB and HSB mice compared to C57Bl mice. Furthermore, PDE 4B1 and 4B3 was unaltered in the striatum of both deer mice and C57Bl mice. This highlights the complexity of cAMP-PDE4 interaction and also emphasizes the difference between PDE4 isoforms from different mouse strains.

Furthermore, results presented thus far indicate that the prefrontal cortex and striatum are important neuroanatomical regions in understanding how these brain regions function in stereotypic deer mice, and such knowledge would assist in shedding more light on the stereotypic aspect of OCD. In fact, the orbitofrontal cortex (OFC) has been demonstrated to be overactive in OCD (Alptekin et al., 2001; Lacerda et al., 2003), and is a region mediating the active expression of emotional response to significant biological stimuli, as well as the inhibition of behavioural response (Rolls, 1999). The OFC seems to play a role in motivational aspects of decision-making. Another large region, the dorsolateral prefrontal cortex (DLPC), plays a key role in the adaptation to changes in environment and in the control of behavioural responses (Dubois et al., 1994). Decreased activity in the DLPC have been shown in OCD patients (Baxter, 1999) which may explain the difficulty to 'stop' compulsive behaviours such as reward-seeking and security motivation.

Subcortical regions are also the sites of frequently reported dysfunctions in OCD where the striatum is seen as a primary site of pathology in OCD (Rauch et al., 1998), The striatum is the main input structure of the basal ganglia and is a key component of the motor system (Kelly, 1999). While the dorsal striatum appears more allied with voluntary motor functions and is involved in the initiation, production, and sequencing of motor behaviour, the nucleus accumbens is more likely an interface between the limbic and the motor system and plays a major role in motivated and goal-directed behaviours as well as the development and expression of addiction (Kelly, 1999). As a result, data implicating the prefrontal cortex and the striatum in stereotypic deer mice adds important construct validity to the model.

In conclusion, these initial behavioural, neurochemical and molecular characterizations of deer mice versus C57Bl mice indicate that raised stereotypic behaviour is characteristic of the deer mouse, and that raised stereotypic behaviour in these animals is associated with reduced expression of select isoforms of the PDE4 family, namely 4A1, 4B4, and 4D1,

which drive a reduced cellular release of cAMP. Furthermore, this was observed in the prefrontal cortex and striatum, two brain regions with well-established association with the neuroanatomical basis of OCD. This model therefore provides excellent face and construct validity for OCD.

Having now established the face and construct validity of the deer mouse model, the next step was to study its predictive validity using the SRI, fluoxetine and the NRI, desipramine. Thereafter, the aim was to characterize the construct validity of the model using selective pharmacological challenges, coupled with western blotting, aimed at establishing the serotonergic and dopaminergic attributes of the model.

7.3 Pharmacological treatment and challenge of stereotypic deer mice

The objective of the present study was to validate spontaneous stereotypic behaviour in the deer mouse as an animal model of OCD. This was performed at three levels, namely face, predictive and construct validity. Pharmacological isomorphism (predictive validity) was evaluated through the differential response of the model to a drug of known efficacy in OCD, namely fluoxetine, and to a drug known to be ineffective in OCD, namely desipramine. Neurochemical parameters investigated following chronic drug treatment include analysis of stereotypic behaviour, cAMP levels, PDE4 enzyme activity, as well as PDE4 expression.

7.3.1 Chronic treatment of deer mice with fluoxetine

The understanding and treatment of OCD may greatly benefit from the availability of an appropriate animal model that closely mimics the behavioural and neurobiological manifestations of the disorder. Recent studies have repeatedly demonstrated that OCD responds selectively to drugs that inhibit the reuptake of 5-HT, while drugs lacking these attributes, such as the standard tricyclic antidepressants desipramine, nortryptiline and amitryptiline, have been shown to be ineffective in randomised controlled studies (Denys, 2006).

This study has demonstrated that spontaneous stereotypic behaviour in deer mice respond in a similar fashion to pharmacological treatment as do specific, repetitive symptoms in

OCD patients (section 3.6.2 – 3.6.5 and summarized in section 4.3.2). Thus in keeping with this, chronic desipramine failed to attenuate stereotypic behaviour in any group of deer mice, while chronic fluoxetine treatment significantly decreased stereotypy in both LSB and HSB mice. In a study performed on stereotyping bank voles (Schoenecker and Heller 2003), chronic treatment with the SRI, citalopram, similarly resulted in a decrease in spontaneous stereotypic behaviour, thus confirming hypolocomotoric effects following administration of a serotonergic agent to high stereotyping animals. The model therefore demonstrates noteworthy predictive validity for OCD.

Distinct changes in molecular parameters linked to antidepressant treatment, including cAMP, may provide new insights into further understanding the pathophysiology of the stereotypic aspect of OCD. This study has chosen to evaluate the cAMP cascade in the deer mouse model, namely cAMP accumulation and expression of PDE4 isoforms in key brain regions of importance to OCD. However, before an in depth discussion on the role of cAMP and PDE4 activity data commences, a few important points should be noted. First, it has been argued that OCD behaviours are performed in a relatively invariant way that is repeated, but that they lack the rhythmic repetitive character of stereotypies (Man et al., 2004). Secondly, at a cognitive level, patients with OCD are able resist the compulsions to perform repetitive acts, while it is suggested that stereotypy's are often performed unconsciously or without resistance (Odberg & Meers, 1987).

The present study therefore cannot assume that the stereotypy engaged in by deer mice are the absolute equivalent to the stereotypy seen in OCD. In addition, the behaviors associated with OCD have been linked to a wide variety of cognitive (Blashfield & Livesley, 1999; Ridley, 1994) and occasionally non-cognitive deficits (Tallis, 1997) including a fundamental impairment of executive function ability, deficits of immediate and secondary memory, and spatial information processing. The disorder is therefore extremely complex, with the presence of diverse neurological manifestations, including neuropsychological symptoms such as cognitive and non-cognitive deficits, neuromotor symptoms such as stereotypy, as well as presenting with prominent symptoms of anxiety.

Neuroimaging studies have also demonstrated the importance of the frontal lobe circuits important in governing cognitive function in OCD (Cosyns & Odberg, 2000). However, although stereotypy per se may not be the absolute equivalent to the stereotypy seen as

part of the complex behavioural phenomenology of OCD, understanding the basis of stereotypy may reveal much about the neurobiology of OCD. Undoubtedly, before employing a putative animal model of stereotypy for application in OCD research, it is essential that the model be carefully validated. The present study has included neurochemical and molecular data from the prefrontal cortex as well as the striatum, which are important constructs in the neurobiology of OCD, in order to support the above-described predictive and face validity of the model for OCD.

Studies have demonstrated adaptations at several levels of the cAMP signal transduction cascade in rodents in response to antidepressant treatment (Nibuya et al., 1996; Ozawa et al., 1991), while the cAMP-CREB cascade is recognised as an important component mediating antidepressant response (Duman, 1998). Preclinical studies have demonstrated that PDE inhibitors incorporate antidepressant-like effects in behavioural models (Griebel et al., 1997; O'Donnel, 1993) and that in clinical trials, rolipram, a specific inhibitor of PDE4, has demonstrated antidepressant efficacy (Fleischhacker et al., 1992).

The prefrontal cortex represents an important anatomical focal point for the neuropathology of OCD, and it is well established that frontal cortical regions is overactive in OCD (Alptekin et al., 2001; Lacerda et al., 2003). In the present study, chronic treatment of deer mice with 10 and 20 mg/kg fluoxetine resulted in a significant decrease in prefrontal cortex cAMP levels in HSB mice (section 5.3 and 5.4). Since levels of this cyclic nucleotide were markedly raised compared to C57Bl mice (section 5.2), that high dose fluoxetine decreased cAMP is a particularly valuable observation concerning construct validity of the model.

5-HT inhibits adenylyl cyclase activity through prefrontal 5-HT_{1A} and 5-HT_{1B} receptors, and both receptors are known to negatively regulate cAMP formation (Raymond et al., 2001). In the striatum however, only a high dose (20 mg/kg) fluoxetine significantly increased cAMP levels. This is in contrast to the effect seen in the prefrontal cortex and may have its origins in the gradual down-regulation of presynaptic 5-HT_{1A} receptors (El Mansari & Blier, 2005), thereby promoting a gradual activation of postsynaptic 5-HT_{1A} receptors mediating activation of adenylyl cyclase and an increase in intracellular cAMP. Of note is that 5-HT_{1A} (Seibel et al., 2003) is thought to be involved in spontaneous

alternation behaviour, which may point towards a significant role for this receptor in the generation of repetitive behavioural pattern in both deer mice and OCD patients.

