THE ROLE OF THE DOPAMINE D3 RECEPTORS IN CUE-INDUCED REINSTATEMENT OF NICOTINE-SEEKING BEHAVIOUR

by

Maram A. T. M. Khaled

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Graduate Department of Pharmacology and Toxicology

University of Toronto

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Dopamine D₃ receptors (DRD₃) are implicated in relapse to drugs. The current study investigated the role of DRD₃ in cue-induced reinstatement of nicotine-seeking in rats. Rats were trained to lever-press for intravenous infusions of nicotine, associated with the illumination of a cue-light, under a fixed-ratio schedule of reinforcement. Following extinction of the behaviour, where lever pressing had no consequences, reinstatement testing was performed by reintroduction of the cues after systemic or local administration (into discrete brain areas) of the DRD₃ selective antagonist SB277011-A. Systemic antagonism of DRD₃ significantly attenuated cue-induced reinstatement of nicotine-seeking. The same effect was observed upon infusions of SB277011-A into the basolateral amygdala or the lateral habenula, but not the nucleus accumbens. The current findings implicate DRD₃ in cue-induced reinstatement of nicotine, delineate some of the neural substrates underlying this role and support a potential for using selective DRD₃ antagonists for the prevention of relapse to smoking.
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<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
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<tr>
<td>BSR</td>
<td>Brain Stimulation Reward</td>
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<td>CeA</td>
<td>Central Nucleus of the Amygdala</td>
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<td>CPA</td>
<td>Conditioned Place Aversion</td>
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<tr>
<td>CPP</td>
<td>Conditioned Place Preference</td>
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<tr>
<td>CS</td>
<td>Conditioned Stimulus / Stimuli</td>
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<td>DRD&lt;sub&gt;1-5&lt;/sub&gt;</td>
<td>Dopamine D&lt;sub&gt;1-5&lt;/sub&gt; Receptors</td>
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<tr>
<td>DS</td>
<td>Discriminative Stimulus</td>
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<tr>
<td>DSM IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, 4&lt;sup&gt;th&lt;/sup&gt; Edition.</td>
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<tr>
<td>FR</td>
<td>Fixed Ratio</td>
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<tr>
<td>IC</td>
<td>Intracranial</td>
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<td>ICSS</td>
<td>Intracranial Self Stimulation</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IVSA</td>
<td>Intravenous Self-Administration</td>
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<td>LHb</td>
<td>Lateral Habenula</td>
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<td>NAcc</td>
<td>Nucleus Accumbens</td>
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<td>NRT</td>
<td>Nicotine Replacement Therapy</td>
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<td>PR</td>
<td>Progressive Ratio</td>
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<td>SA</td>
<td>Self-Administration</td>
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<td>s.c.</td>
<td>Subcutaneous</td>
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<td>VTA</td>
<td>Ventral Tegmental Area</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1  INTRODUCTION

1.1 Statement of the Problem

Tobacco smoking continues to comprise a major health problem in a growing population all over the world and is considered the world single most preventable cause of death (WHO/WPRO, 2006). In addition, tobacco smoking is considered an addictive disorder, as per the Diagnostic and Statistical Manual of Mental disorders, 4th edition (DSM-IV) (American Psychiatric Association, 2000). A high percentage of the attempts to quit, whether self-directed or treatment-guided, are reportedly followed by relapse (Hughes, Gulliver et al., 1992; Hunt, Barnett et al., 1971). Indeed, several factors can trigger relapse to smoking including exposure to the environmental stimuli previously associated with the smoking (drug-associated cues), stress, or re-exposure to nicotine (Brandon, Tiffany et al., 1990; Kassel, Stroud et al., 2003; O'Brien, Childress et al., 1992; Wikler, 1973).

Therefore, it can be concluded that currently available smoking cessation treatments (such as nicotine replacement therapy, bupropion or varenicline) lack a satisfactory outcome in part because they have exhibited a limited ability to prevent relapse to smoking. Consequently, there is a strong need for a better understanding of the neural mechanisms underlying relapse and for the development of therapies to target these specific mechanisms and pathways, in order to prevent relapse to smoking.

Several lines of evidence implicate the dopaminergic system, and particularly the dopamine D₃ receptors (DRD₃), in the processes underlying relapse to drug seeking and drug-cue-associations (Heidbreder, Gardner et al., 2005; Le Foll, Goldberg et al., 2005). The DRD₃
are expressed in areas in the brain strongly implicated in the mechanisms underlying drug reinforcement and stimulus reward associations (Bouthenet, Souil et al., 1991; Diaz, Lévesque et al., 1995; Diaz, Pilon et al., 2000; Le Foll, Diaz et al., 2003). However, no studies have addressed the role of DRD3 in cue-induced reinstatement of nicotine seeking, or investigated the neural pathways underlying such a role. The current availability of compounds that are highly selective to the DRD3, such as SB 277011-A, PG 01037 and BP 897, facilitates further investigation of the role of this receptor in different aspects of nicotine seeking behaviour (Heidbreder and Newman, 2010; Mason, Hassan et al., 2010; Pilla, Perachon et al., 1999; Reavill, Taylor et al., 2000).

1.2 Overall Purpose and Objectives

The purpose of the current study is to investigate the role of the DRD3 in cue-induced reinstatement of nicotine-seeking behaviour in rats. To this end, four experiments were conducted, using the nicotine self-administration / reinstatement paradigm, to assess the effect of the administration of a highly selective DRD3 antagonist (SB 277011-A), both systemically and by local infusions into discrete brain areas, on cue-induced reinstatement of nicotine-seeking behaviour in rats. The effect of local antagonism of the DRD3, in the basolateral amygdala on food taking behaviour was also assessed. In a separate experiment, the ability of cue-lights per se to maintain operant responding when un-associated with the administration of nicotine was investigated.
The objectives of the current study can be summarized as follows:

**Primary objectives:**

1. Evaluating the effect of systemically blocking the DRD$_3$, using the selective DRD$_3$ antagonist SB 277011-A, on cue-induced reinstatement of nicotine-seeking behaviour in rats.
2. Delineating the neural substrates through which the above effect of DRD$_3$ is mediated. The following areas are investigated: the basolateral amygdala (BLA), the nucleus accumbens (NAcc) and the lateral habenula (LHb).

**Secondary Objectives:**

1. Investigating the effect of blocking the DRD$_3$ in the BLA on food-taking behaviour. This was done to exclude non-specific effects of intra-BLA infusion of SB 277011-A on operant behaviour.
2. Investigating the motivational / reinforcing effects of cue-lights per se, and determining whether the cue-induced reinstatement occurs due to previous association of the cue-lights with the infusions of nicotine, or partially due to primary reinforcing effects of the cue-lights themselves.
1.3 Rationale and Hypotheses

Hypothesis I:
Systemic antagonism of the DRD₃ results in the attenuation of cue-induced reinstatement of nicotine-seeking behaviour.

Rationale:
A strong body of evidence supports a role for the DRD₃ in stimulus-reward associations. Several studies have suggested that DRD₃ antagonism can be effective in decreasing the influence of conditioned-environmental stimuli on drug-seeking behaviour, and thereby reducing the tendency to relapse (Heidbreder, Gardner et al., 2005; Le Foll, Goldberg et al., 2007).

Hypothesis II
The effect of the DRD₃ on cue-induced reinstatement is mediated through the BLA and the LHb, but not through the NAcc.

Rationale:
a) The BLA is clearly involved in conditioned reinforcement processes. Several studies, clinical and behavioural, have indicated a strong role for the BLA in the processes underlying cue-associations (Everitt, Parkinson et al., 1999). Moreover, the DRD₃ in the BLA have been found to play a role in cocaine seeking that is maintained by conditioned stimuli (Di Ciano, 2008).
b) The NAcc is an important structure in the brain reward circuitry with an established role in the mechanisms underlying drug seeking, reinforcement and reward (Corrigall, Franklin et
al., 1992; Pontieri, Tanda et al., 1996). Furthermore, the NAcc is one of the areas exhibiting the highest density of expression of the DRD3 (Diaz, Lévesque et al., 1995). The NAcc DRD3 have been shown be involved in stress-induced reinstatement of cocaine seeking (Xi, Gilbert et al., 2004). However, the current study hypothesizes a limited role for the NAcc DRD3 in the modulation of cue-induced reinstatement of nicotine-seeking. This hypothesis is based on various studies indicating dissociation in the role the NAcc plays in primary reinforcement processes and in cue-associations (Grimm and See, 2000).

c) The LHb has been implicated in the mechanisms underlying reward and learning (Lecourtier and Kelly, 2007; Morissette and Boye, 2008). The role of the LHb in these mechanisms is thought to be mediated through an interaction with the dopamine neurons in the ventral tegmental area (VTA) (Herkenham and Nauta, 1979). The expression of the DRD3 in the LHb (Diaz, Pilon et al., 2000) renders such interaction even more interesting. The current study sought to uncover the role of the DRD3 in the LHb in cue-induced reinstatement of nicotine seeking behaviour, and therefore to provide a better understanding of the neuropharmacology of this area.

**Hypothesis III**

**Antagonism of the DRD3 in the BLA has no effect on food taking behaviour.**

**Rationale:**

Several factors can affect operant responding in general. A significant decrease in operant responding is not always specific to behavioural changes as a result of administration of a certain drug, but could be attributed to other non-specific effects such as: sedation, hypolocomotion, anhedonia for reward in general or even an effect on learning the operant behaviour. In order to control for such non-specific effects of the infusion of the DRD3,
antagonist SB 277011-A, or its vehicle, into the BLA, it is crucial to examine operant responses other than cue-induced reinstatement and reinforcers other than nicotine. Food-taking behaviour was chosen as a behavioural response to be assessed as a measure of behavioural and operant responses after local infusion of SB 277011-A into the BLA.

**Hypothesis IV**

Cue lights *per se*, not – previously – associated with nicotine administration, have no primary reinforcing effect that is strong enough to maintain stable responding.

**Rationale:**

In drug intravenous self-administration paradigms, a conditioned stimulus (CS) (initially neutral in itself) is often paired with the drug infusions, thereby gaining motivational salience and becoming a conditioned (secondary) reinforcer. An important feature of such a CS is its ability to reinforce drug-seeking response even in the absence of the primary reinforcer (O'Brien, Childress et al., 1992; Wikler, 1973), i.e. the ability to reinstate extinguished drug-seeking behaviour. The current experiment sought to verify that such ability of CS (cue-lights in this case) is due to former association with the primary reinforcer (nicotine) and not due to inherently salient or primary reinforcing properties of the cue light itself.
1.4 Review of the Literature

1.4.1 Smoking and Nicotine Dependence

Tobacco smoking comprises a major health problem worldwide. Smoking is considered the world’s most preventable cause of death, where around 50% of the estimated smokers (650 million out of 1.3 billion smokers estimated in 2006) are subject to premature death caused by tobacco-related diseases. At the current rate of smoking, the number of smokers is expected to reach 1.7 billion in 2025 (accounting for at least 10 million deaths/year) (WHO/WPRO, 2006).

In Canada, almost one fifth (17%) of Canadians aged 15 and older are current smokers, as per Health Canada’s “Canadian Tobacco Use Monitoring Survey” (CTUMS, 2009). Moreover, tobacco smoking comprises a disease burden as it is responsible for a large number of fatal and disabling diseases; these include lung cancer, chronic bronchitis, emphysema and ischemic heart diseases.

In addition to the high mortality rate and disease burden, tobacco smoking is considered an addictive disorder when it fulfills the definition and the criteria for substance/drug dependence as per the DSM-IV (American Psychiatric Association, 2000). According to this definition, smoking is considered a form of drug dependence where it follows “A maladaptive pattern of substance use, leading to clinically significant impairment or distress”. Some of the criteria of drug dependence that are most commonly evident for nicotine include: a) the appearance of withdrawal symptoms upon cessation of smoking (such as: irritability, depressed mood, difficulty in concentration and anxiety); relief or avoidance of such symptoms can be achieved by smoking (Hughes, Gust et al., 1991), b) persistent failure of trials to quit and, c) continued use despite the knowledge of possible harm occurring due to nicotine consumption.
Tobacco smoke consists of over 400 chemical components including nicotine, carbon monoxide and tar. Many of these components have carcinogenic potential, and although they can all contribute to the addictive properties of tobacco, nicotine is considered the primary psychoactive as well as the main addictive component of tobacco (Dalhamn, 1972; Hoffmann, Adams et al., 1979; Hoffmann, Rivenson et al., 1979; Stolerman and Shoaib, 1991).

Acute administration of nicotine produces a wide range of pharmacological and psychological effects, contributing to the addictive properties of nicotine. Nicotine produces an increase in heart rate, blood pressure and epinephrine and cortisol levels (Stolerman and Jarvis, 1995). Smoking also produces various pleasurable pharmacological effects, such as mild euphoria, increased concentration and heightened alertness (Etter, Bergman et al., 2000; Pomerleau and Pomerleau, 1992; Stolerman and Jarvis, 1995). These effects give nicotine the ability to reinforce and maintain nicotine-seeking behaviour; i.e. positive reinforcing effects. The reinforcing properties of nicotine have been demonstrated in several studies using animal models, where nicotine has been shown to maintain stable self-administration, induce place preference and lower brain reward threshold (thus inducing brain stimulation reward) (Corrigall and Coen, 1989; Fudala, Teoh et al., 1985; Goldberg and Henningfield, 1988). Negative reinforcement is also considered an important factor in the motivational effects of nicotine, as has been demonstrated in humans (Hughes, Gust et al., 1991) and in rats (Epping-Jordan, Watkins et al., 1998). In humans, withdrawal from nicotine produces undesirable symptoms; such as anxiety, irritability and frustration (Hughes, Gust et al., 1991). These negative symptoms are likely to result in the motivation to seek nicotine and, thus, to increase nicotine seeking behaviour (i.e. negative reinforcement). Animal studies have shown similar somatic symptoms to occur in rats following withdrawal from nicotine, whether spontaneous or precipitated (Epping-Jordan, Watkins et al., 1998; Malin, Lake et al., 1992). Moreover, withdrawal from
nicotine has been shown to markedly elevate brain reward thresholds (Epping-Jordan, Watkins et al., 1998).

It is important to note, however, that although the reinforcing effects of nicotine are important for the initiation and maintenance of nicotine-seeking behaviour, these factors are not the only factors in the process of the development of nicotine dependence. Other factors, like smoking conditioning properties, contribute to the smoking behaviour (Caggiula, Donny et al., 2002b; Shahan, Bickel et al., 1999), as will be discussed in detail below.