Results from the present study suggest that regulation of the cAMP system may represent a compensatory response to SRI treatment, and that response with respect to cAMP shows marked differences in the striatum and prefrontal cortex. Brain imaging studies have demonstrated abnormalities in OFC (Lacerda et al., 2003), DLPC (Baxter, 1999), and in the striatum of OCD patients. The DLPC plays a key role in the adaptation to changes in environment and in the control of behavioural responses. Functional neuroimaging have shown decreased activity in the DLPC in patients with OCD (Baxter, 1999) which may explain the difficulty to 'stop' compulsive behaviours such as reward seeking and security motivation. This hyperfrontality may be a direct result of increased activation of the adenyl cyclase – cAMP system in the prefrontal cortex that was attenuated by fluoxetine. Therefore, in the deer mice model the observed decrease in stereotypic behaviour following fluoxetine treatment may most likely be explained by a decrease in cAMP levels. The described increase in cAMP in deer mice may be causally linked to decreased expression of selected PDE4 isoforms and a reduced PDE enzymatic activity, which may represent a target for SRI's. This possibility will now be discussed.

This study has earlier established that deer mice are characterised by decreased PDE4 activity in both the striatum and prefrontal cortex (section 5.2), which explains the raised levels of cAMP noted in these brain regions in treatment naïve deer mice. Treatment of stereotypic deer mice with fluoxetine had diverse effects on PDE4 activity. Thus, 10mg/kg fluoxetine failed to alter PDE4 activity in the prefrontal cortex of both LSB and HSB mice, although high dose fluoxetine decreased PDE4 activity in HSB mice. In the striatum, however, low dose fluoxetine increased PDE4 activity in LSB mice, but decreased said activity in HSB mice, while high dose decreased PDE4 activity in HSB mice (with no effect in LSB).

Since a similar effect on certain PDE4 isoforms was also noted with desipramine, for example both drugs increased PDE 4A expression (see table 6.19), many of the actions on PDE4 are likely to be less representative of the selective anti-OCD action of an SRI and more of a broad based mechanism associated with most antidepressants. However, down-regulation of PDE4 is not normally associated with the decreased cAMP induced by

fluoxetine. Bearing the important negative correlation between PDE4 activity and cAMP levels, data from this study would suggest that fluoxetine-mediated up-regulation of PDE 4A5/8 and PDE 4A1 (in HSB, but especially LSB mice) is more likely responsible for the observed decrease in cAMP following fluoxetine treatment (see tables 6.22, 6.23 and below for discussion). However, this same response was shared by desipramine that also induced a concomitant reduction in cAMP (table 5.1). Clearly, this is not indicative of an OCD-specific action of an SRI. Yet, actions on PDE4 that were selective for fluoxetine include the significant down-regulation of PDE 4D1 in the prefrontal cortex of LSB mice, and thus more likely to be correlated to the selective efficacy of SRI's in OCD.

An explanation for the diverse response to fluoxetine on PDE4 activty could be the severity of pre-existing behavioural and/or the presence of neurochemical abnormalities. Nevertheless, high dose fluoxetine decreases PDE4 activity in both prefrontal cortex and striatum, which may have great importance for its efficacy in treating OCD. Data from the present study indicate that HSB have the highest levels of cAMP and the lowest levels of PDE4 activity compared to LSB and NS mice prior to drug treatment. The results seem to be substantiated by a previous study that suggested that increased stereotypy is due to relative overactive D_1- and underactive D_2-pathways (Presti and Lewis 2005). D_1 receptors are positively coupled to adenylyl cyclase and cAMP production, whereas D_2 receptors are negatively coupled to adenylyl cyclase. Subsequent to fluoxetine treatment, prefrontal cortex cAMP is reduced in HSB alongside a puzzling reduction in PDE4 activity. The relevance of this suggestion will be discussed further in a later section where it has relevance to dopamine challenge studies in the model.

In LSB mice, fluoxetine treatment did not decrease prefrontal cortex PDE4 activity where in fact a trend toward an increase in PDE4 activity was noted. This is in line with what is expected since prefrontal cortex cAMP levels was decreased following fluoxetine treatment. However, irregularities within the CSTC circuit of LSB mice cannot be discounted. Results presented here focus exclusively on the effect of drug treatment on PDE4 enzyme activity, and it is important to realise that PDE4 is not the only phosphodiesterase that utilize cAMP as substrate. Four other family members, namely PDE1, PDE7, PDE8 and PDE10, are expressed in brain tissue and hydrolyze cAMP (table 2.4). Total cAMP levels, and effectively PDE4 activity, are more likely the result of the activities of all four recognized cAMP-utilizing PDE enzymes and it would be an over-

assumption to assume that cAMP is strictly hydrolyzed by PDE4. These divergent data can only be addressed more conclusively with protein expression analysis in the western blot studies.

Moving then to immunoblotting studies, treatment of LSB mice with either a low or high dose fluoxetine resulted in a decrease in prefrontal cortex and striatal PDE4 expression. The only exception was an increase in striatal PDE 4A1, 4A5 and 4A8 expression following 20 mg/kg fluoxetine. This is in contrast to PDE4 activity data for LSB mice which showed an increase in PDE4 activity in LSB mice (table 5.1). From immunoblotting data it is evident that fluoxetine predominantly down-regulates PDE4 expression in the prefrontal cortex of LSB mice. The fact that protein expression data is in contrast to enzyme activity analysis, where the latter indicated a non-significant trend toward up-regulation, would indicate that PDE4 is most likely not the major cAMP hydrolyzing PDE in the prefrontal cortex of deer mice.

Analysis of striatal PDE4 was also important since treatment of LSB mice with 20 mg/kg fluoxetine resulted in an increase in PDE 4A5, 4A8 and 4A1 expression. Even so, of the eleven PDE families, it is PDE10A, and not PDE4 that is highly expressed in this brain region (Fujishige et al., 1999b), particularly in medium spiny projection neurons (MSNs) of the striatum (Seeger et al., 2003). Striatal MSNs function as the principal input side of the basal ganglia that integrate cortical and dopaminergic inputs to facilitate planning and execution of relevant motor and cognitive patterns while suppressing unwanted patterns (Graybiel, 2000). Genetic deletion of striatal PDE10A not only results in behavioural changes in mice (Siuciak et al., 2006a), but pharmacological inhibition also seem to regulate cAMP and cGMP signalling in the MSNs (Siuciak et al., 2006b).

Nonetheless, up-regulation of PDE4A along with a concomitant decrease in cAMP suggests that antidepressant treatment regulates the cAMP system in the striatum of deer mice. Indeed, the up-regulation of the PDE4A isoform seems important since these isoforms is decreased in untreated deer mice (table 6.21). Moreover, this finding becomes more significant when it is considered that a recent study have shown increased expression of PDE4 in the nucleus accumbens of rats treated with fluoxetine (Takahashi et al., 1999), a brain region important in mediating pleasure and reward (section 2.4.1). The mechanisms underlying the up-regulation of PDE4A expression in brain very likely

involves secondary activation of gene expression in response to stimulation of the cAMP pathway. This possibility is supported by studies in cultured cells (human monocytes) that demonstrate that the cAMP system activates PDE4 gene expression (Manning et al., 1996). However, whether this isoform is involved in the preferential response of OCD to SRI's requires further testing, especially since desipramine had similar effects on this isozyme and on cAMP (see below), while PDE4A is also strongly associated with the noradrenergic system. In a recent study, Ye et al. (1997) showed that treatment of rats with desipramine resulted in an increase in PDE4A expression.

Treatment of HSB mice with either low or high dose fluoxetine mainly resulted in a decrease in prefrontal cortex and striatal PDE4 expression. Two noteworthy exceptions were that treatment with 20 mg/kg fluoxetine resulted in a significant up-regulation of striatal as well as prefrontal cortex PDE 4A5 and 4A8. Expression of PDE 4D1 in prefrontal cortex was also increased by the high dose fluoxetine. As explained above, results from the present study suggest that up-regulation of PDE4A may represent a compensatory response to fluoxetine treatment. The selectivity for PDE 4A5, 4A8 and 4D1, but not other isoforms, can be explained by the fact that antidepressants are reported to influence the cAMP system and that PDE4A, 4B and 4D1 are the isoforms seemingly regulated by antidepressant treatment (Dlaboga et al., 2006; Takahashi et al., 1999).