Another major feature of nicotine dependence is relapse to smoking. Relapse can be defined as the return of nicotine-seeking / nicotine-taking behaviour after a period of abstinence (See, Fuchs et al., 2003). Although a large percentage of smokers indicate their wish to quit smoking and actually try to quit smoking, the rates of relapse remain very high (Fiore, 2000) (Hunt, Barnett et al., 1971), and the long-term abstinence rates (6 months) remain less than 10% (Hughes, Gulliver et al., 1992). Several factors can predispose to the relapse to smoking, including stress (Kassel, Stroud et al., 2003), re-exposure to nicotine (Brandon, Tiffany et al., 1990) and exposure to the environmental stimuli (cues) that were previously associated with smoking (O'Brien, Childress et al., 1992; Wikler, 1973). Cue-induced relapse is the main interest in the current study and has been addressed by the work demonstrated throughout the present thesis.

As defined by See and colleagues, craving is “the extensive desire for a specific object or experience”, and can be considered the primary factor motivating relapse (See, Fuchs et al., 2003). In humans, the exposure to stimuli reliably associated with different drugs of abuse has been shown to induce cravings and increased motivation towards drug seeking (Carter and Tiffany, 1999; Childress, 1993; O'Brien, Childress et al., 1992; O'Brien and McLellan, 1996). Moreover, the exposure to smoking-related cues causes regional activation in the brains of
abstinent human smokers (Goudriaan, De Ruiter et al., 2010). Numerous studies have indicated a similar effect in animals, where the reintroduction of conditioned stimuli previously associated with the intake of the drug has been shown to significantly reinstate drug-seeking behaviour after its extinction in their absence (Davis and Smith, 1976; See, Grimm et al., 1999).

Currently, a few medications are available as aids for smoking cessation. However, several limitations exist to the effective use of these medications in smoking cessation therapy and prevention of relapse. Nicotine replacement therapy (NRT), bupropion and varenicline constitute the first line of currently available smoking cessation therapies.

- NRT is available as nicotine gum, patch, spray, inhaler or lozenge. This line of treatment is effective in alleviating withdrawal symptoms; however, its efficacy in decreasing the reinforcing effects of nicotine remains limited (Moolchan, Robinson et al., 2005).

- Bupropion, which was originally used as an antidepressant, has been shown to decrease the reinforcing effects of nicotine and to alleviate withdrawal symptoms. However, several side effects of bupropion should be taken into consideration, such as increased risk of seizures and depression (Mooney and Sofuoglu, 2006).

- Varenicline is an α4β2 nicotinic receptor partial agonist, which improves withdrawal symptoms and mood in abstinent smokers (Rollema, Chambers et al., 2007). However, in some cases, varenicline use has been reportedly associated with suicidal thoughts and depression.

The fact that long term abstinence rates remain low and relapse rates remain high (as mentioned above), despite the current availability of therapeutic agents for smoking cessation, indicates the limited success of the currently available medications. In other words, the currently available therapies may be either less specific or less effective than it is to be hoped for. Investigation of novel therapeutic agents should, therefore, take into account an extensive understanding of the neurobiological mechanisms of nicotine dependence and relapse and
specifically target these mechanisms and pathways. Such understanding can be reached by the integration of different research approaches and models including preclinical models, genetic studies and clinical trials.

In the current study, an animal model of nicotine self-administration / reinstatement has been utilized in order to assess the role of the DRD3 in cue-induced relapse to nicotine seeking. To better understand the parameters and utility of the paradigm used in the current study, an overview of the animal models used in studying various aspects of drug-taking / drug-seeking behaviour is covered in the next section. This will be followed by a review of the neurological mechanisms of nicotine dependence and cue-induced relapse. Subsequently, a detailed literature review will be provided highlighting the studies investigating the role of the dopaminergic system and receptors in substance dependence in general, with a special focus on nicotine.

1.4.2 Animal Models of Drug-seeking and Drug-taking Behaviours

Before proceeding with a background on the specific mechanisms involved in nicotine dependence and cue-induced relapse, it is important to discuss one of the established approaches in studying drug-seeking / drug-taking behaviours, and one which was also used in the current study, i.e. animal modelling. Although it may be difficult to mimic the entire aspects of a certain human condition in animal models, the utility of such models remains substantial for understanding specific mechanisms underlying this condition. For many psychiatric disorders, as well as drug addiction, the use of animal models has long been established as a measure of specific signs and symptoms or certain behaviours related to psychiatric disorders and / or substance use (Markou, Weiss et al., 1993). Thus, animal models gain both construct and predictive validity not only providing a better understanding of these complex disorders, but
also allowing the investigation of the potential utility of different pharmacological substances as therapeutic agents for these disorders (Ebel, 1961; Willner, 1984).

Different methods can be used to classify animal models used for studying drug use, as in regards of the behaviour they model, the property of the drugs of abuse they evaluate or the type of validity they exhibit, etc.

Animal models have been developed to mimic the different stages of progression of drug use from the stage of drug taking to withdrawal to relapse and craving. For purposes of simplification, these stages will be used to highlight some examples of the animal models for studying drug taking / drug seeking behaviours in Table 1.1.
Table 1.1
Examples of animal models for the study of drug-seeking / drug-taking behaviours

<table>
<thead>
<tr>
<th>Stage of drug-taking behaviour</th>
<th>Examples of animal models</th>
<th>Utility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Models of Drug</td>
<td>Drug self-administration</td>
<td>* Assessment of the primary reinforcing / rewarding effects of drugs. * Measures - Primary reinforcing effects, - Reinforcing efficacy and, - Motivational effects of the drugs.</td>
<td>(Caine and Koob, 1993; Collins, Weeks et al., 1984; Schindler, Panlilio et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>* Fixed ratio schedule (FR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Progressive ratio schedule (PR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Second order schedule</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain stimulation reward (BSR)</td>
<td>* Assessment of the reward value.</td>
<td>(Kornetsky and Esposito, 1979)</td>
</tr>
<tr>
<td></td>
<td>Conditioned place preference (CPP)</td>
<td>* Assessment of primary reward and the conditioning of drug reinforcement.</td>
<td>(van der Kooy, 1987)</td>
</tr>
<tr>
<td></td>
<td>Drug discrimination</td>
<td>* Assessment of the interoceptive cue state produced by the drug.</td>
<td>(Holtzman, 1985)</td>
</tr>
<tr>
<td>Models of Craving and Relapse</td>
<td>Resistance to extinction following self-administration</td>
<td>* Measures motivational effects of the drug.</td>
<td>(Schuster and Woods, 1968)</td>
</tr>
</tbody>
</table>
### Table 1.1 (Continued)

**Examples of animal models for the study of drug-seeking / drug-taking behaviours**

<table>
<thead>
<tr>
<th>Stage of drug-taking behaviour</th>
<th>Examples of animal models</th>
<th>Utility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Models of Craving and Relapse</strong> (Continued)</td>
<td>* Second order schedules of reinforcement</td>
<td>* Measure drug-seeking behaviour that is maintained by the presentation of drug-associated cues.</td>
<td>(Everitt and Robbins, 2000; Schindler, Panlilio et al., 2002)</td>
</tr>
</tbody>
</table>
| **Models of Withdrawal** | * Conditioned place aversion (CPA)  
* Intracranial self-stimulation (ICSS) | * Assessment of the aversive effects of withdrawal.  
* Assessment of the changes in the reward circuitry over the course of the addiction process.  
* Understanding the neurobiological mechanisms of the drugs of abuse, and the counter-adaptive mechanisms that drive the addiction process. | (Kornetsky and Esposito, 1979; Stinus, Le Moal et al., 1990) (Holtzman, 1985) |

Reinstatement models have been developed primarily to assess the ability of different factors to trigger reinstatement of an extinguished drug-seeking behaviour in abstinent animals, and have since been used to study the neurobiological mechanisms underlying the relapse phenomenon. Despite some procedural differences between the animal models of reinstatement and the actual situation of relapse in humans, these models have shown a strong validity for the study of relapse processes, over time. This validity is exhibited by the correspondence between the outcomes of different studies assessing triggers of relapse to drug seeking after a period of withdrawal (Breiter, Gollub et al., 1997; Shiffman, Paty et al., 1996; Sinha, 2001), also see (Shaham, Shalev et al., 2003) for a review on reinstatement models.
Since the current study is mainly dealing with cue-induced relapse to nicotine seeking, it is important to review the different types of cue-reinstatement, as well as other methods that are used for studying the conditioning properties of drugs and conditioned reinforcement in general. Different types of modelling of conditioned stimuli have been described in the literature.

One of the earliest types of conditioned stimuli to be developed and the most commonly used in animal models is the **discrete conditioned stimulus (CS)**, where the administration of the drug is accompanied by the contingent presentation of a discrete cue, commonly an auditory or a visual stimulus. Such association not only results in an increase in the level of responding of the animals to the administered drug, but also imparts motivational salience onto the CS (Lesage, Burroughs et al., 2004). The CS, therefore, becomes a conditioned reinforcer able to reinstate extinguished drug-seeking behaviour in the absence of the drug as the primary reinforcer (Davis and Smith, 1976). The reinforcing properties of the CS seem to be dependent upon contingency of their presentation with the administration of the drug where non-contingent presentation of the CS was found to have little effect on the reinstatement of drug-seeking behaviour (de Wit and Stewart, 1981; Di Ciano, Underwood et al., 2003; Fuchs, Tran-Nguyen et al., 1998; Grimm and See, 2000).

**Discriminative stimuli (DS)** are a different type of conditioned stimuli that were more recently used in cue-reinstatement models. The presentation of the DS (which could be a tone or a light) precedes the administration of the drug and predicts the availability of the drug (Ettenberg, MacConell et al., 1996; Weiss, Maldonado-Vlaar et al., 2000). The reintroduction of the DS was shown to reliably reinstate extinguished drug-seeking behaviour (Gracy, Dankiewicz et al., 2000; McFarland and Ettenberg, 1997). Interestingly, unlike discrete conditioned stimuli (CS), contingency of DS presentation with the responding was found to be not critically important for influencing drug-seeking behaviour (Di Ciano, Underwood et al., 2003).
Another method used for modelling the drug-associated-stimuli-induced reinstatement of drug-seeking behaviour is the **context-induced reinstatement**, where context refers to the physical environment of the test chambers. In this paradigm drug-seeking behaviour is extinguished in a context that is different from the one where the drug had been administered. Upon returning the animals to the original drug-associated context, the drug-seeking behaviour was found to be significantly reinstated (Crombag, Grimm et al., 2002).

Cue-induced reinstatement models are not the only method used to assess the conditioning properties of drugs or stimulus-reward associations. Another paradigm that is commonly used for this purpose is the **appetitive pavlovian conditioning** paradigm. In this paradigm behavioural autoshaping is first established where the presentation of a stimulus (for example: visual) precedes the delivery of reward (usually food), in a different location within the test chamber. This presentation is non-contingent upon the animal’s responding. As a result, the animal develops a conditioned response of approaching the stimulus predictive of the reward, prior to collecting the reward itself. Pavlovian to instrumental transfer can then be performed where the delivery of the reward becomes contingent upon the animal’s responding, thereby concerning active behaviour and its consequences (Bussey, Everitt et al., 1997). This paradigm can be used, not only to assess the conditioning properties of different drugs, but also to study the different mechanisms and pathways regulating different stages of cue-association learning (Everitt, Parkinson et al., 1999).

**Second order schedules of reinforcement** have also been used to assess the conditioning properties of drugs through measuring the level of responding to the self-administered drug which is maintained by the presentation of drug-associated cues (Arroyo, Markou et al., 1999; Spear, Muntaner et al., 1991). Briefly, in this paradigm, a brief (usually visual) stimulus is presented upon completion of a component or unit of the schedule (for
example a certain number of lever presses or nose pokes i.e. [FR\textsubscript{y};S]). Upon completion of the full schedule (after a certain number of the above responses; i.e. FR\textsubscript{y}[FR\textsubscript{y};S]), the stimulus is introduced together with the drug (primary reinforcer). Testing can be performed in two stages; a drug free interval (where the responding is maintained by the presentation of the stimulus alone) and a second interval where drug is available.

It is important to note that the method of presentation of the conditioned stimuli may vary in the above models and that the above procedures may seem to be modelling different aspects of the stimulus-reward association process (i.e. discrete CS vs. contextual cues vs. pavlovian conditioning, etc.). The reinstatement triggered by each type of the above stimuli may even be mediated through different neuronal substrates within the brain stimulus-reward circuitry. Nonetheless, the integrated knowledge of the data collected using all of these paradigms remains crucial for further understanding the different mechanisms underlying the conditioning process and, therefore, cue-induced relapse.

In the current study, a discrete conditioned stimulus (cue-light) was used as a conditioned reinforcer, where self-administered nicotine infusions were accompanied by the contingent presentation of a cue-light under a fixed ratio schedule of reinforcement. After extinction of the nicotine self-administration behaviour (in the absence of both nicotine and the cue-light), the cue-light was reintroduced to achieve reinstatement of nicotine-seeking behaviour. The effect of DRD\textsubscript{3} antagonism (systemic and local) on cue-induced reinstatement of nicotine-seeking behaviour was then assessed.
1.4.3 Neurocircuitry of Nicotine Reinforcement and Reward

Although the work in the current thesis mainly addresses conditioning properties of nicotine and cue-induced reinstatement of nicotine seeking, it remains of critical importance to highlight the basics of the neurocircuitry underlying the primary reinforcing properties of nicotine, and the mechanisms regulating nicotine seeking behaviour.