The regional specificity for up-regulation of PDE 4D1, i.e. significantly increased expression of this isoform in the prefrontal cortex of HSB mice and the significant decrease in expression in the striatum, indicates markedly different patterns of use for this isoform in response to increased cAMP. This isoform therefore plays different roles in responding to antidepressant treatment in the prefrontal cortex and striatum. The striatum is the main input structure of the basal ganglia and is a key component of the motor system (Kelly, 1999). The prefrontal cortex on the other hand plays a key role in the adaptation to changes in environment and in the control of behavioural responses (Dubois et al., 1994). This may be over-speculation, but a specific PDE isoform or set of isoforms may well be associated with a specific behavioural response, just as specific neuropsychological features are associated with a specific neuroanatomical region.

Present results indicate that the increase in prefrontal cortex PDE 4D1 expression is accompanied by not only a reduction in cAMP levels, but also a decrease in both PDE4

activity and stereotypy (behavioural response). The prefrontal cortex is the dominant regulator of stereotypic behaviour since this structure exerts significant control over behavioural responses that have their origin in deeper-seated regions of the brain, such as the striatum (Dubois et al., 1994). This would imply that the striatum is 'forced' to interpret signals from the prefrontal cortex even though it operates independently with different levels of cAMP-driven responses and accompanying cAMP hydrolyzing PDEs. Therefore, abnormalities may be present within one or both of these structures in deer mice, but with the degree of abnormality in this pathway reflective of the degree of stereotypy in a certain population, e.g. high abnormality associated with HSB mice.

7.3.2 Chronic treatment of deer mice with desipramine

Treatment of deer mice with desipramine served as a 'negative control' during the pharmacological validation of the deer mice model. The response to desipramine, or more importantly, non-response to the drug during the behavioural study, was an important aspect of the predictive validation process. Data from the present study indicate that stereotypic behaviour in the deer mice is unaffected by chronic desipramine treatment (section 4.2.2). This is in line with recent studies that have demonstrated that OCD responds selectively to drugs that inhibit the reuptake of 5-HT, while drugs lacking these attributes, such as the standard tricyclic antidepressants have been shown to be ineffective in OCD (Denys, 2006).

Desipramine significantly reduces prefrontal as well as striatal cAMP in both LSB and HSB mice (section 5.2 – 5.4). This is in contrast to previous studies using rat models that indicate postsynaptic β-adrenoceptor involvement in the action of this drug, and which demonstrated an increase in cAMP (Ye et al., 2000). It should however be emphasized that a recent study (Dlaboga et al., 2006) have shown that mice and rats may not only use different PDE4 subtypes in the same signal transduction pathway, but also that differential compartmentalization of PDE4s are involved in pathways affected by antidepressants. Since β-adrenergic receptors stimulate cAMP production (Tausig & Gilman, 1995), it is plausible that chronic treatment of deer mice with desipramine can be expected to be through stimulation of α_2-adrenoceptors (section 2.9.5.1), which are negatively coupled to adenylyl cyclase. It could also be plausible that β-adrenoceptors are

in fact adenylyl cyclase inhibitory in deer mice. In support of this, a recent study by Lefkowitz et al. (2002), demonstrated that β_2-adrenoceptors could go through reprogramming, which serves to change specificity of this receptor from G_s (stimulatory) to G_i (inhibitory). The possibility exists that a switch from G_s to G_i, and hence an increase in cAMP production could be present in deer mice. However, since desipramine failed to modify stereotypy in these animals strongly argues against the simple assumption that attenuation of the cAMP response in these two brain regions, as shown by fluoxetine as well, is a major role player in attenuating stereotypy in these animals. This suggests that additional targets of action may be involved in the selective ability of fluoxetine to reduce stereotypy.

In general, clinically used antidepressants enhance noradrenergic-mediated and 5-HT-mediated neurotransmission, either by inhibiting monoamine reuptake, inhibiting their catabolism or by blocking inhibitory, presynaptic α-adrenoceptors (Frazer, 1997). Desipramine specifically inhibits noradrenaline reuptake by binding to noradrenaline transporters, thereby increasing the amount of noradrenaline available in the synaptic cleft (Frazer, 1997). The significant attenuation of desipramine on striatal and prefrontal cortex cAMP levels can therefore be attributed to the effect this drug has on noradrenergic pathways, and may involve down-regulation of receptor binding or inhibition of its subcellular signalling to adenylyl cyclase.

However, like with fluoxetine, desipramine treatment produced a mixture of PDE4 activity results. In fact, desipramine treatment in many ways mirrored fluoxetine treatment in that it increased striatal PDE 4A1, 4A5, and 4A8 expression in deer mice, especially LSB mice. Since PDE4 is a widely recognised drug target for antidepressants and so of importance in depression, these responses are not representative of a critical therapeutic target for OCD. Thus, chronic treatment of LSB mice with 10 mg/kg desipramine significantly reduced prefrontal cortical expression of PDE 4B1, 4B3, 4B4, and 4D4 (section 6.4.1). Increasing the dosage did not significantly alter PDE4 expression in the prefrontal cortex of LSB mice, although a non-significant trend towards down-regulation was noted. In HSB mice, treatment with a low dose desipramine significantly reduced expression of PDE 4D1 and 4D4 (section 6.4.2) in prefrontal cortex, whereas 20 mg/kg desipramine significantly increased expression of PDE 4A1, 4D1 and 4D4 (section

6.4.4) in this region. A noteworthy feature following desipramine treatment, was the prominence of PDE4D isoforms. In most cases, these changes were replicated by fluoxetine.

A select few PDE4 isozymes were targeted by fluoxetine with desipramine having no effect, including the 4D1 isoform in LSB mice and HSB mice (10 and 20mg fluoxtine) and the 4B4 isoform in HSB mice (20mg fluoxetine). LSB and HSB deer mice are characterized by raised cAMP levels in the prefrontal cortex and striatum, and that fluoxetine but not desipramine reverses this behaviour. This suggests that at least some of the clinical efficacy of fluoxetine may rest in its ability to selectively modulate certain PDE isoforms that drive a reduction in cAMP in these two brain regions, and in this manner reverse stereotypic behaviour.

Indeed, the prominence of PDE 4D isoforms in the present study is substantiated by previous studies (Baillie et al., 2003) which have shown that this isoform regulates phosphorylation of the β-adrenoceptor by interacting with β arrestin (Baillie et al., 2003). The switch from a decrease to an increase in PDE 4D1 and 4D4 expression in the prefrontal cortex of HSB mice following an increase in desipramine dosage could most likely be explained by the reprogramming that is said to occur in β_2-adrenoceptors. (Lefkowitz et al., 2002). In addition, this possible reprogramming may explain data generated from fluoxetine treatment of HSB mice. The solitary difference is that desipramine treatment, in addition to the PDE 4D isoforms, also increased prefrontal cortex PDE 4A1 expression.

In a recent study, Zhang et al. (2002) suggested that PDE 4D appears to be necessary for classical antidepressant drugs to produce their behavioural effects. It was also suggested that PDE 4A and 4B subtypes are regulated at the transcriptional level and that PDE 4D may be regulated predominantly by phosphorylation rather than altered expression. However, this may also be due to these drugs affecting the synthesis side of signal transduction systems, e.g., indirect stimulation of β-adrenoceptors. If anything, reductions in cAMP catabolism might be expected to increase the sensitivity to and effectiveness of these antidepressants. These data would indicate that while an exact role is unclear, it appear that PDE 4D is involved in the signalling pathways affected by antidepressants.

The clear difference between the striatal and prefrontal cortex is also highlighted by desipramine treatment of deer mice. In LSB mice, 20 mg/kg desipramine significantly increased striatal PDE 4A1, 4A5 and 4A8 (section 6.4.3). In HSB mice, only PDE 4A5 and 4A8 seem to be affected in the striatum (section 6.4.4). This observation is agreement with recent studies (Dlaboga et al., 2006; Zhang et al., 2002) that suggest that PDE 4A subtypes increases after repeated desipramine treatment. In the prefrontal cortex however, PDE 4A isoform expression is unaffected by desipramine treatment, with 4A1 being the only exception.