Nicotine exerts its actions through binding to nicotinic acetylcholine receptors (nAChR) which are expressed peripherally as well as centrally in the brain. Specifically, activation of the nAChR is critically involved in the reinforcing actions of nicotine, where the administration of nAChR antagonist has been shown to block self-administration of nicotine (Corrigall, Coen et al., 1994; Corrigall, Franklin et al., 1992). The neurocircuitry involved in mediating the reinforcing effects of nicotine appears to be complex, and to involve integrating multiple neurochemical systems. However, the mesolimbic dopamine system is believed to be the main system implicated in mediating the reinforcing / rewarding effects of nicotine. This is supported by several lines of evidence, first, the nAChR are densely expressed in the VTA, which is the area containing the cell bodies of the dopamine neurons, as well as the NAcc, at the terminals of the dopamine neurons (Clarke and Pert, 1985; Schwartz, Lehmann et al., 1984). Second, nicotine administration increases the firing of dopamine neurons in the VTA (Clarke and Pert, 1985; Pidoplichko, DeBiasi et al., 1997). Third, activation of the nAChR by nicotine (systemically administered or locally infused in the VTA or NAcc) induces dopamine release (Nisell, Nomikos et al., 1994). This action was antagonised by concomitant intra-VTA, but not intra-NAcc, infusions of the nicotinic antagonist mecamylamine (Nisell, Nomikos et al., 1994). It is worth mentioning that this effect of nicotine on dopamine release is a common effect shared by different drugs of abuse (Pontieri, Tanda et al., 1996). Furthermore, studies have shown that
dopaminergic lesions, by the infusion of 6-hydroxydopamine in the NAcc (Corrigall, Franklin et al., 1992), or systemic antagonism of dopamine receptors (D₁ and D₂) (Corrigall and Coen, 1991) significantly reduce nicotine self-administration. In addition, nAChR antagonism in the VTA has resulted in the attenuation of nicotine self-administration as well as nicotine-induced potentiation of brain stimulation reward (Corrigall, Coen et al., 1994; Panagis, Kastellakis et al., 2000).

Although the dopaminergic system is a key player in modulating the effects of nicotine, it is not the only component in the neurocircuitry underlying these effects. A role has been suggested for other neurochemical systems in the brain, including the cholinergic (Lanca, Adamson et al., 2000), glutamatergic (McGehee, Heath et al., 1995), GABAergic (Kalivas, Churchill et al., 1993), serotonergic (Ribeiro, Bettiker et al., 1993), opioid (Pierzchala, Houdi et al., 1987) and cannabinoid systems (Cohen, Kodas et al., 2005; Forget, Hamon et al., 2005; Le Foll, Forget et al., 2008). However, the involvement of the above systems in the mechanisms underlying nicotine reward and reinforcement is also believed to be through an interaction with the dopaminergic system.

1.4.4 Craving and Cue-induced Relapse

Cue-induced relapse and craving are prominent features of nicotine dependence as well as of dependence on other addictive substances. Drug craving is a term that applies to an intense desire to obtain and consume the drug. This desire is not only driven by the urge to gain the pleasurable and rewarding effects of the drug, but also to avoid experiencing the negative effects of withdrawal. Craving is commonly elicited by the exposure to the environmental stimuli that were previously associated with the intake of the drug (Carter and Tiffany, 1999; Childress, 1993; O'Brien, Childress et al., 1992; O'Brien and McLellan, 1996) and results in an increase in
the motivation for drug seeking ultimately ending in relapse (See, Fuchs et al., 2003). Repeated and effective pairing of the drug taking behaviour with certain stimuli has been shown to gain motivational salience to these stimuli (See, Fuchs et al., 2003). Over time, the conditioned stimuli themselves become secondary reinforcers increasing the actual drug taking behaviour (Caggiula, Donny et al., 2002b), and inducing drug seeking behaviour in cases of abstinence; thus gaining control over drug seeking behaviour (Everitt and Wolf, 2002).

Although the reinforcing effects of nicotine, such as euphoria, are more subtle than other drugs, the abuse potential of nicotine and rate of relapse remain comparably high (Goldberg, Spealman et al., 1981). A possible explanation for this discrepancy is the presence of other non-pharmacological factors that gain incentive salience through their association with nicotine intake, further reinforce nicotine-seeking behaviour and predispose to the high rates of relapse encountered in human smokers. Indeed, several animal studies have indicated a strong role for the environmental stimuli associated with nicotine intake in maintaining nicotine-seeking behaviour (Donny, Caggiula et al., 1999; Rose and Corrigall, 1997). In 2001, Caggiula and co-workers demonstrated an important role for nicotine-associated cues in maintaining nicotine self-administration in rats (Caggiula, Donny et al., 2001). In their study, the rats self-administered nicotine that was not paired with cues at a much lower rate than they self-administered cue-paired nicotine (Caggiula, Donny et al., 2001). Moreover, following a period of extinction, the reintroduction of nicotine together with its associated cues successfully reinstated nicotine-seeking behaviour to a higher degree than that resulting following the reintroduction of nicotine alone.

Taken together, a strong body of evidence supports an important role for nicotine-associated cues in maintaining nicotine seeking, as well as in relapse.
1.4.5 Neurocircuitry of Cue-induced Relapse – Role of the BLA and NAcc

The neural mechanisms underlying the processes of conditioned reinforcement and its role in relapse, and the specific areas in the brain involved in these processes have been the focus of a large body of research.

In humans, imaging studies have revealed a relation between the dopaminergic system and cue-induced cravings. In 2006, using PET imaging, Wong and colleagues found an increase in dopamine receptor occupancy in the dorsal striatum of frequent cocaine users following the exposure to cocaine-related cues (Wong, Kuwabara et al., 2006). This increase was directly proportional to the intensity of craving experienced by the subjects as a result of exposure to the cues (Wong, Kuwabara et al., 2006). Similarly, in the same year, another study found an increase in extracellular dopamine in the dorsal striatum, upon exposure to cocaine-related cues (Volkow, Wang et al., 2006). Moreover, drug-induced increase in extracellular striatal dopamine was insufficient to produce craving, in cocaine abusers, unless it was accompanied by the presentation of cocaine-associated cues (Volkow, Wang et al., 2008).

Animal models have also widely investigated the neurocircuitry of conditioned reinforcement and cue-induced relapse. The amygdala and the NAcc and their dopaminergic innervations have long been viewed as key structures underlying the processes of reinforcement and relapse. However, the literature shows that these two areas have differential effects on different aspects of relapse. Moreover, subdivisions of the amygdala and the NAcc have exhibited differential functional roles in modulating conditioned reinforcement and cue-induced relapse. Although substantial advancements have been made in the field of studying the neural substrates mediating cue-induced relapse, the exact mechanisms regulating this process remain to be fully elucidated. Moreover, some areas in the brain, which may have also an important role in this process, remain understudied in this regard, such as the LHb.
The basolateral region (BLA) as well as the central nucleus (CeA) of the amygdala have been shown to be involved in reward-related processes and conditioned reinforcement. The role of the BLA in conditioned reinforcement is supported by numerous studies. First, the presentation of drug-associated conditioned stimuli resulted in an increase in cFos expression in the BLA. In a study by Brown and colleagues, pairing of cocaine injections with a cocaine-paired environment produced an increase in cFos expressions in the BLA and LHb among other areas, whereas the NAcc did not exhibit such an increase (Brown, Robertson et al., 1992). A similar increase in cFos expression was observed in the BLA upon the reintroduction of a DS which was previously predictive of the availability of intravenous cocaine (Ciccocioppo, Sanna et al., 2001). The latter effect was significantly blocked by the administration of DRD1 antagonists (Ciccocioppo, Sanna et al., 2001). Secondly, BLA lesions disrupted the responding to different types of conditioned stimuli. Bilateral excitotoxic lesions in the BLA, using infusions of N-methyl-D-aspartate (NMDA), disrupted the responding of rats to conditioned stimuli that were previously paired with the administration of water, and attenuated the potentiation of conditioned reinforcement responses by intra-NAcc infusions of D-amphetamine (Cador, Robbins et al., 1989). NMDA lesions of the BLA also disrupted responding using a pavlovian second order conditioning. In this case, a light paired with food administration was unable to acquire secondary reinforcing properties, in the BLA lesioned rats, and was therefore unable to reinforce the acquisition of a second order conditioning, by the presentation of tone-light pairings in the absence of food (Hatfield, Han et al., 1996). In another study, bilateral NMDA lesions of the BLA resulted in the attenuation of reinstatement response of cocaine seeking induced by the presentation of discriminative cues (Meil and See, 1997). Similarly, quinolinic acid lesions of the BLA impaired conditioned place preference for cocaine without an effect on cocaine-induced hyperlocomotion, indicating a role for the BLA only in stimulus-reward conditioning rather than the unconditioned psychomotor effects of cocaine (Brown and
Moreover, BLA lesions inhibited the acquisition of a response to conditioned stimuli, indicating that an intact BLA is important for the acquisition as well as the expression of the reinstatement (Everitt, Parkinson et al., 1999; Kruzich and See, 2001).

The dopaminergic input to the BLA, in specific, has been strongly implicated in conditioned reinforcement processes. In 1998, Tran-Nguyen and co-workers demonstrated an elevation in dopamine levels in the amygdala in rats upon returning to the chambers where they self-administered cocaine following a month of withdrawal (Tran-Nguyen, Fuchs et al., 1998). Furthermore, intra-BLA infusions of DRD₁ agonists and antagonists and DRD₂-like antagonists have been shown to modulate conditioned fear expression (Lamont and Kokkinidis, 1998) and retention (Guarraci, Frohardt et al., 2000; Guarraci, Frohardt et al., 1999), as well as the acquisition of cue-association learning and responses for conditioned stimuli (Berglind, Case et al., 2006; See, Kruzich et al., 2001). More recently, in 2008, Di Ciano demonstrated an attenuation of cocaine-seeking behaviour that is maintained by the presentation of cocaine-associated cues, under a second order schedule of reinforcement, in rats treated with intra-BLA infusions with the selective DRD₃ antagonist SB 277011-A (Di Ciano, 2008). Infusion of the same ligand into the NAcc or dorsal striatum was without effect on cocaine seeking under the same second order schedule (Di Ciano, 2008).

Dissociation has been shown in the roles of the CeA versus the BLA in conditioned learning processes. For example, Everitt and co-workers first trained the rats to associate the presentation of a conditioned light stimulus (CS) with the delivery of food until the rats developed a conditioned response to the presentation of the CS. In their study, bilateral lesions of the CeA, but not the BLA, were shown to disrupt the acquisition of appetitive pavlovian conditioning (Everitt, Parkinson et al., 1999). Conversely, whereas BLA lesions attenuated conditioned reinforcement responses, lesions of the CeA were ineffective in this regard (Everitt, Parkinson et al., 1999). The CeA and BLA also exhibited different roles in modulating fear
conditioning (Killcross, Robbins et al., 1997). Therefore, it can be concluded that each of these subdivisions is responsible for a certain aspect of the conditioning process and stimulus-reward association learning.

The NAcc also plays a complex role in the regulation of reward-related processes. A marked dissociation can be observed in the roles played by the NAcc shell and the NAcc core in the conditioning process. For example, an increase in cFos expression occurs in the NAcc shell following the exposure to cocaine-paired stimuli, but not in the NAcc core (Neisewander, Baker et al., 2000). On the other hand, NAcc neurons in both subregions show indistinguishable firing rates during a DS task (Jones, Day et al., 2010b). Lesions of the NAcc core significantly attenuated pavlovian conditioning as well as conditioned reinforcement responses, but had no effect on the potentiation of conditioned reinforcement induced by intra-NAcc infusions of D-amphetamine (Everitt, Parkinson et al., 1999). The opposite was true for the NAcc shell (Everitt, Parkinson et al., 1999). The dopaminergic input to the NAcc is believed to be of critical importance to the role played by the NAcc in reward processing. A closer investigation of the dopaminergic innervations to the NAcc, revealed that dopamine concentrations in the NAcc exhibit subsecond fluctuations at rest (Wightman, Heien et al., 2007), which are potentiated by the administration of drugs of abuse (Stuber, Roitman et al., 2005). Dopaminergic depletion from the NAcc, as a whole, prevented the formation of a pavlovian conditioning response (Everitt, Parkinson et al., 1999). In addition, intra-NAcc infusions of D-amphetamine, an indirect dopamine agonist, increased conditioned responses (Everitt, Parkinson et al., 1999).

Conversely, Neisewander and colleagues reported no change in the NAcc dopamine levels upon the reintroduction of cocaine-associated cues (Neisewander, Le et al., 1996). Similarly, the contingent presentation of cocaine-associated cues under a second order schedule was without effect on NAcc dopamine release in rats (Ito, Dalley et al., 2000) and in rhesus monkeys (Bradberry, Barrett-Larimore et al., 2000). Moreover, the presentation of amphetamine-
associated CS was found to have no effect on NAcc dopamine signal (Di Ciano, Blaha et al., 2001).

Therefore, it can be concluded, by the preponderance of evidence to date, that the BLA is a focal structure in the modulation of stimulus-reward-associations and conditioned reinforcement. It can also be concluded that, although some changes have been reported in the NAcc as a consequence of exposure to drug-associated conditioned stimuli, these changes may not reflect a critical role for the NAcc in the regulation of stimulus-reward associations, or conditioned reinforcement processes.

1.4.6 The Mesocorticolimbic Dopamine System

The dopaminergic system arises primarily from the dopamine neurones located in the VTA, and the sustantia nigra pars compacta, and projecting to the NAcc, amygdala and prefrontal cortex. These structures are in turn interconnected through glutamatergic pathways. Moreover, cholinergic fibres connect the above structures to basal forebrain structures and pedunculopontine nucleus and lateral dorsal tegmentum (Kelley, 2002). The dopamine neurons also innervate other areas such as the bed nucleus of the stria terminalis and the lateral septum [a detailed review has been presented in (Di Chiara, 1995)]. Therefore, the mesolimbic dopamine system appears to be situated in the centre of a circuit containing the structures most implicated in reward processing and the mechanisms underlying the regulation of reinforcements.

The dopaminergic system has long been believed to have a key role in the expression of goal directed behaviour of drugs of abuse, including nicotine, as well as natural rewards (Carboni, Imperato et al., 1989; Corrigall, Franklin et al., 1992; Di Ciano and Everitt, 2004). Moreover, a clear involvement has been demonstrated for the dopaminergic system in the
conditioned reinforcing properties of drugs of abuse and their associated stimuli (Schultz, 1998; Stuber, Klanker et al., 2008). Dopamine has also been implicated in the development of behavioural sensitization, which occurs following the repeated administration of drugs of abuse and non-drug stimuli (Kalivas and Stewart, 1991).

A large body of evidence supports the role of the dopaminergic system in cue-associations. For example, in 1981, Miller and colleagues demonstrated, using albino rats, an increase in the neuronal firing of the dopamine neurons in the VTA, and substantia nigra in response to conditioning task using either a DS or a CS associated with the availability of reward in the form of chocolate milk (Miller, Sanghera et al., 1981). Similarly, phasic neuronal responses were recorded in the monkey dopamine neurons in response to conditioned stimuli (Schultz, Apicella et al., 1993). Moreover, in 2008, Stuber et al. observed that CS predictive of sucrose availability (in an appetitive pavlovian task) not only resulted in an increase in the firing of the dopamine neurons, but also increased the synaptic strength over these neurons (Stuber, Klanker et al., 2008). Additionally, dopaminergic neurons are believed to mediate reward-related error signals (Ljungberg, Apicella et al., 1992). In other words, dopaminergic neuronal firing has been shown to increase following the presentation of an unexpected reward and to cease following the omission of an expected reward (Fiorillo, Newsome et al., 2008).

In summary, the mesocorticolimbic dopamine system is believed to be of major importance in regulating reward processing and conditioned reinforcement. This suggests an important role for pharmacological agents modulating the dopamine pathways and receptors, as potential therapeutic agents for the treatment of drug addiction and relapse to drug seeking.
1.4.7 Dopaminergic Receptors

Dopamine exerts its action through binding to dopamine receptors (DRD$_1$ – 5). Dopamine receptors are a type of G-protein coupled receptors and are mainly expressed in the central nervous system. Dopamine receptors have been classified into two subgroups based on their structural homology, affinities to pharmacological ligands and their downstream effects on cyclic AMP (cAMP); namely DRD$_1$-like and DRD$_2$-like receptors (Seeman and Van Tol, 1994). Table 1.2 depicts the main differences between the two subgroups. A detailed review on different types of dopaminergic receptors can be found in (Le Foll, Gallo et al., 2009).

Table 1.2
The differences between the dopamine receptor subtypes

<table>
<thead>
<tr>
<th>DRD$_1$-like Dopamine Receptors</th>
<th>DRD$_2$-like Dopamine Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD$_1$ and DRD$_5$</td>
<td>DRD$_2$, DRD$_3$ and DRD$_4$</td>
</tr>
<tr>
<td>Activation of the members of this group stimulates adenylyl cyclase and cAMP</td>
<td>Activation of the members of this group inhibits adenylyl cyclase and cAMP</td>
</tr>
<tr>
<td>Show low to moderate affinity for antipsychotic drugs</td>
<td>Show moderate to high affinity for antipsychotic drugs</td>
</tr>
</tbody>
</table>

The main focus of the current study is the investigation of the role of the DRD$_3$ in cue-induced reinstatement of nicotine-seeking behaviour. However, a brief overview of the role of other dopamine receptors in drug addiction will be presented before reviewing the available literature on the involvement of the DRD$_3$ in drug addiction.
1.4.7.1 The Dopamine D₁ Receptor (DRD₁)

The DRD₁ was first cloned by Dearly and colleagues in 1990 (Dearry, Gringrich et al., 1990) and is encoded by an intronless gene on chromosome 5 (Sunahara, Niznik et al., 1990). In addition, it is the most abundantly expressed dopamine receptor. The DRD₁ mRNA is expressed in all the areas of the nigrostriatal and mesocorticolimbic dopamine system (Fremeau, Duncan et al., 1991). The DRD₁ is also found in distinct striatal areas expressing substance P, dynorphin and co-expressing the DRD₃ (Diaz, Lévesque et al., 1995; Diaz, Pilon et al., 2000).

Several studies have indicated an involvement of the DRD₁ in the rewarding effects of drugs of abuse, as well as their locomotor effects. Animal studies have shown that systemic blockade of the DRD₁ attenuates nicotine self-administration (Corrigall and Coen, 1991), morphine self-administration (Glick and Cox, 1975), heroin intake (Gerrits, Ramsey et al., 1994) and alcohol-seeking behaviour, and decreases the reinforcing effects of cocaine in rodents (Koob, Le et al., 1987) and in non-human primates (Bergman, Kamien et al., 1990). Moreover, local infusions of DRD₁ antagonists into the NAcc shell blocked the acquisition of nicotine-induced conditioned-place preference (Spina, Fenu et al., 2006), decreased the reinforcing effects of cocaine (Bari and Pierce, 2005) and attenuated context-induced reinstatement of heroin seeking (Bossert, Poles et al., 2007). In the BLA, DRD₁ seem to be more involved in cue and cocaine-priming induced reinstatement of cocaine seeking (Norman, Norman et al., 1999; See, Kruzich et al., 2001).

Taken together, the aforementioned studies indicate that the DRD₁ are implicated in different aspects of drug-seeking behaviour, and can be potential targets for the treatment of drug addiction.
1.4.7.2 The Dopamine D2 Receptor (DRD2)

The DRD2 was the first of the dopamine receptors to be cloned in 1988 (Bunzow, Van Tol et al., 1988). It is also abundantly expressed in the brain in all the dopaminergic areas (Bouthenet, Souil et al., 1991). The areas showing the highest levels of expression of the DRD2 mRNA are the dopaminergic neurons in the VTA and substantia nigra, where the DRD2 acts as an autoreceptor (Dickinson, Sabeti et al., 1999; Mercuri, Saiardi et al., 1997). An increase in the DRD2 expression has been found to occur following 6-hydroxy-dopamine lesions in rats (Gerfen, Engber et al., 1990), as well as following chronic antipsychotic drug treatment (Martres, Sokoloff et al., 1992).

The involvement of the DRD2 in drug-seeking behaviours appears to be of a complex nature. In human smokers, DRD2 blockers increase and DRD2 agonists decrease smoking behaviour (Caskey, Jarvik et al., 1999). Animal studies have shown that a DRD2 antagonist decreases nicotine self-administration (Corrigall and Coen, 1991), and the intracranial administration of a DRD2 agonist significantly reduced intracranial self-administration of nicotine (Ikemoto, Qin et al., 2006). Similarly, discrepant effects have been observed on heroin-seeking behaviour, where both DRD2 agonists and antagonists were shown to decrease heroin taking (Hemby, Smith et al., 1996; Rowlett, Platt et al., 2007). On the other hand, only DRD2 antagonists were able to block morphine-induced conditioned place preference, whereas the agonists had the opposite effect (Kuribara, 1995; Rezayof, Zarrindast et al., 2003).

Under a low ratio requirement schedule (FR5), the administration of DRD2 blockers reduced the rewarding effect of cocaine (increasing self-administration behaviour), whereas the DRD2 agonists produced the opposite effect (Caine and Koob, 1994; Caine, Negus et al., 2002). However, under schedules of high response requirements (such as PR schedules and FR15)
DRD_2 antagonists decreased cocaine self-administration which has been explained as due to a decrease in the reinforcing effects of cocaine (Caine and Koob, 1994; Hubner and Moreton, 1991). Moreover, DRD_2 knock-out mice showed a higher rate of cocaine self-administration than their wild type controls, suggesting the DRD_2 may be needed to limit the rate of self-administration of the high doses of cocaine (Caine, Negus et al., 2002).

1.4.7.3 The Dopamine D_4 Receptor (DRD_4)

The DRD_4 was cloned in 1991 by Van Tol et al. (Van Tol, Bunzow et al., 1991). In comparison to other dopamine receptors, the density of the DRD_4 expression in the brain is relatively low. The areas exhibiting the highest density of expression of the DRD_4 mRNA include the retina, cerebral cortex, amygdala, pituitary, cerebellum and hypothalamus (Cohen, Todd et al., 1992; Mei, Griffon et al., 1995; Valerio, Belloni et al., 1994).

Very few preclinical studies have been conducted investigating the role of the DRD_4 in drug addiction. A role is suggested for the DRD_4 in the discriminative effects of nicotine (Brioni, Kim et al., 1994) and morphine-induced withdrawal symptoms (Mamiya, Matsumura et al., 2004). However, further studies are needed to uncover the exact role of the DRD_4 in nicotine dependence and drug addiction in general.

1.4.7.4 The Dopamine D_5 Receptor (DRD_5)

Sunahara and co-workers were the first to characterize the DRD_5 in 1991 (Sunahara, Guan et al., 1991). The expression of the DRD_5 in the rat brain appears to be restricted to the hippocampus, parafascicular thalamic nucleus and the mammillary bodies (Mamiya, Matsumura et al., 2004; Meador-Woodruff, Mansour et al., 1992). The structural homology between the DRD_1 and the DRD_5 has comprised a difficulty in assessing the exact areas of expression of the
DRD₃. Similarly, the lack of ligands that are selective to the DRD₃ has rendered it difficult to investigate its role in drug addiction using preclinical studies.

1.4.7.5 The Dopamine D₃ Receptor (DRD₃)

The DRD₃ was first characterized in 1990 by Pierre Sokoloff and co-workers (Sokoloff, Giros et al., 1990). Of all the dopamine receptor subtypes, the DRD₃ has the highest binding affinity to endogenous dopamine. Unlike the DRD₁ and the DRD₂, the DRD₃ has a restricted pattern of expression in the brain (Bouthenet, Souil et al., 1991; Diaz, Lévesque et al., 1995; Diaz, Pilon et al., 2000; Le Foll, Diaz et al., 2003; Le Foll, Schwartz et al., 2003). The areas exhibiting the highest areas of expression of the DRD₃ in the rat brain include the islands of Calleja, the olfactory tubercle and the NAcc (Sokoloff, Giros et al., 1990). Nonetheless, the DRD₃ was shown to be expressed in other brain areas, with varying densities, including the medial prefrontal cortex, ventral pallidum, BLA and LHb (Bouthenet, Souil et al., 1991). In addition, high levels of the DRD₃ mRNA are found throughout the dopaminergic system including the VTA, bed nucleus of stria terminalis, CeA and NAcc (Diaz, Pilon et al., 2000). Therefore, the DRD₃ appears to be expressed in brain areas that are strongly implicated in the reinforcing effects of drugs of abuse, and reward processing (Koob, 1992).

There is a growing body of evidence implicating the DRD₃ in drug addiction, and especially in the processes underlying relapse. In humans, the density of the DRD₃ has been shown to be elevated in long-term cocaine abusers, and in the brains of cocaine overdose victims (Segal, Moraes et al., 1997; Staley and Mash, 1996). Animal studies also revealed a selective increase in the DRD₃ expression in the brains of rats and mice treated with nicotine (Le Foll, Diaz et al., 2003; Le Foll, Schwartz et al., 2003), cocaine (Le Foll, Francès et al., 2002; Neisewander, Fuchs et al., 2004), morphine (Spangler, Goddard et al., 2003) and alcohol
(Vengeliene, Leonardi-Essmann et al., 2006). Such changes were not observed in DRD\textsubscript{1} or DRD\textsubscript{2} (Le Foll, Diaz et al., 2003; Le Foll, Schwartz et al., 2003).

Various pharmacological approaches have been explored to study the function of the DRD\textsubscript{3} and to modulate the DRD\textsubscript{3} transmission. Different pharmacological agents have been used as DRD\textsubscript{3} ligands. A special interest has arisen in DRD\textsubscript{3} partial agonists and antagonists as potential therapeutic agents especially for reducing relapse (Heidbreder, Gardner et al., 2005; Heidbreder and Newman, 2010). However, a lack of selectivity to the DRD\textsubscript{3} has always comprised an obstacle to the understanding of the functions of this receptor as most of these ligands exhibited a partial affinity to the DRD\textsubscript{2} as well.

Nevertheless, advances in the medicinal chemistry have recently resulted in the development of a group of drugs that have a higher selectivity to the DRD\textsubscript{3} than the DRD\textsubscript{2}. The first selective ligand to be tested was the DRD\textsubscript{3} partial agonist BP 897, which has 70 fold selectivity for DRD\textsubscript{3} over DRD\textsubscript{2} (Pilla, Perachon et al., 1999). This was rapidly followed by the development of the highly selective DRD\textsubscript{3} antagonist SB 277011-A exhibiting a 100 fold selectivity for the DRD\textsubscript{3} over the DRD\textsubscript{2} (Reavill, Taylor et al., 2000), which was used throughout the current study. In addition to the higher selectivity exhibited by SB 277011-A at DRD\textsubscript{3} over DRD\textsubscript{2}, it has no agonist activity, and exhibits similar potencies at rat and human DRD\textsubscript{3}. SB 277011-A has good bioavailability after oral administration and good CNS penetration and its plasma half life in rats is two hours (Reavill, Taylor et al., 2000). Moreover, at the range of doses that are selective to DRD\textsubscript{3}, SB 277011-A is non-cataleptogenic (up to 78.8 mg/kg), doesn’t induce hyperprolactenemia and has no effect on locomotor activity per se, and no alteration in stimulant-induced hyperactivity (up to 42.3 mg/kg) (Reavill, Taylor et al., 2000). In the current study SB 277011-A was used as the selective DRD\textsubscript{3} antagonist. The range of doses of SB 277-11-A used in the current study was chosen based on previous studies where the same range of
doses has been shown to block nicotine-induced place preference and reactivity to nicotine-associated stimuli (Le Foll, Schwartz et al., 2003)(Pak, Ashby et al., 2006).

Currently, more compounds are available that have a high potency and selectivity for the DRD$_3$ such as NGB 2904, PG 01037 (Mason, Hassan et al., 2010) and others (see (Heidbreder and Newman, 2010) for a review on the advances in DRD$_3$ antagonists as pharmacotherapeutics for addiction). Such advancements in this field will result in better understanding of the mechanisms through which the DRD$_3$ are involved in drug seeking and relapse.