It should however be emphasized that the present study focuses on stereotypic deer mice and that not only is basal PDE4 expression different from non-stereotypic mouse strains, it is possible that the difference in regional expression could be accounted for by the fact that each region express different compliments of PDE4 isoforms (Houslay & Kolch, 2000). Indeed, treatment of mice with an anxiolytic (diazepam) highlighted the fact that some PDE isoforms, such as PDE 4A, are more sensitive to the same drug than others, e.g. PDE 4B (Cherry et al., 2001). Results from the present study suggest that up- or down-regulation of PDE4 activity may represent a compensatory response to antidepressant treatment in general and may not be definitively linked to a role in OCD.

7.4 Serotonergic function in stereotyping deer mice

An important aspect of the present study was to extend the construct validity of the model by investigating the contribution of selected serotonergic pathways and receptors in spontaneous stereotypic behaviour in the deer mice model. Although controversial (Goodman et al., 1995; Khanna et al., 2001), the non-selective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist, mCPP (Barnes & Sharp, 1999), has been found to exacerbate obsessive compulsive symptoms in OCD patients (Zohar et al., 1987; Goodman et al., 1995), underscoring the role of 5-HT and 5-HT_2 receptors in OCD. It was therefore hypothesized that subacute administration of mCPP would result in the exacerbation of spontaneous stereotypic behaviour and that chronic pretreatment with the SRI, fluoxetine but not the NRI, desipramine, would reverse mCPP-induced behaviours. This would further confirm the face and predicative validity of the model described earlier, but also extend current knowledge on the role of 5-HT in the aetiology and symptomology of OCD.

Contrary to the above hypothesis, treatment of deer mice with mCPP resulted in a significant decrease in stereotypic behaviour in both LSB and HSB mice (section 5.5 and 5.6). The precise mechanism whereby this decrease in stereotypic behaviour in deer mice is evoked remains speculative, especially since mCPP is a relatively broad spectrum serotonergic receptor agonist, which complicates any prediction of how this drug will act (Barnes & Sharp 1999). The present study would suggest that 5-HT_{2A} and 5-HT_{2C} receptors play an important role in this response. However, there is significant ambiguity concerning the role of the 5-HT_2 receptor as both 5-HT_{2C} agonists (Hollander et al., 1992; Zohar & Insel 1987) and 5-HT_{2C} antagonists (Ramasubbu et al., 2000) have been shown to induce and/or exacerbate symptoms of OCD. In addition, 5-HT_{2A} (Brocco et al., 1998; Guimaraes et al., 1997) and to a lesser extent, 5-HT_{2C} receptors, have been implicated in the regulation of anxiety (Dhonnchadha et al., 2003). Nevertheless, these studies strongly implicate the involvement of either or both 5-HT_2 receptors in stereotypic behaviour in the deer mouse model. It is however, the observations with fluoxetine prior to mCPP challenge that provide important clues to the underlying neurobiology of spontaneous stereotypy in deer mice.

Chronic fluoxetine pre-treatment significantly reversed mCPP-induced hypolocomotion in LSB deer mice, and partially reversed it in HSB mice. This was not achieved with desipramine, which is an important finding. It is interesting that chronic fluoxetine alone reduced stereotypy, as did sub-acute mCPP alone, concluding that either direct or indirect 5-HT agonism alone will attenuate stereotypy. However, that fluoxetine pretreatment reversed mCPP-induced stereotypy seems paradoxical since an additive effect may have been a more reasonable expectation. Thus, while it is recognised that fluoxetine increases 5-HT release through desensitization of terminal presynaptic 5-HT autoreceptors (Bergqvist et al 1999; El Mansari and Blier 2006), and in this manner creates a milieu of desensitized terminal 5-HT autoreceptors and increased 5-HT levels thereby allowing concommittant mCPP treatment to more effectively activate 5-HT_{2A} and 5-HT_{2C} receptors, this does not explain why fluoxetine pretreatment reversed mCPP effects.

Fluoxetine, however, is an antagonist of the 5-HT_{2C} receptor (Ni & Miledi, 1997) and one of the principle receptor binding cites for mCPP. After chronic fluoxetine dosing prior to mCPP administration, it is suggested that high occupancy of the 5-HT_{2C} receptor by fluoxetine will prevent effective binding of mCPP to this receptor thereby attenuating its

effects on stereotypic behaviour. Indeed, chronic treatment with fluoxetine has been shown to up-regulate 5-HT_{2C} receptor binding sites (Laakso et al., 1996) confirming a powerful binding of the drug to this receptor.

Reversing drug-induced effects on stereotypy, as demonstrated with fluoxetine prior to mCPP, may underlie different mechanisms to that which underlies naturalistic stereotypy, such as a more direct involvement of the 5-HT_{2C} receptor described above. Chronic pre-treatment of deer mice with 20 mg/kg fluoxetine prior to mCPP challenge significantly increased stereotypic behaviour in both LSB and HSB mice (section 5.7 and 5.8), suggesting that the effects of fluoxetine at 5-HT_{2C} receptors may have importance in the therapeutic effect of fluoxetine in OCD. Indeed, 5-HT_{2C} receptor knockout mice engage in compulsive behaviour akin to that observed in OCD (Chou-Green et al., 2003), thus arguing in favour of a 5-HT_{2C} mediated mechanism in stereotypic behaviour that is reversed by chronic SRI treatment.

The separate fluoxetine and mCPP data would extend this premise, suggesting that increasing 5-HT activity through either indirect means via blocking of the 5-HT transporter (with SRI), or by directly stimulating post-synaptic 5-$HT_{2A/C}$ receptors (with mCPP) would both mediate more effective 5-HT_{2A} and 5-HT_{2C} receptor signalling and an attenuation of stereotypy. Indeed, in a study performed on stereotyping bank voles (Schoenecker & Heller, 2003), treatment with a SRI, citalopram, similarly resulted in a decrease in spontaneous stereotypic behaviour, thus confirming hypolocomotoric effects following administration of a serotonergic agent to high stereotyping animals. In the present study, treatment of stereotypic deer mice with mCPP allowed the opportunity to explore construct validity of the deer mice model. From the behavioural data, it is plausible to implicate 5-HT, and largely the 5-$HT_{2A/2C}$ receptors in the stereotypic behaviour of deer mice.

Moving on to the neurochemical aspects and their possible role in construct validity, of the model, of importance here is the fact that both LSB and HSB mice present with significantly raised levels of cAMP in both striatum and prefrontal cortex, as well as present with an important negative correlation with PDE4 activity in these two brain regions. Consequently, realistic contruct validity would require reversal of these two parameters in a parallel fashion. In the present study, fluoxetine had a mixture of effects

on striatal and prefrontal cortex cAMP levels in stereotypic deer mice. This was not strictly shared with desipramine, which tended to have the opposite effect on cAMP in both the striatum and prefrontal cortex in both LSB and HSB mice. With regards their action on PDE activity, as with cAMP, treatment of deer mice with fluoxetine or desipramine resulted in dissimilar outcomes (see section 7.3.2). It is evident from the present study that up- or down-regulation of PDE4 activity may represent a compensatory response to antidepressant treatment in general and may not be definitively linked to a role in OCD.

PDEs provide a sophisticated system for organising cAMP signal transduction in specific intracellular compartments, for allowing integration with other signalling systems and for controlling the kinetics and reaction characteristics of cAMP signal transduction. At this point it is still uncertain whether the changes/alterations in cAMP levels reflect a primary condition or a secondary response to upstream (e.g. receptor) perturbations. In this regard, subacute drug challenges may improve or extent construct validity of the model.

Subacute challenge of deer mice with mCPP resulted in a significant increase in striatal cAMP levels in LSB mice although not in HSB mice. mCPP is a non-selective 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptor agonist, although it is thought that this drug acts primarily through the 5-$HT_{2A/C}$ receptor. The 5-HT_{2A} receptor does not typically regulate cAMP formation in most cells or tissues, but it has been shown to both stimulate and diminish cAMP accumulation in neuronal cell lines (Berg et al., 1994). Although controversial, stimulation of 5-HT_{2A} would explain the increase in striatal cAMP levels. The more likely explanation however may be that 5-HT_{1A} receptors activate adenylyl cyclase, since these receptors are more traditionally associated with effects on cAMP (Raymond et al., 2001). Based on this evidence and other studies where 5-HT_{1A} agonists (Zohar & Insel, 1987) have been used, 5-HT_{1A} receptors may perhaps play a crucial role in the compulsive behaviour seen among patients with OCD.