Numerous studies have used animal models, to investigate the role of DRD$_3$ ligands in different aspects of drug seeking and drug taking behaviours. The most important studies in this regard are summarized in Table 1.3.
Table 1.3

The role of DRD3 partial agonists and antagonists in different aspects of drug seeking and drug taking behaviours

<table>
<thead>
<tr>
<th>Reinforcer</th>
<th>BSR</th>
<th>SA</th>
<th>Reinstatement Models</th>
<th>Second order</th>
<th>CPP</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nicotine</strong></td>
<td>SB 277011-A, NGB 2904: Attenuation</td>
<td>BP 897: No effect on FR. SB 277011-A: No effect on FR. High doses of SB 277011-A decrease SA on PR</td>
<td>SB 277011-A: Attenuation of nicotine primed reinstatement. BP 897: No effect on cue-induced reinstatement.</td>
<td>N/A</td>
<td>BP 897, ST 198 and SB 277011-A: Block the expression of CPP.</td>
<td>(Andreoli, Tessari et al., 2003; Khaled, Farid Araki et al., 2010; Le Foll, Sokoloff et al., 2005; Pak, Ashby et al., 2006; Ross, Corrigall et al., 2007)</td>
</tr>
<tr>
<td><strong>Cocaine</strong></td>
<td>SB 277011-A, NGB 2904: Attenuation</td>
<td>SB 277011-A, NGB 2904: No effect on FR, lowering PR break point</td>
<td>SB 277011-A, NGB 2904: Attenuation of cue-,stress- and cocaine-induced reinstatement</td>
<td>SB 277011-A and BP 897: Attenuation. Intra-BLA infusion of SB 277011-A: Attenuation</td>
<td>SB 277011-A: Block acquisition and expression.</td>
<td>(Cervo, Burbassi et al., 2005; Cervo, Cocco et al., 2006; Di Ciano, Underwood et al., 2003; Pilla, Perachon et al., 1999; Vorel, Ashby et al., 2002; Xi, Gilbert et al., 2004; Xi, Gilbert et al., 2005; Xi, Newman et al., 2006)</td>
</tr>
</tbody>
</table>
Table 1.3 (Continued)
The role of DRD3 partial agonists and antagonists in different aspects of drug seeking and drug taking behaviours

<table>
<thead>
<tr>
<th>Reinforcer</th>
<th>BSR</th>
<th>SA</th>
<th>Reinstatement Models</th>
<th>Second order</th>
<th>CPP</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methamphetamine</td>
<td>SB 277011-A, NGB 2904, BP 897 and PG01037: Attenuation</td>
<td>PG01037: No effect on FR CJB090 and PG01037 attenuate SA under PR</td>
<td>PG01037: Attenuation of cue-induced reinstatement</td>
<td>N/A</td>
<td>N/A</td>
<td>(Higley, Spiller et al., 2010; Orio, Wee et al., 2010; Xi and Gardner, 2007)</td>
</tr>
<tr>
<td>Opiates</td>
<td>NGB 2904: Attenuation of heroin induced BSR</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>SB 277011-A, BP 897: Block morphine, heroin induced CPP</td>
<td>(Ashby, Paul et al., 2003; Francès, Le Foll et al., 2004; Xi and Gardner, 2007)</td>
</tr>
<tr>
<td>Food</td>
<td>N/A</td>
<td>SB 277011-A: Attenuation of food SA under FR, no effect on PR NGB 2904: No effect on FR</td>
<td>SB 277011-A: No effect on cue-induced reinstatement</td>
<td>SB 277011-A: No effect</td>
<td>SB 277011-A: No effect</td>
<td>(Cervo, Cocco et al., 2006; Di Ciano, Underwood et al., 2003; Thanos, Michaelides et al., 2008; Vorel, Ashby et al., 2002)</td>
</tr>
</tbody>
</table>

**BSR:** Brain stimulation reward, **SA:** Self-administration, **CPP:** Conditioned place preference, **FR:** Fixed ratio schedule, **PR:** Progressive ratio schedule, **SB 277011-A, NGB 2904, ST 198, PG01037 and CJB090** are selective DRD3 antagonists and **BP 897** is a selective DRD3 partial agonist. **N/A:** No available data.
It can be concluded from the above table (Table 1.3) that the DRD₃ antagonists seem to be involved in some, but not all, aspects of drug taking and drug seeking behaviour. Indeed, selective DRD₃ antagonists have a strong potential as therapeutic agents in the field of drug addiction, and specifically for the prevention of relapse. Such potential arises from several integrating factors, and when taken into consideration, render the DRD₃ selective antagonists promising as successful therapeutic agents in this field. These factors include:

1. The DRD₃ antagonists do not produce many of the side effects that may interfere with daily activities. For example, the DRD₃ antagonists have no effect on locomotor activity, whether spontaneous or stimulus-induced, nor on motor coordination (Reavill, Taylor et al., 2000). Notably, the DRD₃ antagonists cause no impairment in attention, learning or memory (Millan, Di Cara et al., 2007; Sarter and Parikh, 2005). In fact, the DRD₃ antagonists seem to be involved in a mechanism that enhances cognition, attention and learning (Glickstein, Desteno et al., 2005; Sarter and Parikh, 2005). Moreover, the selective DRD₃ antagonists are not cataleptogenic and do not produce hyperprolactenemia (Reavill, Taylor et al., 2000). Finally, social interaction is enhanced with blocking the DRD₃ (Millan, Loiseau et al., 2008), and helplessness, anhedonia and despair were not observed in knock-out mice lacking the DRD₃ (Chourbaji, Brandwein et al., 2008).

2. The use of the DRD₃ receptor antagonists to prevent relapse, triggered by different factors, in animal models has proven to be highly successful (Table 1.3). However, this class of drugs has been shown to have little or no effect on the actual drug taking behaviour (Table 1.3). Therefore, it seems that the DRD₃ antagonists target a specific aspect of drug addiction, which is relapse. Indeed, the decision to stop using a certain drug depends primarily on the readiness and motivation of the individual to quit. Although some medications have shown some usefulness in this aspect, it remains critical to control the newly abstinent individuals’ urge to relapse. Especially at this stage, the DRD₃ antagonists present an optimal choice for
the prevention of relapse. It is important to note, however, that there is no evidence that the use of the DRD3 antagonists will be helpful in alleviating the withdrawal symptoms of drugs of abuse. Further studies are needed to investigate this possibility.

3. The DRD3 antagonists appear to have no significant effect on natural reinforcers, such as food taking behaviour. However, a potential utility for the DRD3 antagonists has been suggested by the fact that SB 277011-A was shown to affect food taking in a rat model of obesity (Thanos, Michaelides et al., 2008).

4. Preclinical studies have clearly indicated that DRD3 antagonists do not have an abuse potential. When substituted for cocaine, SB 277011-A and NGB 2904 (two highly selective DRD3 antagonists) could not maintain stable self-administration on their own.

Therefore, based on the available data from preclinical studies, it can be concluded that the DRD3 antagonists present a relatively safe and reliable option for the prevention of relapse to drug use. The extrapolation of the above findings to humans requires the conduction of a series of clinical studies to validate the utility of these agents in substance dependant individuals.
1.5 Restatement of the Hypotheses

Hypothesis I
Systemic antagonism of the DRD$_3$ results in the attenuation of cue-induced reinstatement of nicotine-seeking behaviour.

Hypothesis II
The effect of the DRD$_3$ on cue-induced reinstatement is mediated through the BLA and the LHb but not through the NAcc.

Hypothesis III
Antagonism of the DRD$_3$ in the BLA has no effect on food taking behaviour.

Hypothesis IV
Cue lights *per se*, un-associated with nicotine administration, have no primary reinforcing effect that is strong enough to maintain stable responding.
Chapter 2 METHODOLOGY

2.1 Animals

Male, Long–Evans rats (Charles River, Canada) experimentally naive at the start of the study and initially weighing 250–275 g were used for all experiments. All rats were individually housed in a temperature controlled environment on a 12-hour reversed light/dark cycle (lights off 07:00 am). Prior to any experimental manipulation, animals were given a minimum of 7 days to habituate to the colony room, during which time they were weighed, handled and received unlimited access to both food and water. Subsequently, upon beginning the experimental training, the rats were food-restricted to 20 g of chow per day, in order to maintain their body weight throughout the experiment. All the experimental procedures described in this thesis were carried out in compliance with the guidelines of the Canadian Council on Animal Care (compatible with the US ‘Guide for Care and Use of Laboratory Animals’), and were reviewed and approved by the institutional Animal Care Committee.

2.2 Apparatus

Self-administration sessions were carried out in commercially available experimental chambers (Med Associates, USA) (see Forget et al. 2009 for details), encased in sound-attenuating boxes and equipped with two levers, a house-light and two cue-lights, one located above each lever. In each experiment, for half the animals the left lever was active and the right was inactive and for the other half the opposite applied. Session start was signalled by the illumination of the house-light and presentation of the levers. Rapid delivery of the self-administered drug (approximately 1-s delivery time) was achieved with infusion pumps (Med Associates Model PHM-104). The volume of infusion was adjusted according to the weight of
the rat, in the range of 28-45 µl. Pressing on the active lever resulted in the delivery of nicotine (30 µg/kg per infusion) when schedule requirements were met, accompanied by extinction of the house-light and illumination of the cue-light above the active lever. Pressing on the inactive lever was recorded, but had no consequences.

2.3 Experimental procedures

2.3.1 Experiment (1): Systemic Administration of SB 277011-A and Cue-induced Reinstatement of Nicotine-seeking Behaviour

The experimental procedures for the current experiment are schematically shown in Figure 2.1.

2.3.1.1 Food Training

Techniques for initial training were similar to those previously reported (Corrigall and Coen, 1989; Forget, Coen et al., 2009; Khaled, Farid Araki et al., 2010). The training was performed in 1-hour daily sessions for 5 days where the animals were trained to press the lever for food reinforcement on a continuous reinforcement schedule. Under this schedule, each press on the active lever resulted in the delivery of a 45-mg food pellet. During these training sessions, the house-light was on, but the lever light was off.

2.3.1.2 Intravenous Catheter Implantation Surgery

Once trained, the animals were subjected to surgery for intravenous catheter implantation. Surgical procedures for implantation of chronic intravenous catheters were conducted using strict aseptic technique and were ethically approved by the institutional Animal Care Committee. Surgery was performed under anaesthesia induced by xylazine (10 mg/kg i.p.) and ketamine hydrochloride (90 mg/kg i.p.). Incision sites were infiltrated with the local
anaesthetic Marcaine (0.125%). Buprenorphine was given for post-operative analgesia (0.03 mg/kg s.c.). Catheters (constructed in the laboratory prior to surgery) were driven through the scapular incision, under the skin and under the rat’s right shoulder to be implanted into the right jugular vein. Catheters were sutured in place and further secured using cyano-acrylic glue. Incision sites were closed, and a single dose of penicillin (30 000 U i.m.) was administered at the completion of surgical procedures. Surgical procedures were similar to those previously reported (Corrigall and Coen, 1989; Forget, Coen et al., 2009; Forget, Hamon et al., 2009). Also, please see the attached standard operating procedure (SOP) for intravenous catheterization surgery (Appendix II) for details.

Animals were allowed to recover for a 1-week period before drug self-administration sessions were begun.

2.3.1.3 Nicotine Intravenous Self-administration (IVSA) Procedures

Acquisition of nicotine self-administration was performed under a Fixed Ratio (FR) schedule of reinforcement at a unit dose of 30µg/kg per infusion of nicotine base. Self-administration training was carried out in daily 1 hour sessions. Pressing on the active lever for a fixed predetermined number of times resulted in an infusion of nicotine followed by a time-out period of 1 minute, during which the house-light was extinguished and the cue-light above the active lever was illuminated. Pressing on the inactive lever had no scheduled consequences. Rats were randomly assigned the right or the left lever as the active lever. During the first week of acquisition, response requirement was FR1, i.e., each active lever press during the time-in period resulted in the delivery of one infusion. Response requirements were then gradually increased to reach a final value of FR5, by which time the self-administration behaviour was stable after 15- to 20-days history of training. Self-administration sessions occurred mostly 5 days a week. Rats were considered to have acquired stable nicotine self-administration when
they received a minimum of 10 infusions per 1-hour session and had <20% variation in the number of infusions earned per session during three consecutive sessions. Moreover, by the end of the FR5 training phase, all the rats pressed on the active lever more than twice the number of times they pressed the inactive lever. Once stability was achieved, the rats were carried into extinction and reinstatement testing as described below.

2.3.1.4 Extinction Training and Cue-induced Reinstatement of Nicotine-seeking

After the end of the 3- to 4-week acquisition training under the FR schedule, an extinction phase was conducted by withholding nicotine and its associated cues (house-light stays on and cue-light stays off throughout the session). Responses on the active or inactive levers were recorded, but had no consequence. An extinction criterion was established for each animal individually and was defined as total active lever responses being < 20 active lever presses or <15% of the mean active lever responding maintained during the last 3 days of FR5 for nicotine, whichever is less. The extinction criterion had to be reached for two consecutive days in order to conduct the testing. Nicotine-trained rats (n=8) were tested for the effect of SB 277011-A (i.p.) on cue-induced reinstatement of nicotine-seeking behaviour at the following doses: vehicle (10% hydroxpropyl-β-cyclodextrin in sterile water), or SB 277011-A at 1, 3 and 10 mg/kg. All tests were carried out in a counter-balanced within-subject design. After each test, extinction was re-established until the extinction criterion was obtained for at least two consecutive days. Reinstatement tests were conducted under conditions identical to that of self-administration, except that the responses on the active lever (on a FR5 schedule) resulted in contingent presentation of the cues (light above the active lever on and house-light off for 60 s) without nicotine availability (no infusions). Responses on the inactive lever were recorded without consequences. Each testing session lasted 1 hour.
Figure 2.1
Schematic Diagram for the Experimental Procedures Performed in Experiment 1

IV: Intravenous, IVSA: Intravenous self-administration
2.3.2 Experiments (2-4): Local Infusion of SB 277011-A into Discrete Brain Areas and Cue-induced Reinstatement of Nicotine-seeking Behaviour

The experimental procedures for the local infusion experiments are schematically shown in Figure 2.1.

2.3.2.1 Food Training

The food training phase was the same as that described in section 2.3.1.1.

2.3.2.2 Intravenous Catheter Implantation Surgery

Procedures for intravenous catheter implantation surgery were the same as described in section 2.3.1.2.

2.3.2.3 Nicotine Self-administration Procedures

Procedures for intravenous nicotine self-administration were the same as described in section 2.3.1.3 with the following exceptions: A fixed ratio schedule of reinforcement was followed for 15 days (FR1: 5 days, FR2: 3 days and FR5: 7 days). After that the rats were subjected the intracranial cannula implantation surgeries. After a period of recovery (7-10 days), IVSA sessions were resumed for 2-3 days, depending on catheter patency. This was done to verify that the surgical procedure did not have an effect of the self-administration behaviour. However, in a few rats (n=4), resumption of the IVSA sessions was not possible due to catheter blockade during the recovery phase.

2.3.2.4 Intracranial Cannula Implantation Surgery

Following the acquisition of nicotine self-administration, surgeries were performed for the implantation of intracranial cannulae into: the basolateral amygdala (BLA), the nucleus accumbens (NAcc), or the lateral habenula (LHb). Stereotaxic surgical procedures were
conducted using the same regimen of anesthetics, analgesic, and antibiotic described in section 2.3.1.2. Strict aseptic technique was observed in all procedures, which were also ethically approved by the institutional Animal Care Committee.

Animals were positioned in stereotaxic frame and guide cannulae (22 gauge, Plastics One) were bilaterally implanted according to the following coordinates (being lowered to 2.0 mm above the target site): BLA: –2.5 AP, ±5 ML and –6.6 DV, NAcc: +1.6 AP, ±1.7 ML and -4.7 DV (using a 6° off-vertical angle of approach), and LHb: –3.6 AP, ±2.5 ML and –3.1 DV (using a 20° off-vertical angle of approach), according to the atlas of Paxinos and Watson 1998 (Paxinos and Watson, 1998). Cannulae were anchored to small screws, threaded partially into the skull, with dental acrylic. Cannulae-occluders (28 gauge, Plastics One) were inserted into the guide cannulae immediately after the surgery to maintain patency and restrict the entry of foreign material into the brain in between the infusions. The occluders extended 2.0 mm lower than then the cannulae; i.e. reaching the target site. This was done to ensure that no damage would occur upon the introduction of the microinjectors in the first testing sessions which might have had an effect on the responding or interpretation of the testing results. Please see the attached standard operating procedure (SOP) for intracranial cannulae implantation surgery (Appendix III) for details.

Following the surgery, rats were allowed to recover for 7-10 days before resuming experimental procedures.

**2.3.2.5 Procedures for Local Infusion of SB 277011-A and Cue-induced reinstatement of Nicotine-seeking**

Extinction training was carried out following recovery from the surgical procedures, and re-stabilization of the self-administration behaviour (where applicable). Upon stabilization of
the extinction behaviour, according to the criterion previously mentioned (section 2.3.1.4), rats were randomized to receive intracranial microinfusions of the vehicle or different doses of SB 277011-A, according to a counterbalanced within-subject design.

Microinjectors (28 gauge, Plastics One) were connected to 50 μl Hamilton syringes via polyethylene tubing (inner diameter 0.38 mm; Plastics One, Roanoke, Virginia) filled with sterile water, separated by a bubble from either vehicle or SB 277011-A. Microinjectors were inserted into the guide cannulae, after removal of the occluders, and were 2.0 mm longer than the guide cannulae, i.e. extending to the target site. A volume of 0.5 μl of SB 277011-A (0.01, 0.1 or 1 μg/0.5 μl) or vehicle (10% DMSO in 10% w/v hydroxyl propyl-β-cyclodextrin in sterile water) was infused on each side over the course of 1 minute using a microinfusion pump (Harvard Apparatus, Model 22, SouthNatick, Massachusetts), 5–10 minutes before a test session. The microinjectors were left in place for 1 minute after the infusion, to allow for diffusion, after which the microinjectors were removed, and occluders were replaced. Reinstatement testing was conducted using the same procedures described in section 2.3.1.4, and rats were required to maintain stable extinction levels for at least 2 consecutive days before the next testing session. At the end of the experiment, each rat had received a total of 4 microinfusions with at least 2 days in between one testing session and the next.
Schematic Diagram for the Experimental Procedures Performed in Experiments 2-4

**IV:** Intravenous, **IVSA:** Intravenous self-administration, **IC:** Intracranial
2.3.3 Experiment (5): Local Infusion of SB 277011-A into the Basolateral Amygdala and Food-taking Behaviour

In this experiment, the rats (n=6) were trained similarly to the nicotine self-administration experiments, except for the following differences: Completion of the required ratio (FR1, FR2 or FR5) was rewarded with a 45 mg sucrose pellet instead of an infusion of nicotine; therefore, no intravenous surgery was needed in these rats. After stabilization of the responding on the active lever on FR5 (5-7 days), the rats were subjected to intracranial surgery for implantation of guide cannulae into the BLA, following the same procedure and the same coordinates previously described in section 2.3.2.4. 7-10 days were allowed for recovery, following which the rats were again trained on FR5 schedule to ensure that there was no residual effect of surgery on the level of responding. The behaviour was considered to be stable if the difference between any 2 consecutive days was less than 20%. In this case, the rats were ready for testing. On the testing day, vehicle or the highest dose of SB 277011-A (1μg/0.5μl/side) was bilaterally infused into the BLA using the same techniques described in section 2.3.2.5, 5 minutes prior the start of the testing session. The effect of SB 277011-A or vehicle on food taking behaviour was assessed.

2.3.4 Experiment (6): Responding Maintained by Light Cues

A control experiment was performed to evaluate the responding maintained by the light-cues presentation only. The training was identical as the nicotine self-administration experiments, except that there was no surgery for catheter implantation and no nicotine delivery. Rats (n=8) were first trained to respond for food (lever light off, as mentioned above) on a continuous reinforcement schedule for 5 days. Then, responding of rats for cue presentation only (without food delivery) was measured under a FR schedule of reinforcement. Session duration was 60 minute, and a time-out period of 1 minute followed each ratio completion on
the active lever during which time the house-light was extinguished and the cue-light above the active lever was illuminated. During the first week of acquisition, response requirement was FR1. Response requirements were then gradually increased to reach a final value of FR5.

2.4 Drugs

Nicotine hydrogen tartrate (Sigma-Aldrich, USA) was dissolved in saline, the pH was adjusted to 7.0 (±0.2), and the solution was filtered through a 0.22-μm syringe filter (Fisher Scientific, USA) for sterilization purposes. All nicotine doses are reported as free base concentrations. Nicotine was administered intravenously in a volume of 100 ml/kg per infusion. SB 277011-A (trans-N-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide) was provided by Dr Steven Goldberg (NIH, NIDA, IRP), and synthesized by Dr Jenő Varga and Dr József Gaál (MegaPharma Kft., Budapest, Hungary), as part of an ongoing research collaboration on involvement of dopamine D3 receptors in drug reward mechanisms. For the systemic administration experiment (Experiment 1), SB 277011-A was dissolved in 10% w/v hydroxypropyl-β-cyclodextrin in sterile water and sonicated for 30 minutes. The drug was administered intraperitoneally (i.p.), 30 min prior to testing in a volume of 2 ml/kg. For the local infusion experiments (Experiments 2-5), SB 277011-A was dissolved in 10% DMSO in 10% w/v hydroxypropyl-β-cyclodextrin in sterile water. The drug was locally infused into the specified areas in the brain, 5 minutes prior to testing, in a volume of 0.5 μl / side.

Figure 2.3
Structure of SB 277011-A (Reavill, Taylor et al., 2000)
2.5 Histological Examination

Upon completion of the behavioural testing, rats were overdosed with pentobarbital (approximately 350 mg/kg, i.p.) and 0.5 μl of cresyl violet dye was infused bilaterally into the target area using microinjectors inserted into the guide cannulae (procedure identical to the procedure for microinfusion of the drug described in section 2.3.2.5). The rats were then decapitated. Brains were dissected out, and flash-frozen in methyl butane on dry ice. Brains were stored at –65 to –80°C until sectioning. Serial coronal sections (25 μm-thick) were obtained using a cryostat at –25°C. Close to the target area, every 4th-5th section was thaw mounted on pre-cleaned glass slides, and the sections were examined for verification of the placement. Acceptable histology required that the tip of the injector bilaterally lay within the target area.

2.6 Data Analysis

The number of active and inactive lever presses and the number of nicotine infusions were recorded and analysed. A one-way analysis of variance (ANOVA) with repeated measurements was used to analyze the effects of SB 277011-A (systemic or locally infused) or vehicle on cue-induced reinstatement, or on food taking behaviour. Newman–Keuls post-hoc test was used to assess the differences between individual means (baseline, vehicle and different doses of SB 277011-A).

To assess the responding, on active and inactive levers, maintained by cues only, a two-way ANOVA with repeated measurements (factors being time and levers), was performed followed by LSD post-hoc test.
Chapter 3  RESULTS

3.1  Experiment (1) Effect of SB 277011-A (1, 3, 10 mg/kg i.p.) on cue-induced reinstatement of nicotine-seeking

All the animals (n=8) achieved significant self-administration of nicotine over 4 weeks of acquisition (5 d under FR1, 3 d under FR2, 12 d under FR5 schedules). Significant extinction was also achieved after 12 sessions of extinction [the mean number of active lever presses dropped from 132.56 (±18.67) over the last 3 days of acquisition to 15.56 (±3.23) during the last 3 days of extinction] (see Figure 3.1 which is also representative of a typical acquisition curve obtained under the schedule of reinforcements applied in all the nicotine self-administration experiments mentioned below). Figure 3.2 presents data related to the effects of SB 277011-A, or vehicle, on the responding during reinstatement testing. A one-way ANOVA with repeated measurements showed a significant group effect of treatment (SB 277011-A or vehicle) (F4,28=8.99, p<0.01). Neuman–Keuls post-hoc tests revealed that significant reinstatement was obtained in the vehicle-treated group as compared to the extinction baseline (average of active lever responding on the days before the testing) (p<0.01) and that all the doses of SB 277011-A tested (1–10 mg/kg i.p., 30 min before the session) significantly reduced reinstatement of nicotine-seeking behaviour (p<0.01), compared to responding in the vehicle-treated group (Figure 3.2). No significant difference was observed between the effects of the different doses of SB 277011-A (p>0.05, Neuman–Keuls post-hoc tests). Moreover, neither the vehicle nor the drug had a significant effect on inactive lever pressing (F4,28=1.51, p>0.1 with one-way ANOVA) (Figure 3.2).
Figure 3.1
This figure represents a typical acquisition curve in one of the groups of rats included in the current study.

A) The level of responding during acquisition (under a fixed ratio (FR) schedule of reinforcement) and extinction phases of nicotine self-administration. Data are expressed as mean (±SEM) of the number of active (–●--) and inactive (–○--) lever presses. B) The mean number (±SEM) of reinforcements (nicotine infusions) obtained during the acquisition phase (FR1-5).
Systemic Administration of SB 277011-A significantly attenuates cue-induced reinstatement of nicotine-seeking behaviour

**Figure 3.2**
This figure represents the effect of SB 277011-A (1, 3, 10 mg/kg), or vehicle (0), on the mean number (± SEM) of active (●) and inactive (○) lever presses under conditions for cue-induced reinstatement of nicotine-seeking. Significant reinstatement was obtained in the vehicle-treated group (††† p<0.005 vs. baseline (BSL) with two-tailed t test). SB 277011-A significantly reduced cue-induced reinstatement at all the doses tested (n=8) (** p<0.01 vs. vehicle with repeated-measures ANOVA). No effect of vehicle or drug was seen on inactive lever presses.

Figure from (Khaled, Farid Araki et al., 2010), with permission.
3.2 Experiment (2): Effect of Local Infusion of SB 277011-A into the BLA on Cue-induced Reinstatement of Nicotine-seeking Behaviour

Similarly to experiment (1), animals in this group (n=7) achieved stable nicotine self-administration after 3 weeks of training (including 7 days on FR5). Neither the intracranial surgery nor the period of recovery had a significant residual effect on the level of responding on the active lever. The mean number of active lever presses was 171.3 (± 1.8) during the 3 days of FR5 prior to surgery and became 169.3 (± 17.5) during the 3 days of FR5 following the surgery and recovery (for rats n=5). However, for 2 rats, this could not be verified due to blockade of the intravenous catheter during the recovery period. The 2 rats were run on extinction directly after the recovering from the surgeries. Nicotine self-administration behaviour was successfully extinguished after 9 days of extinction (the mean number of active lever presses dropped to 13.3 (± 5.9) over the last 3 days of extinction). The mean number of lever presses exerted upon the reintroduction of nicotine-associated cues was subjected to statistical analysis to evaluate the effect of intra-BLA infusion of SB 277011-A on cue-induced reinstatement of nicotine-seeking behaviour. A one-way ANOVA with repeated measurements showed a significant group effect of treatment (SB 277011-A or vehicle) on the number of active lever pressing (F4,24=8.01, p<0.01). Neuman-Keuls post-hoc test revealed that significant reinstatement was obtained in the vehicle-treated group compared to the extinction baseline (average of active lever responding on the days before the testing) (p<0.01) and that all the doses of SB 277011-A tested (0.01, 0.1 and 1 μg/μl/side infused into the BLA 5 min before the session) significantly reduced reinstatement of nicotine-seeking behaviour (p<0.01), compared to responding in the vehicle-treated group (Figure 3.3). No significant difference was observed between the effects of the different doses of SB 277011-A (p>0.05). Neither the vehicle nor the drug had a significant effect on inactive lever pressing (F4,24=1.62, p=0.2 with one-way ANOVA) (Figure 3.3).
Intra-BLA Administration of SB 277011-A abolishes cue-induced reinstatement of nicotine-seeking behaviour

![Graph showing the effect of SB 277011-A on lever presses under nicotine-cue-induced reinstatement conditions.](image)

**Figure 3.3**
This figure represents the effect of SB 277011-A (0.01, 0.1, 1 μg/μl/side), or vehicle (0), locally infused into the BLA, on the mean number (± SEM) of active (●) and inactive (○) lever presses under conditions for cue-induced reinstatement of nicotine-seeking. Significant reinstatement was obtained in the vehicle-treated group (††† p<0.05 vs. baseline with two-tailed t test). SB 277011-A significantly reduced cue-induced reinstatement at all the doses tested (n=7) (** p<0.01 vs. vehicle with repeated-measures ANOVA). No effect of vehicle or drug was seen on inactive lever presses.
3.3 Experiment (3): Effect of Local Infusion of SB 277011-A into the NAcc on Cue-induced Reinstatement of Nicotine-seeking Behaviour

In this group of rats (n=8), stable self-administration was also achieved after 3 weeks of training (including 7 days on FR5). No effect of intracranial surgery or recovery was noted on the active lever responding following the recovery period. In the last 3 days prior to cannula-implantation surgery, the level of active lever responding under FR5 schedule was 114.2 (±14.1). Following 7-10 days of recovery, the level of mean active lever responding under FR5 schedule was 126 (±19.2). Re-stabilization of the self-administration behaviour was conducted for 2 sessions and in only 6 out of 8 rats, due to issues with intravenous catheter blockade. Successful extinction of nicotine self-administration was achieved after 9 sessions of extinction training, during the last 3 sessions of which the number of active lever responding declined to 23.5 (±11.4).

A one-way ANOVA with repeated measurements was also used in this experiment to examine the effect of intra-NAcc infusion of SB 277011-A on cue-induced reinstatement of nicotine seeking. The one way repeated measures ANOVA showed a significant group effect of treatment (SB 277011-A or vehicle) (F4,28=11.74, p<0.01). Neuman–Keuls post-hoc test revealed that significant reinstatement was obtained in the vehicle-treated group, as well as the groups treated by different doses of SB 277011-A (0.01-1 μg/μl/side), compared to the extinction baseline (p<0.05). Moreover, no significant difference in the level of reinstatement was shown between the vehicle-treated group versus the groups treated with different doses of SB 277011-A (0.01-1μg/μl/side) (p>0.05) (Figure 3.4).