Although saline plus mCPP induced cAMP attenuation was more pronounced than that induced by acute mCPP challenge alone (to control for injection stress) in the striatum, chronic pre-treatment with fluoxetine to both LSB and HSB mice appeared to strengthen mCPP induced reduction in cAMP, resulting in a further decrease in striatal cAMP levels (section 5.8.1 and 5.8.2). Pre-treatment with desipramine similarly resulted in a further

decrease in cAMP levels. From this data, it is evident that adenylyl cyclase is inhibited by the mCPP challenge, but that pre-treatment with fluoxetine or desipramine is unable to reverse these effects. In contrast to striatal levels, fluoxetine pre-treatment in LSB mice, but not desipramine, significantly reversed mCPP-induced suppression of prefrontal cortex cAMP (section 5.7.1 and 5.7.2). In HSB mice, however, only desipramine succeeded in marginally reversing mCPP-induced suppression of prefrontal cortex cAMP. However, it may be plausible that prefrontal 5-HT_{1B} stimulation by mCPP is also taking place, since this receptor is known to negatively regulate cAMP formation (Raymond et al., 2001).

Chronic fluoxetine treatment results in an increase in 5-HT release, mainly due to desensitization of terminal 5-HT autoreceptors (Bergqvist et al., 1999; El Mansari & Blier, 2005). It is also interesting that fluoxetine is a 5-HT_{2C} receptor antagonist (Ni and Miledi 1997) and that after chronic treatment has been shown to up-regulate 5-HT_{2C} receptor binding sites (Laakso et al 1996). Therefore, fluoxetine may regulate other 5-HT receptors as well and because of its indirect action, creates an environment that would mediate more effective 5-HT receptor signalling (via mCPP stimulation) and hence the present increase in prefrontal cAMP levels. Noradrenergic stimulation of prefrontal cortex in HSB mice furthermore would seem to create an adenylyl cyclase inducing milieu in these mice such that desipramine slightly increases mCPP associated cAMP. The fact that injection stress associated with mCPP administration has a marked inhibitory effect on prefrontal cortex cAMP of its own (see Fig 5.15: saline plus mCPP compared to acute mCPP) complicates an accurate interpretation of how antidepressant pre-treatment is altering the effects of mCPP.

Treatment of LSB mice with subacute mCPP significantly increased prefrontal PDE4 activity (section 5.6.1), whereas in HSB mice mCPP decreased PDE4 activity (section 5.6.2). Striatal PDE4 activity was not influenced by subacute mCPP in either LSB and HSB mice. No clear relationship emerged from cAMP and PDE4 activity data following subacute mCPP challenges, indicating that PDE4 is not the only enzyme involved in cAMP hydrolysis in these regions, but that other cAMP utilizing PDE families might be responsible for changes in second messenger levels. Although PDE4 has a critical role in hydrolyzing cAMP associated with the noradrenergic system (Whalin et al., 1989), other

PDE families present in the brain such as PDE1, PDE7, PDE8 and PDE10 may also stimulate or inhibit adenylyl cyclase (Siuciak et al., 2006; Whalin et al., 1989).

Both chronic fluoxetine and desipramine reduced striatal PDE4 activity in LSB and HSB deer mice compared to saline plus mCPP (section 5.8), with little to no effect on cAMP levels noted in HSB mice. In contrast, only fluoxetine pre-treatment significantly suppressed PDE4 activity compared to saline plus mCPP in the prefrontal cortex of deer mice (section 5.7.1). Chronic saline plus mCPP furthermore significantly increased PDE4 activity in the striatum of HSB mice compared to baseline, while not having any effect on the prefrontal cortex of deer mice. That these results differ from that obtained with subacute mCPP, highlight a possible role of injection stress. Nevertheless, the chronic treatment studies (saline/fluoxetine/desipramine) suggest antidepressant effects on mCPP challenge. The response of PDE4 activity to mCPP and its reversal with fluoxetine but not desipramine pre-treatment further consolidate the role of the 5-HT system in this response. In this regard, 5-HT represents a major regulator of behaviour in deer mice driven by cAMP-PDE4 dependent pathways.

7.5 Dopaminergic function in stereotyping deer mice

It must be considered that while serotonergic agents, such as SRIs represent the treatment of choice for OCD, treatment non-response is common and on average only 50% of patients show an adequate response to treatment with these agents (Goodman et al., 1991). This very likely represents effects subsequent to, or in addition to, hyposerotonergia involving other signalling systems active within the CSTC circuit, one in particular being dopamine. In these instances, it is often the case that in SRI-resistant cases, SRI treatment is combined with a neuroleptic (Denys, 2006).

Since neuroleptics effective in this regard act on the D_2 receptor family, as a further validation, the study evaluated the response of deer mice to a dopaminergic challenge using the selective D_2/D_3 receptor agonist, QNP. Moreover, the effects of chronic high dose fluoxetine or desipramine treatment on QNP-associated stereotypy and prefrontal cortex/striatal cAMP and PDE4 activity were studied. In addition, to further establish a role for dopamine in these results, western blotting for expression of the highly

dopamine-associated protein, Darpp-32, was carried in the prefrontal cortex and striatum of stereotypic deer mice with and without chronic fluoxetine and desipramine treatment.

Subacute QNP of deer mice resulted in a significant decrease in stereotypic behaviour in both LSB and HSB mice (section 5.5 and 5.6). Chronic pre-treatment of deer mice with 20 mg/kg fluoxetine prior to a subacute QNP challenge effectively reversed the effects of QNP on stereotypic behaviour in LSB mice. A similar response was noted in HSB mice, although it narrowly missed statistical significance. Fluoxetine treatment exerts diverse effects on dopamine activity, including an increase in dopamine release in the striatum (Benloucif et al., 1993; McMahon & Cunningham, 2001), but also a pronounced inhibitory action on the synthesis and release of striatal dopamine in the substantia nigra and on presynaptic dopamine projections within the striatum and cortex via 5-HT_{2C} receptor activation (Kapur & Remington, 1996). The latter action results in a reactive up-regulation of postsynaptic dopamine receptors (Ashby et al., 1995) that very likely underlie the reversal in QNP effects observed here.

While a trend towards reversal was evident, that fluoxetine failed to have a marked effect in QNP challenged HSB mice is intriguing, but very likely indicates a greater degree of dopaminergic imbalance present in these animals requiring a higher dose and or longer duration of treatment. If changes to dopamine functions are indeed secondary to the serotonergic effects of the SRI, and since pre-synaptic orbitofrontal cortex 5-HT auto-receptors require a longer SRI treatment period to desensitise than in other brain regions (Blier & Tremblay 2006), weakened effects on dopaminergic pathways can similarly be expected following an inadequate treatment period.

Moving on to the effects of drugs on cAMP levels and PDE4 activity, subacute challenge of deer mice with SKF (D_1 agonist) resulted in a significant increase in striatal cAMP levels in stereotypic deer mice (section 5.6). Similarly, subacute challenge with QNP (D_2 agonist) significantly increased striatal cAMP levels. Interestingly, in the deer mice, D_2-type receptors decrease adenylyl cyclase activity, but recent studies have indicated a biphasic action of QNP across dose and time (Van Hartesveldt, 1997). The dose used in this particular study (5 mg/kg) seems to stimulate adenylyl cyclase, which result in increased striatal cAMP levels and may explain the increase in cAMP levels. In the prefrontal cortex, SKF significantly decreased cAMP, whereas QNP significantly

increased second messenger levels in this brain region. The reduction in prefrontal cortex cAMP by SKF is in line with 'normal' D_2 signalling in that this receptor is generally known to inhibit cAMP production, whereas QNP treatment confirm that D_1 signalling seem to be functioning properly. Subacute treatment with these dopaminergic drugs therefore indicates a possible anomalous cAMP system, or anomalous regulators of the cAMP system, for example upstream receptors in the striatum. Results therefore indicate possible irregularities that may be present in the CSTC circuit of stereotypic deer mice.