Neither the vehicle nor the drug had a significant effect on inactive lever pressing (F4,28=0.25, p=0.95 with one-way ANOVA) (Figure 3.4).
Intra-NAcc Administration of SB 277011-A Has No Effect on Cue-induced Reinstatement of Nicotine-seeking Behaviour

![Graph showing the effect of SB 277011-A on lever presses](image)

**Figure 3.4**

This figure represents the effect of SB 277011-A (0.01, 0.1, 1 μg/μl/side), or vehicle (0), locally infused into the NAcc, on the mean number (± SEM) of active (–●–) and inactive (–○–) lever presses under conditions for cue-induced reinstatement of nicotine-seeking. Significant reinstatement was obtained in the vehicle-treated group as well as the group treated with SB 277011-A (*** p<0.05 vs. baseline with one way ANOVA). Moreover, the level of active lever responding in the SB 277011-A – treated group was not significantly different from the vehicle-treated group (ns, p>0.05 with one way ANOVA). No effect of vehicle or drug was seen on inactive lever presses.
3.4 Experiment (4): Effect of Local Infusion of SB 277011-A into the LHb on Cue-induced Reinstatement of Nicotine-seeking Behaviour

In this group of rats (n=7), stable nicotine self-administration was achieved after 14 days of training (including 6 days of FR5). Due to technical difficulties with maintaining catheter patency during the intracranial surgery and recovery periods, re-stabilization of responding was carried out for one session only following the recovery from the cannulae implantation surgery. Nonetheless, no notable change was observed in the number of active lever responding prior to or after the surgery and recovery. The mean number of active lever presses was 124.9 (± 19.1) during the last 3 days of FR5 preceding the surgery and 107.1 (± 13.9) after the recovery from the surgery. Successful extinction was achieved after 7 sessions of extinction training, where the mean number of active lever responding declined to 17.9 (± 3.3) during the last 3 days of extinction.

To assess the effect of intra-LHb infusion of SB 277011-A on cue-induced reinstatement of nicotine-seeking behaviour, the mean number of lever responding resulting from the reintroduction of the cues was analysed similarly to the above experiments. A one way ANOVA with repeated measurements revealed a significant group effect of treatment (SB 277011-A or vehicle) on the number of active lever presses (F4,24=6.20, p<0.05). Neuman-Keuls post-hoc test revealed that significant reinstatement was obtained in the vehicle-treated group compared to the extinction baseline (p=0.01) and that all the doses of SB 277011-A tested (0.01, 0.1 and 1 μg/μl/side infused into the LHb, 5 min before the session) significantly reduced reinstatement of nicotine-seeking behaviour (p<0.05), compared to responding in the vehicle-treated group (Figure 3.5). No significant difference was observed between the effect of different doses of SB 277011-A (p>0.05). Neither the vehicle nor the drug had a significant effect on inactive lever pressing (F4,24=0.84, p=0.51 with one-way ANOVA) (Figure 3.5).
Intra-LHb Administration of SB 277011-A attenuates cue-induced reinstatement of nicotine-seeking behaviour

Figure 3.5
This figure represents the effect of SB 277011-A (0.01, 0.1, 1 μg/μl/side), or vehicle (0), locally infused into the LHb, on the number of active (●--) and inactive (○--) lever presses under conditions for cue-induced reinstatement of nicotine-seeking. Significant reinstatement was obtained in the vehicle-treated group (†† p=0.01 vs. baseline with two-tailed t test). SB 277011-A significantly reduced cue-induced reinstatement at all the doses tested (n=7) (** p<0.05 vs. vehicle with repeated-measures ANOVA). No effect of vehicle or drug was seen on inactive lever presses.
3.5 Experiment (5): Effect of Local Infusion of SB 277011-A into the BLA on Food-taking Behaviour

In this group of rats (n=5), stable acquisition of food taking behaviour was achieved after training similar to that described in the above experiments (including 5 days on FR5). No effect of surgery or recovery was observed on the number of lever presses, or the number of reinforcements (food pellets) obtained under FR5 schedule of reinforcements. Figure 3.6 shows the level of responding on active and inactive levers as well as the number of reinforcements obtained prior to and after the intracranial surgery and period of recovery.

A one way ANOVA with repeated measurements showed no effect of the infusion of SB 277011-A (1 μg / 0.5 μl / side) or vehicle, into the BLA, on the number of active (F2,17 = 2.23, p = 0.15) or inactive (F2,17 = 0.22, p = 0.8) lever presses under FR5 schedule (Figure 3.7A and B). Similarly, no effect of the infusion of SB 277011-A (1 μg / 0.5 μl / side) or vehicle, into the BLA, was found on the number of reinforcements obtained by the rats under FR5 schedule (F2,17 = 0.01, p = 0.98, with one way ANOVA with repeated measurements) (Figure 3.7C).
Figure 3.6
This figure represents the responding of the rats for food under FR5 schedule of reinforcement. No marked change is observed in the level of active lever responding, or the number of reinforcements in the period following intracranial surgery and recovery as compared to the period preceding the surgery.

A) The level of responding during the acquisition phase (under a fixed ratio 5 (FR5) schedule of reinforcement) of food self-administration, prior to and following the cannulae implantation surgery and recovery. Data are expressed as mean (±SEM) of the number of active (●) and inactive (○) lever presses. B) The mean number (±SEM) of reinforcements (food pellets) obtained during the acquisition phase (FR5).
Intra-BLA Administration of SB 277011-A Has No Effect on Food-taking Behaviour

Figure 3.7
This figure represents the effect of intra-BLA infusion of SB 277011-A (1μg / 0.5 μl / side), or vehicle, on food-taking behaviour. No effect is observed as a result of DRD₃ antagonism on the number of active lever responding (A) or the number of reinforcements obtained (C) as compared to baseline responding under FR5 schedule of reinforcements. Also, no effect of vehicle or drug is seen on inactive lever presses (B). (n=5, ns, p>0.05). Data are expressed as the mean (+SEM) of active (A) or inactive (B) lever presses or reinforcements (Food pellets obtained, C).
3.6 **Histological Examination (Experiments 2-5):**

The location of the tips of the microinjectors within the discrete brain areas investigated in the current study is shown in Figure 3.8, Figure 3.9 and Figure 3.10. Rats were only included in the study when the histological analysis showed that the tips of the microinjectors, as evident by mechanical damage as well as dye infusion, were bilaterally placed within the target site. Microscopic examination revealed no damage, in the cellular tissue of the microinfusion field, other than the mechanical damage caused by the penetration of the guide cannulae and microinjectors (data not shown).

Figure 3.8 shows microinjector tips placement in the BLA, for rats used in the nicotine cue-induced reinstatement experiment (n= 7, open circles) and for rats used in the food seeking experiment (n=5, closed circles), Figure 3.9 shows microinjector tips placement in the NAcc (n= 8), and Figure 3.10 shows microinjector tips placement in the LHb (n=6).
Location of the microinjector tips in the BLA of the rats included in the nicotine cue-induced reinstatement experiment (–○–) and the food taking experiment (–●–) plotted on coronal sections of the rat brain taken from the atlas of (Paxinos and Watson, 1998). The numbers refer to the anterior-posterior coordinates relative to the bregma. The arrows (←→) indicate the target area.
Cannulae Placement in the NAcc

Location of the microinjector tips in the NAcc of the rats included in the nicotine cue-induced reinstatement experiment (○—) and the food taking experiment (●—) plotted on coronal sections of the rat brain taken from the atlas of (Paxinos and Watson, 1998). The numbers refer to the anterior-posterior coordinates relative to the bregma. The arrows (➡️) indicate the target
Location of the microinjector tips in the LHB plotted on coronal sections of the rat brain taken from the atlas of (Paxinos and Watson, 1998). The numbers refer to the anterior-posterior coordinates relative to the bregma. The arrows (→) indicate the target area.
3.7 Experiment (6): Responding maintained by light stimuli only

In this experiment, after the first few days of training, the responding on the active and inactive levers became indistinguishable (Figure 3.11). A two-way ANOVA with repeated measurements showed no significant effect of levers (F_{1,14}=1.01, p=0.33), a significant effect of time (F_{19,266}=8.04, p<0.05) and significant lever x time interaction (F_{19,266}=2.49, p<0.05). Post-hoc analysis, using LSD post-hoc test, revealed the effect of day 1 only to be approaching significance (p=0.057).

Cue-lights per se are Unable to Maintain Stable Responding

![Figure 3.11](image)

This figure represents responding on the active (●) and inactive (○) levers during 1-h sessions in which completion of the ratio requirement on the active lever resulted in illumination of a cue-light followed by a 60-s time-out period and no delivery of nicotine. Data are expressed as means (± S.E.M) of the number of lever presses under FR1, FR2 and FR5 schedules of reinforcement. After a few days of training, responding on the active and inactive levers is indistinguishable. Cues alone are unable to maintain significant responding on the active vs. inactive lever (p=0.33).

Figure from (Khaled, Farid Araki et al., 2010), with permission.
3.8 Sample Size

The total number of animals used in the current study is 111 rats, 14 of which were used for practicing the techniques of intracranial surgery and cannula placement. Table 3.1 indicates the number of rats excluded from the study and the reasons for exclusion.

Table 3.1

<table>
<thead>
<tr>
<th>Number of rats</th>
<th>Reason for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Death: either for surgical (n=7) or other (n=4) reasons.</td>
</tr>
<tr>
<td>22</td>
<td>Catheter-related issues (including: blockade, failure, pain on flushing, etc.).</td>
</tr>
<tr>
<td>5</td>
<td>Failure of extinction</td>
</tr>
<tr>
<td>8</td>
<td>Cannula-related issues (including: blockade, bending, dislocation).</td>
</tr>
<tr>
<td>6</td>
<td>Aberrant cannula placement as evident by histological examination.</td>
</tr>
</tbody>
</table>
4.1 General Discussion

The main objective of the current study is to investigate the involvement of the dopamine D₃ receptors in cue-induced reinstatement of nicotine-seeking behaviour. To this end, the following stepwise approach was used:

Step 1- Evaluating the effect of systemically blocking the DRD₃ on cue-induced reinstatement of nicotine-seeking behaviour.

Step 2- Delineating the neural substrates through which the above effect of DRD₃ on cue-induced reinstatement of nicotine-seeking behaviour is mediated.

In the current study, nicotine intravenous self-administration paradigm was applied (experiments 1-4), followed by extinction training and then reinstatement testing. In this paradigm, the rats were trained to lever press for nicotine under gradually increasing fixed ratio (FR) schedule of reinforcement (FR1 to FR5). Upon completion of schedule requirements, the rats were rewarded by an infusion of nicotine (30μg/kg/infusion in a volume of 28-45μl according to the weight of the rat), which was accompanied by the illumination of a cue light. Such association imparts some reinforcing properties upon the cue light, which is originally neutral in itself. Thereby, it gains secondary motivational salience and develops the ability to reinstate extinguished nicotine-seeking behaviour in the absence of the primary reinforcer; i.e., nicotine in this case.

Self-administration training was carried out for a minimum of 15 sessions in the above-mentioned experiments. During these sessions the rats obtained around 15 – 20 infusions of nicotine / session, and accordingly the same number of cue presentations, adding up to around 225 – 300 cue-nicotine pairings during the course of the study. The length of training period and the adequacy of the number of infusions / cue presentations earned possibly strengthened the
nicotine-cue association; thus, resulting in a robust reinstatement of the behaviour upon the reintroduction of the cues after extinction.

In the current study a highly selective DRD₃ antagonist (SB 277011-A) was used to assess the effect of blocking DRD₃ on cue-induced reinstatement of nicotine-seeking behaviour, both systemically and locally. The systemic administration of SB 277011-A significantly reduced cue-induced reinstatement of nicotine-seeking behaviour. Moreover, the same effect was observed when SB 277011-A was infused into the BLA and LHb, but not in the NAcc.

Secondary objectives of the current study include:

1- Investigating the effect of blocking BLA DRD₃ on food-taking behaviour. This was done to exclude non-specific effects of intra-BLA infusion of SB 277011-A on operant behaviour or general activity of the rats.

2- Investigating the motivational effects of cue-lights per se, and determining whether the cue-induced reinstatement occurred due to previous association of the cue-lights with the infusions of nicotine, or partially due to primary reinforcing effects of the cue-lights themselves.

4.1.1 Systemic Administration of SB 277011-A Attenuates Cue-induced Reinstatement of Nicotine-seeking Behaviour

In the present experiment, nicotine, at the dose of 30μg/kg/infusion, supported vigorous self-administration under a fixed ratio schedule of reinforcement in Long-Evans rats; which is comparable to previously reported studies (Corrigall, 1999; Donny, Caggiula et al., 1995; Forget, Coen et al., 2009; Paterson, Froestl et al., 2004; Paterson, Semenova et al., 2003; Shoaib, Schindler et al., 1997).
The major finding of the present experiment is that the selective DRD$_3$ antagonist (SB 277011-A) blocks cue-induced reinstatement of nicotine seeking (Figure 3.2), suggesting a role for the DRD$_3$ in the regulation of this process. Such a role is supported as the current findings are consistent with previous studies indicating that antagonism at DRD$_3$ attenuates reactivity to nicotine-associated stimuli under pavlovian conditioning procedures (Le Foll, Schwartz et al., 2003; Pak, Ashby et al., 2006), and blocks the expression of nicotine-induced place preferences (Le Foll, Sokoloff et al., 2005; Pak, Ashby et al., 2006). It is important to note that the range of doses producing the inhibition of reinstatement in the present experiment (i.e. 1, 3 and 10 mg/kg) is very similar to previous experiments [i.e. 1, 3 and 10 mg/kg in (Pak, Ashby et al., 2006) and 3 and 10 mg/kg in (Le Foll, Schwartz et al., 2003)].

Interestingly, in the range of doses that are clearly DRD$_3$ selective (such as the doses used in the present study), SB 277011-A had no effect on nicotine self-administration under a fixed ratio (FR) schedule of reinforcement (Andreoli, Tessari et al., 2003), or under a progressive ratio (PR) schedule of reinforcement (Ross, Corrigall et al., 2007). Moreover, in the range of doses tested, SB 277011-A had no effect on locomotor activity (Reavill, Taylor et al., 2000) nor on responding for sucrose under a second order schedule of reinforcement (Di Ciano, Underwood et al., 2003). This suggests that the effect observed in the current study is rather specific to cue-induced reinstatement without an effect on nicotine-taking behaviour and that is not due to other non-specific effects.