Subacute challenge of LSB mice resulted in a significant decrease in striatal PDE4 activity, whereas this drug had the opposite effect in the prefrontal cortex. Treatment of deer mice with SKF on the contrary significantly decreased prefrontal cortex PDE4. Results are somewhat mixed and difficult to explain, but it must be kept in mind that there are more than 50 PDE4 isoforms and it is possible that these dopaminergic drugs will target some isoforms over others and in so doing determine total PDE4 activity.

Saline plus QNP significantly lowered cAMP levels in the striatum of LSB and HSB mice, which was significantly shored up by chronic pre-treatment with fluoxetine or desipramine prior to QNP in HSB mice (section 5.7.3), suggesting a reinforcement of the attenuating effects of QNP on this second messenger. In both drug treatments, this was correlated with a significant increase in striatal PDE4 activity. A similar trend was noted in HSB mice, although effects on cAMP were not significant, while only fluoxetine significantly reversed the decreased PDE4 activity induced by QNP. While the observed decrease in striatal cAMP may be because of adenylyl cyclase inhibition by dopamine D_2 receptors (section 2.9.2.2), these data provide strong evidence for increased PDE4 activity in this response.

In the prefrontal cortex, fluoxetine pre-treatment but not desipramine significantly reversed reduced cAMP levels evoked by QNP in LSB but not in HSB mice (section 5.7.3 and 5.7.4). In both HSB and LSB mice, this was accompanied by a significant increase in PDE4 activity following fluoxetine (in LSB and HSB mice) and desipramine (in HSB mice). As mentioned earlier in the discussion, results from the present study suggest that regulation of the cAMP system may represent a compensatory response to drug treatment (Nibuya et al., 1996). Furthermore, the difference in regional expression could be accounted for by the fact that each region expresses different compliments of PDE4

isoforms (Houslay & Kolch, 2000) and data generated from the present study could be substantiated by future protein expression studies.

Typically, D_1 and D_2 receptors exert opposing actions on intracellular signalling molecules and they often have disparate physiological effects. In a study by Denys and co-workers (Denys et al., 2004), abnormalities in the binding potential of the dopamine D_2 receptor was shown in OCD patients, which suggest an involvement of the dopaminergic system in the pathophysiology of OCD. However, D_1 receptors are also implicated in the pathophysiology of OCD. As was pointed out in an earlier section (section 2.7.3.3.1), D1CT-7 transgenic mice that engage in episodes of perseverance or repetition, was engineered to express a neuro-potentiating cholera toxin transgene in a subset of D_1 receptor-expressing neurons (Campbell et al., 1999). Furthermore, several lines of evidence suggest that concurrent activation of D_1 and D_2 receptors is required for certain dopamine -mediated responses, particularly stereotyped behaviour (Fetsko et al., 2003). A very important molecule in facilitating, and even to some extent regulating dopamine responses, is Darpp-32.

Darpp-32 is localized, with few exceptions, to regions that receive dopaminergic innervation, however, moderate levels of Darpp-32 are also found throughout the neocortex and in several sub-regions of the hypothalamus (section 2.9.6). Darpp-32 function is primarily regulated by altering the phosphorylation state of one or more of its regulatory threonine or serine residues (Greengard et al., 1998). Darpp-32 and its regulation is of keen interest in this study since this molecule is not only a modulator of the cAMP/PKA signalling pathway (section 2.9.6.1.1), but also in view of the fact that changes in its phosphorylation reinforces the effects of neurotransmitters, neuromodulators and drugs that regulate the cAMP pathway.

In addition, Darpp-32 plays an important role in the direct and indirect signalling pathways within the CSTC circuit (section 2.4.2), a pathway thought to be important in the pathophysiology of OCD. Darpp-32 phosphorylation on residue Thr75 was shown to be significantly increased in the prefrontal cortex and striatum of untreated stereotypic deer mice compared to C57Bl (section 6.2.1 and 6.2.2). This would seem to indicate a link between pre-existing levels of stereotypic behaviour and phoshorylation of Darpp-32

on Thr75, with higher levels of phosphorylation in stereotypic deer mice, and low levels of Thr75 Darpp-32 phosphorylation in NS and C57Bl mice.

Measuring dynorphin and enkephalin content in dopaminergic synapses has suggested that increased stereotypy is due to relative overactive D_1- and underactive D_2-pathways (Presti and Lewis 2005). The presence of a hyperactive D_1-pathway is substantiated by present data that indicate increased Thr34 phosphorylation of striatal Darpp-32 in HSB mice (section 6.2.2). The increase in Darpp-32 phosphorylation on both Thr residues in the striatum is more difficult to explain. It is plausible to hypothesize that even though D_1 signalling is overactive (increase in Thr34 phosphorylation); D_2 signalling is attempting to compensate for this imbalance (increase in Thr75 phosphorylation). However, it is plausible that structural or functional impairments in deer mice D_2 receptor signalling may prevent a return to D_1/D_2 signalling balance and as a result stereotypic behaviour continues to take place.

Deer mice, particularly high stereotypic deer mice, present with a unique pattern of Darpp-32 phosphorylation. In non-stereotypic C57Bl mice activation of D_1 receptors classically decrease phosphorylation of Darpp-32 at Thr75 by a process that likely involves the PKA-dependent activation of a specific isoform of PP-2A (Nishi et al., 2000). Thus, any increase in dopaminergic transmission via D_1 receptors will lead to a decrease in phosphorylation of Darpp-32 on the Thr75 residue, reduce inhibition of PKA and thereby facilitate signalling via the PKA/Thr34-Darpp-32/PP-1 cascade (Nishi et al., 2000). Even though increased levels of cAMP have been noted in stereotypic deer mice, the classic PKA/Thr34-Darpp-32 pathway is not the pathway followed in deer mice, indicating a possible break down in dopamine-Darpp-32 signalling.

The effect of fluoxetine on Darpp-32 phosphorylation was also investigated since a change in the phosphorylation state of this molecule, through distinct signalling pathways, increase inhibition of PP-1, a major serine/threonine protein phosphatase in the brain (section 2.9.6.1). Treatment of LSB mice with fluoxetine resulted in a significant increase in Darpp-32 phosphorylation on Thr75 in the prefrontal cortex, something that was not observed in HSB mice. Therefore, phosphorylation of Thr75-Darpp-32 in HSB mice was actually decreased compared to basal levels. In LSB mice, phosphorylated Thr75-Darpp-32 may also be on the decline, but not as much compared to HSB mice. In this instance,

the phrase 'the tallest trees catches the most wind' might be appropriate since HSB mice, with the highest degree of abnormality, respond at both behavioural and neurochemical level, the best to fluoxetine treatment.

Fluoxetine treatment furthermore increased striatal Thr34 phosphorylation in the striatum of LSB mice and had the opposite effect in HSB mice. HSB mice had the highest level of striatal Thr34-Darpp-32 under basal conditions (section 6.2.2), and data would seem to indicate that fluoxetine treatment decreased this phosphorylation state. Phosphorylation of Thr34 is associated with D_1 receptor signalling (Walaas & Greengard, 1984), the same pathway thought to be overactive in deer mice (Presti and Lewis 2005). Differential response to fluoxetine could once more be explained by the severity of pre-existing behavioural and/or the presence of neurochemical abnormalities, with the greatest abnormalities being restored first or reacting faster to treatment.

In a recent study on C57Bl mice, Darpp-32 phosphorylation on both Thr34 and Thr75 was unaltered following chronic fluoxetine treatment (Svenningson et al., 2002b). The study did however show an increase in Darpp-32 mRNA and protein in response to fluoxetine, indicating that these chronic changes may be relevant to the delayed onset of therapeutic efficacy of the drug. Nonetheless, the present study does indicate that regulation of Darpp-32 phosphorylation should be taken into account when animals are chronically treated with antidepressants.

The present study furthermore explored the effects of chronic desipramine treatment on stereotypic deer mice. Treatment of LSB mice with 10 mg/kg desipramine increased striatal Thr34-Darpp-32 (section 6.4.1), and had the opposite effect in HSB mice (section 6.4.3). Particularly interesting is the observation that Thr75-Darpp-32 is increased in the striatum of HSB mice. An increase in Darpp-32 phosphorylation at Thr75 is associated with D_2 receptor signalling (Nishi et al., 2000). Even though behaviour is not affected by desipramine, this drug still affects receptors as well as the cAMP system. It is plausible, that as with fluoxetine, desipramine treatment influence the D_1 and D_2 pathways, but that fluoxetine, because of its 5-HT regulatory properties, affect behaviour. Once again, regional differences in protein expression, protein phosphorylation and disparity according to pre-existing behavioural abnormalities are highlighted by drug treatment of stereotypic deer mice.

In the deer mice, the noradrenergic system would appear to play a key role at altering molecular parameters such as cAMP levels and PDE4 activity levels, but not so at the behavioural level. Indeed, SRI's and not NRI's are effective in reducing stereotypic behaviour in the deer mouse model, indicating that the 5-HT system not only plays a critical role in regulating stereotypic behaviour, but also modulates various parameters at the molecular level that may have a causal link to this behaviour. This is none more evident when considering what happens in OCD following SRI treatment

In OCD patients, only the potent SRIs are consistently effective (section 2.6.1). For instance, fluoxetine has been demonstrated to significantly attenuate OCD symptoms (Zohar & Insel, 1987). In addition, the tricyclic antidepressant (TCA) clomipramine, which is a potent 5-HT reuptake inhibitor, produces an anti-OCD effect, but relapse takes place when patients are switched to the TCA desipramine, which is a selective NRI (Leonard et al., 1991). Evidently then, the anti-OCD effect unlike the antidepressant response, rests largely on the inhibition of the 5-HT reuptake process.

Data suggest that therapeutic response to an SRI in OCD is the result of decreased 5-HT release and that this decrease is the result of desensitization of the presynaptic 5-HT_{1D} autoreceptor (El Mansari & Blier, 1995) and postsynaptic 5-HT_{1A} receptors in the OFC (El Mansari & Blier, 2005), but also in other regions of the CSTC circuit. In the present study, fluoxetine reversed the mCPP-induced decrease in stereotypic behaviour in deer mice, indicating the effectiveness of SRI treatment in this model. Moreover, the inhibitory effect of 5-HT is most likely also mediated by postsynaptic 5-HT_2 receptors such as 5-HT_{2A} and 5-HT_{2C} receptor (El Mansari & Blier, 2005).

Taken together, the activation of 5-HT_2 receptors mediates the effect of an SRI following long-term administration. The sensitivity of 5-HT_{1A} receptors is attenuated (El Mansari & Blier, 2005), whereas that of 5-HT_2 receptors remains unaltered following SRI treatment. Indeed, the regulatory role of 5-HT in the deer mice is most likely based on increased tonic activation of postsynaptic 5-HT_{1A} receptors which may be attributable in part to a desensitization of α_2-adrenoceptors as a result of elevated noradrenergic activity. A concise summary of all the conclusions reached in the present study is provided in chapter eight along with ideas for future research.

8. Conclusions & future work

8.1 Conclusions

The aim of the present study was to validate spontaneous stereotypic behaviour of the deer mouse (*Peromyscus maniculatus bairdii*), as a potential animal model of OCD. A well-validated deer mice model may address many of the current limitations and considerations described in the literature review section. The model was assessed on three criteria, namely face, predictive, and construct validity. Face validity was performed through detailed behavioural characterisation of deer mice and compared to a mouse strain known not to present with stereotypic behaviour, but that does present with high locomotor activity, in this case the C57Bl mouse. Moreover, the extent and nature of stereotypic behaviour within a population of deer mice was studied and analysed in order to assess inter-individual variation.

Pharmacological isomorphism was evaluated through the differential response of the model to drugs of known efficacy in OCD, in this case SRI versus drugs ineffective in OCD namely NRI. Construct validity was studied through evaluation of the CSTC circuit cAMP-pathways, particularly cAMP levels, PDE4 activity and PDE4 expression, in naïve and SRI/NRI treated deer mice. Construct validation was further studied using dopamine and 5-HT challenges and their effect on behaviour and neurochemistry, as described above, and whether these could be altered by chronic SRI or NRI treatment. Moreover, construct validity with respect to dopamine was also carried out through assessment of Darpp-32 phosphorylation.

Validation studies were preceded by comparing stereotypic deer mice to a non-stereotypic strain, namely C57Bl. Disparity between untreated deer mice and C57Bl were compared at both the behavioural and molecular level. Deer mice engaged in clear, stereotypic behavioural patterns such as backward somersaulting, repetitive jumping, and patterned running. Deer mice furthermore executed stereotypic behaviour at various rates and could accordingly be classified as LSB, HSB or non-stereotypic mice. In contrast, while C57Bl mice displayed high levels of locomotor activity, these animals did not perform any stereotypic behaviours characteristic of deer mice.

Comparison of the two mouse strains not only highlights the unique stereotypic behaviour of deer mice, but also contributes to the face validity of the model. In this regard, deer

mice engage in stereotypic behaviour at different rates, much the same as OCD patients perform compulsions at different rates.

At the molecular level, the degree of stereotypy could be linked to the amount of cAMP under basal conditions. HSB mice had the highest cAMP levels compared to LSB, non-stereotypic and C57Bl mice. Deer mice also presented with the highest measured PDE4 enzyme activity. Stereotypic deer mice furthermore expressed lower quantities of the short form PDEs namely PDE 4B4 and 4D1 as well as the noradrenergic associated 4A5 and 4A8 isoforms compared to C57Bl mice. Untreated stereotypic deer mice moreover presented with increased levels of Thr75-Darpp-32 compared to C57Bl and non-stereotypic deer mice. Taken together, deer mice, particularly LSB and HSB mice, can be unmistakably distinguished from C57Bl at both the behavioural and molecular level. These marked differences do suggest differences in function in these brain regions, particularly locomotor function as has been described with stereotypy and may shed light on the stereotypic aspect in OCD.

Given the selective response of OCD to SRIs compared to NRIs, behavioural response to chronic administration of a SRIs and a NRI was investigated. To this end, fluoxetine, but not desipramine, significantly reduced stereotypic behaviour compared to vehicle-treated animals in both low and high stereotypy animals. Seeing as serotonin and dopamine also seem to play important roles in OCD, the prospective functions of these two neurochemicals in deer mice were explored by means of subacute challenge studies with the serotonergic agent, mCPP and the dopaminergic agents, QNP and SKF.

Subacute mCPP and QNP challenges both evoked a significant suppression of stereotypic behaviours, while high dose fluoxetine, but not desipramine, reversed the suppressive effects of mCPP and QNP in LSB (significance reached) and HSB mice (trend to significance). Subacute challenge with SKF also reduced stereotypic behaviour, but this was only significant in HSB mice. Since a number of studies have focussed on the role of D_1 receptor mediated signalling in deer mice, the present study opted to explore the contribution of D_2 receptors in stereotypic deer mice. Taken together, the present study provides substantial evidence that spontaneous stereotypic behaviour in the deer mice presents with significant predictive validity, while evidence for the involvement of

dopamine D_2, 5-HT_{2A} and 5-HT_{2C} receptors in stereotypic behaviour demonstrates noteworthy construct validity.

To explore construct validity of the model, changes in various molecular parameters were investigated. Chronic drug treatment and subacute challenges clearly highlight the disparity between LSB and HSB mice. This discrepancy is plainly seen at the molecular levels in HSB mice where the cAMP pathway (cAMP levels and PDE4 activity) is down-regulated following drug treatment or challenge. Accordingly, the severity of pre-existing behavioural and/or neurochemical abnormalities is not only a factor to be utilized for behavioural classification, but may also be the basis for differential molecular responses. This important finding in the deer mice model has greater value, since it may elucidate why some treatment regimes are efficacious in OCD, and others are not.

In support of behavioural results, data collected on regional levels of cAMP furthermore implicate 5-HT_{1A}, 5-HT_{1B} as well as 5-HT_{2C} in stereotypic behaviour in the deer mice. Moreover, analysis of protein expression indicate an increase in noradrenergic associated PDE 4A1, 4A5, 4A8 isoforms and the so-called short 4D1 in the prefrontal cortex of HSB mice as well as the striatum of LSB mice. Differences in regional expression of PDEs are most likely explained by the need for each region to express its own compliment of functionally relevant PDE4 isoforms. Differential regulation of Darpp-32 phosphorylation on Thr34 and Thr75 implicate an imbalance along the D_1/D_2 pathway in the CSTC circuit of deer mice. Taken together, it is concluded that PDE4 isoforms have an important role to play in the cAMP pathway of deer mice and that expression of the various isoforms is very particular, while abnormalities in the D_1/D_2 pathway is likewise confirmed by irregular Darpp-32 phosphorylation.

In conclusion, serotonin seems to play an important role in regulating stereotypic behaviour as well as molecular parameters in the deer mouse model. Indeed, augmentation of stereotypic behaviour following fluoxetine treatment and mCPP challenge is supported by corresponding augmentation of cAMP levels and PDE4 activity. In this regard, 5-HT represents a major regulator of behaviour and molecular pathways in deer mice driven by cAMP-PDE4 dependent pathways. The present study provide behavioural and pharmacological evidence that spontaneous stereotypic behaviour in the deer mice present with significant face, predictive and construct validity

as an animal model of OCD. It is therefore an effective animal model that will contribute greatly to research regarding diverse aspects of OCD pathology.

8.2 Future research

8.2.1 5-HT, 5-HT receptors and 5-HT transporters in deer mice

Data from the present study confirm that serotonin plays an important role in regulating stereotypic behaviour and range of molecular parameters in deer mice. During brain development, the 5-HT system controls neuronal specification, differentiation, and phenotype maintenance. While formation and integration of these neural networks is dependent on the action of multiple proteins, genetically controlled variability in the expression of the 5-HT transporter is critical to the development and plasticity of distinct neurocircuits. Indeed, the 5-HT transporter has been implicated in OCD (Torres & Caron, 2002; Lesch et al., 1996). It would be advantageous to explore both structural (e.g. screen for genetic variations) and functional (e.g. determine transporter density and affinity) characteristics of the 5-HT transporter in the deer mice.

In addition, it would be valuable to explore the role of specific 5-HT receptors in deer mice. Since 5-HT_{1A}, 5-HT_{1B} as well as 5-$HT_{2A/C}$ have been implicated in stereotypic deer mice, more selective 5-HT receptor agonists and antagonist could be employed. The effect of ketanserin (5-$HT_{2A/C}$ antagonist), buspirone (5-HT_{1A} agonist) or even sumatriptan (5-HT_{1D} agonist) on stereotypic behaviour and various molecular parameters may offer further insight into this model as well as OCD.

It must be considered that while serotonergic agents, such as SRIs represent the treatment of choice for OCD, treatment non-response is common and on average only 50% of patients show an adequate response to treatment with these agents (Goodman et al., 1991). In addition, some patients may initially respond well to treatment, but may experience relapse of symptoms at a later stage. The underlying mechanism of symptoms relapse may be an interesting avenue to explore. A straightforward protocol to follow would be to treat deer mice with an SRI (e.g. fluoxetine) for an adequate period, i.e. until a decrease of symptoms is noted. Thereafter proceed with the withdrawal of treatment until relapse of symptoms appears. In all instances, measurement of behaviour and

molecular parameters may offer useful insight into underlying mechanisms of symptom relapse.

8.2.2 D_1/D_2 signalling at the molecular level and other dopamine receptors

The present study, supported by previous findings, has shown that increased stereotypy is due to relative overactive D_1- and underactive D_2-pathways (Presti and Lewis 2005), increased cAMP levels as well as increased variable phosphorylation of striatal Darpp-32 in regions of the CSTC circuit (present study). In addition to the implicated dopamine receptors (D_1 and D_2), it may be useful to explore the role of dopamine D_4 receptors. In fact, recent studies have found a correlation between increases in D_4 receptors in caudate-putamen and motor hyperactivity (Zhang et al., 2001). D_1/D_2 signalling balance at the molecular level may also be explored further, particularly in the CSTC circuit of deer mice since the present study and previous findings (Presti et al., 2002) have indicated involvement of the dopamine system.

8.2.3 Involvement of various other neurotransmitters and neurochemicals in deer mice

Behavioural and molecular data confirmed the indisputable role of 5-HT in deer mice. Neurotransmitters are neither static nor isolated in their distribution in the brain and it is through these interactions that the CNS performs its vital role in sustaining life as well enabling humans and animals to engage in complex behavioural phenomena such as stereotypic behaviour. Effective communication is achieved through neurochemical interaction and this allows neurochemicals to modulate, regulate, and mediate each other's activity.

Apart from 5-HT, other neurotransmitters such as dopamine, noradrenaline, glutamate, and GABA may be critically involved in regulating stereotypic behaviour in deer mice. A number of neurochemical interactions thought to be important in OCD was highlighted in earlier sections (section 2.9.9.1 – 2.9.9.5). It may well be that these are also implicated in stereotypy of deer mice. In this regard, radioligand studies exploring NMDA receptor density and affinity may be useful. Indeed, NMDA have been shown to markedly elevate cAMP levels in rats (Zhang et al., 2000) and a recent study has shown heightened

glutamate release in certain topographic groups (repetitive jumpers) of stereotypic deer mice (Presti et a., 2004). Furthermore, a recent study (Suvarna & O'Donnell, 2002) suggested a possible link between NMDA, cAMP and PDE4.

Another interesting avenue to explore in the deer mice model is that of corticotrophin-releasing hormone (CRH) and its various receptors in the brain, particularly in regions such as the hypothalamus. Exploration of the role of CRH-1 receptors, which is said to regulate immediate response to stress, and CRH-2 receptors, which feature in the coordination of slow responses and aimed at facilitating recovery of homeostasis (De Kloet & Derijk, 2004), may be valuable. Experience from this study has indicated that HSB mice are easier to agitate than LSB mice. It is plausible that severity of pre-existing stereotypic behaviour could be associated with a decrease in the ability to reduce anxiety or stress, which in turn implicate the possible involvement of CRH and CRH receptors.

8.2.4 Characterization the cAMP signalling pathway in deer mice

The cAMP signaling pathways is a signal transduction system comprising a sequence of molecules whose function is to govern the generation, degradation, and the biological response of cAMP inside the cell. It might be useful to explore the cAMP pathway at the molecular level in deer mice since findings from the present study implicate some irregularities in this pathway. In fact, a recent study found significantly lower catalytic PKA subunits in platelets of OCD patients compared to healthy controls (Perez et al., 2000). It might also be useful to explore protein expression, function, and regulation of other molecules of the cAMP pathway such as adenylyl cyclases (the enzymes responsible for cAMP production), PKA (molecule directly phosphorylated by cAMP), brain-derived neurotrophic factor (BDNF; a transcription factor and key target for cAMP-mediated phosphorylation) and cAMP response element-binding protein (CREB). The regulatory and functional contribution of adenosine receptors such as the postsynaptic adenosine A_1 receptor may perhaps also be useful.

Since PDE4 is not the only phosphodiesterase responsible for cAMP hydrolysis, the role of other PDE families in cAMP regulation in deer mice could be investigated. An ideal candidate is PDE10A, since genetic deletion of striatal PDE10A not only resulted in behavioural changes in mice (Siuciak et al., 2006a), but pharmacological inhibition also

seem to regulate cAMP and cGMP signalling in the striatum of mice (Siuciak et al., 2006b).

8.2.5 Delineate the precise role and location of PDE4 isoforms in deer mice

Results form the present study indicates that expression of PDE4 isoforms is different from that of a non-stereotypic strain. PDE4 expression has been shown to be compartmentalized (Beavo, 1995). Since antibodies are readily available for most of the PDE4 isoforms, compartmentalization of different isoforms may be explored by confocal microscopy. In addition, the exact function of PDE4 isoforms is also unknown. In this instance, the generation of knock-out deer mice may be useful. A less expensive alternative, although more labour intensive, may be to prepare primary cell cultures from deer mice embryos. From there the precise function of PDE4 isoforms can be explored by making use of RNAi (RNA interference) technology.

8.2.6 Characterize Darpp-32 expression and regulation

Darpp-32 has a key role in many neurotransmitter pathways throughout the brain (Greengard 2001). Darpp-32 function is primarily regulated by altering the phosphorylation state of one or more of its regulatory Thr or Ser residues. The present study has shown that regulation of Darpp-32 is indeed altered in deer mice. In this regard, it may be useful to explore Darpp-32 regulation by focussing on both threonine and serine phosphorylation. In addition, it may also be valuable to determine whether total Darpp-32 expression or mRNA levels are modified in deer mice. In this instance, changes in Darpp-32 phosphorylation may be correlated with stereotypic behaviour.

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