Several lines of evidence strongly suggest that at the above mentioned doses, SB 277011-A is acting at the DRD$_3$ and not at the DRD$_2$. First, SB 277011-A is highly selective DRD$_3$ antagonist, with 100 folds higher affinity to DRD$_3$ than DRD$_2$ (Reavill, Taylor et al., 2000). Secondly, the doses selected and used here are far below those that produce effects typically mediated by DRD$_2$ blockade, such as inhibition of spontaneous locomotion or
stimulant-induced hyperlocomotion (up to 42.3 mg/kg SB 277011-A) and induction of cataleptogenic effects or increase of plasma prolactin levels (up to 78.8 mg/kg SB 277011-A) (Reavill, Taylor et al., 2000). Therefore, the above effects appear to be mediated specifically through the DRD3 and not through the DRD2.

It is interesting to note that a similar effect on cue-induced reinstatement of nicotine-seeking behaviour was not obtained using the DRD3 partial agonist BP 897, which failed to attenuate the reinstatement to nicotine seeking induced by previously associated cues (Khaled, Farid Araki et al., 2010). This suggests that DRD3 partial agonists and antagonists are affecting nicotine reinstatement differently. Previous studies have reported a dissociation of effects between DRD3 partial agonists and antagonists. First, subtle differences between the effects of BP 897 and SB 277011-A on responses maintained under second order schedule of reinforcement for cocaine have been reported (Di Ciano, Underwood et al., 2003; Pilla, Perachon et al., 1999). However, both ligands disrupted the responding during the first interval (drug free), whereas only SB 277011-A decreased responding during the second-interval (after cocaine infusion) (Di Ciano, Underwood et al., 2003; Pilla, Perachon et al., 1999). Secondly, although, several studies found that the effects obtained with DRD3 partial agonists and antagonists on reactivity to drug associated cues are convergent (Aujla, Sokoloff et al., 2002; Gyertyan, Kiss et al., 2007; Heidbreder, Gardner et al., 2005), mixed findings have also been reported with DRD3 partial agonists. A study comparing the effects of BP 897 and SB 277011-A on cocaine-induced place conditioning found an absence of effect of BP 897 at doses ranging from 0.3 to 3 mg/kg, whereas 3 mg/kg SB 277011-A was effective to block cocaine-induced place conditioning (Cervo, Burbassi et al., 2005). Similarly, a discrepancy in the effects of
DRD₃ blockade on opiate-induced place conditioning has been reported (Duarte, Lefebvre et al., 2003; Francès, Smirnova et al., 2004; Vazquez, Weiss et al., 2007).

Another explanation is that the stimulation of the DRD₃ due to the partial agonist profile may participate in the absence of effect on reinstatement, as stimulation of dopamine receptors could produce reinstatement of drug-seeking (Bachtell, Whisler et al., 2005) and stimulation of DRD₃ potentiates DRD₁-mediated responses (Le Foll, Schwartz et al., 2000; Marcellino, Ferre et al., 2008).

Nonetheless, it appears that complete blockade of the DRD₃, using an antagonist rather than a partial agonist, is required to achieve significant reduction in the cue-induced reinstatement of nicotine-seeking behaviour.

Taken together, the above findings indicate an important role for the DRD₃ in the modulation of cue-induced reinstatement processes. This role appears to be specific to reinstatement and not to nicotine-taking behaviour, and is specifically mediated through the DRD₃.

The present findings, along with the findings that SB 277011-A has been shown to significantly decrease the reinstatement of nicotine-seeking behaviour induced by nicotine priming (Andreoli, Tessari et al., 2003), support the hypothesis that blockade of DRD₃ by the selective antagonist SB 277011-A could be effective to decrease relapse to nicotine-seeking.

4.1.2 Local Infusions of the DRD₃ Antagonist SB 277011-A

The current group of experiments set out to identify some of the neural substrates through which the effects of DRD₃, on cue-induced reinstatement of nicotine-seeking behaviour, are mediated. Based on compelling evidence of their involvement in reward-related processes
(discussed in detail below), three areas of interest were chosen, namely; the BLA, the NAcc and the LHb. The role of the DRD3 in each was investigated in relevance to cue-induced reinstatement of nicotine seeking. In addition, the effect of blocking the DRD3 in the BLA on food taking behaviour was evaluated. The findings of the current set of experiments will be discussed in detail in the following sections.

4.1.2.1 DRD3 Antagonism in the BLA, Abolishes Cue-induced Reinstatement of Nicotine-seeking Behaviour, but Has No Effect on Food Intake.

The major finding of the current experiments is that blockade of the DRD3, using local infusions of the DRD3 antagonist SB 277011-A in the BLA, significantly and dose-dependently reduced cue-induced reinstatement of nicotine-seeking behaviour (Figure 3.3). Although significant levels of reinstatement were achieved in the vehicle treated group, shown by an increase in the number of active lever responding relative to extinction baseline levels, upon the infusion of SB 277011-A, dose-dependent reduction in the level of reinstatement was observed. Such levels of reinstatement were not only significantly lower than those observed in the vehicle treated group, but were also comparable (when the higher doses of SB 277011-A were used) to the levels of responding during extinction. In addition, no changes were observed in the levels of inactive lever pressing. This suggests a significant role for the DRD3 that are located in the BLA in the modulation of the cue-induced reinstatement response. The lack of an effect of the DRD3 blockade on inactive lever pressing suggests that the attenuation of reinstatement observed was not due to non-specific effects of the drug.

Indeed, it is highly unlikely that the blockade of reinstatement observed in the current experiment is attributed to motor deficits, since the infusion of SB 277011-A into the BLA, at 4μg/0.3μl/ side (i.e. 4 times higher than the highest dose used in the current study), did not have any effect on rat spontaneous locomotor activity (Di Ciano, 2008). Moreover, neither the
infusion of the highest dose of SB 277011-A (1μg/0.5μl/ side) nor the vehicle had an effect on food taking behaviour under FR5 schedule of reinforcement (Figure 3.7); further discussed below. This does not only support that, at the range of doses tested in the current study, SB 277011-A caused no locomotor deficits, but also excludes the possibility of other non-specific effects like general anhedonia, generalized weakness or sedation which may result in alteration in the pattern of the rats’ operant responding. This possibility is further excluded by the fact that DRD3 antagonists enhance social interaction (Millan, Loiseau et al., 2008) and the evidence from recent studies indicating the lack of the DRD3, in knock-out mice, show no impairments in animal models of anhedonia or helplessness (Chourbaji, Brandwein et al., 2008).

Additionally, it is rather unlikely that the effects of the DRD3 blockade, observed in the current study, are due to a disruption of other factors, which may have an effect on the form of responding, tested under the current paradigm, such as an effect on cue-light perception, memory or learning, or due to a decrease in attention. In fact, recent studies have shown that blocking the DRD3 receptors may play an important role in enhancing some aspects of cognition including memory, attention and learning (Millan, Di Cara et al., 2007; Sarter and Parikh, 2005). This role is mediated through increasing the extracellular levels of acetylcholine, a neurotransmitter critically involved in the above processes (Millan, Di Cara et al., 2007; Sarter and Parikh, 2005), in the frontocortical regions of the brain (Lacroix, Hows et al., 2003). Moreover, DRD3 antagonists were shown to improve learning deficits in memory impaired rats (Laszy, Laszlovszky et al., 2005).

The effects observed in the current study are, therefore, most likely to be attributed to a specific effect on the ability of conditioned stimuli, previously paired with the administration of nicotine, to reinstate extinguished nicotine-seeking behaviour in the absence of nicotine.
(primary reinforcer). This result indicates the importance of DRD3 in the BLA in mediating the effects of conditioned reinforcers.

The BLA was chosen as a primary target for the current study based on a strong and established body of evidence implicating the BLA in the mechanisms underlying the process of conditioned reinforcement. The amygdaloid complex is traditionally considered as an important structure in the limbic system. It has been reported, however, that although the BLA is a subcortical structure, it also has quasicortical properties (Carlsen and Heimer, 1988). The BLA receives dopaminergic innervation from the VTA (Brinley-Reed and McDonald, 1999), a connection which is of special interest in the current study. These dopaminergic inputs target dopamine receptors expressed in the BLA (DRD1,2 or 3) (Gaspar, Bloch et al., 1995; Rosenkranz and Grace, 1999), and contribute to the role of the BLA in the regulation of responding to reinforcing stimuli (Nakano, Lenard et al., 1987).

Efferent projections from the BLA include dense innervation of most of the striatum, and it is even considered that projections from the BLA constitute the main amygdalostriatal projections (Kelley, Domesick et al., 1982). Being a corticolimbic structure and a part of the corticostriatal system, especially in connection to the mesolimbic dopamine system, suggests a strong role for the BLA in the motivational processes required for goal-directed behavioural responses.

Clinical studies have indicated a role for the amygdala in cue-association and cravings. In humans, activation of the amygdala was observed upon the presentation of cocaine-related cues, which was also associated with subjective reports of cravings (Childress, Mozley et al., 1999). In addition, signal changes in response to drug-associated cues were observed in the amygdala using functional magnetic resonance imaging (fMRI), as well as an increase in
regional cerebral metabolic rate for glucose, which strongly correlated with measures of cravings (Breiter and Rosen, 1999; Grant, London et al., 1996).

Behavioural studies, using animal models, also reported a clear involvement of the BLA in conditioned reinforcement processes. An increase in cFos expression in the amygdala was shown after the presentation of a cocaine-associated environment (Brown, Robertson et al., 1992). Moreover, bilateral BLA lesions disrupted the responding of rats to conditioned reinforcers previously associated with water (Cador, Robbins et al., 1989), second order conditioning (Hatfield, Han et al., 1996), conditioned place preference for cocaine (Brown and Fibiger, 1993), and abolished the reinstatement of cocaine-seeking behaviour induced by the reintroduction of conditioned stimuli previously associated with cocaine (Meil and See, 1997). BLA deactivation also decreased discriminative stimulus-evoked conditioned responses to sucrose, without an effect on sucrose taking or consumption (Jones, Day et al., 2010a). Additionally, although BLA lesions were without an effect on Pavlovian conditioning, rats with bilateral excitotoxic lesions in the BLA were unable to acquire a new response to conditioned reinforcement (pavlovian to instrumental transfer of control over behaviour by a conditioned stimulus) (Everitt, Parkinson et al., 1999), or responding under a second order schedule of cocaine self-administration (Whitelaw, Markou et al., 1996).

More specifically, the dopaminergic innervation to the BLA has been implicated in the processes of stimulus-reward associations (Ciccocioppo, Sanna et al., 2001). Previous studies have suggested a role for the DRD1 in the BLA in conditioned reinforcement, where the infusion of a DRD1 antagonist in the amygdala disrupted conditioned fear expression (Lamont and Kokkinidis, 1998), and the infusion of a DRD1 agonist in the amygdala potentiated conditioned fear retention (Guarraci, Frohardt et al., 1999), while intra-amygdalar infusion of raclopride (a
mixed D₂/D₃ antagonist) attenuated it (Guarraci, Frohardt et al., 2000). Moreover, the DRD₁ antagonist SCH-23390, but not the mixed D₂/D₃ antagonist raclopride, attenuated conditioned reinstatement of cocaine seeking, but not cocaine seeking *per se* (when unassociated with cues) (See, Kruzich et al., 2001). This lack of effect of raclopride on cue-induced reinstatement could be explained by its low selectivity for the DRD₃. Nonetheless, a high dose of raclopride, infused in the BLA prior to a single classical cue-association session, was able to block the acquisition of cocaine-cue association (as evident by a failure of reinstatement induced by cues thereafter) to a degree comparable to the DRD₁ antagonist SCH-23390 (Berglind, Case et al., 2006).

In the current study, SB 277011-A was chosen due to its high selectivity to DRD₃, which facilitates the investigation of the specific roles of this receptor without being influenced by actions on other dopamine receptors. It is therefore highly unlikely that the effects shown in the current study are due to an action on either DRD₁ or DRD₂.

The present findings are in line with a recent study showing that the infusion of SB 277011-A (4μg/0.3μl/ side) into the BLA reduced cocaine seeking that is maintained by the presentation of cocaine-associated cues, under a second order schedule of reinforcement (Di Ciano, 2008). The discrepancy in the effective dose between the two studies can be explained by the difference in the drug acting as a primary reinforcer (nicotine *versus* cocaine). A similar difference was noticed in systemic studies, where SB 277011-A was able to block cue-induced reinstatement of nicotine seeking at the doses of 1, 3 and 10 mg/kg (results shown above), whereas doses of 10, 20 and 30 mg/ kg were required to block cue-induced reinstatement of cocaine seeking or cocaine seeking under a second order schedule of reinforcement (Cervo, Cocco et al., 2006). Another explanation could be the use of a different vehicle for dissolution of SB 277011-A. In the current experiment, 10% DMSO in 10% w/v hydroxypropyl-β-
cyclodextrin in sterile water was used as a vehicle for SB 277011-A, instead of 10% w/v hydroxypropyl-β-cyclodextrin in sterile water alone. This was resorted to due to issues with the solubility of the compound, which would have comprised difficulties in procedures for microinfusions. A much better solubility was obtained upon adding a low concentration of DMSO (10%) to the vehicle, which, perhaps, rendered it possible to observe an effect at such low doses, as was seen in the current experiment. It is important to mention that careful histological examination of the brain sections was performed to ensure that no damage has occurred as a result of the infusion of DMSO (data not shown).

Structures adjacent to the BLA, especially the central nucleus of the amygdala (CeA), may have an important role in processes underlying drug seeking and motivation. However, the two areas have different roles in the processes underlying stimulus-reward associations, and the control over the effect of conditioned stimuli on behavioural responses (as evident by differential effects on conditioned suppression, appetitive pavlovian conditioning, conditioned reinforcement and its potentiation by intra-NAcc D-amphetamine) (Everitt, Parkinson et al., 1999; Killcross, Robbins et al., 1997). The effects shown in the current study are more in line with a more evident role for the BLA than the CeA in controlling conditioned stimuli-induced reinstatement. Furthermore, at the volume of infusion used in the present study (0.5 μl / side), diffusion of the drug was limited to the BLA and not to the adjacent structures. This was confirmed by localization of the dye, infused into the rat brains immediately before extraction, to the BLA and not the adjacent structures. Therefore, it is not likely that the effects of SB 277011-A on cue-induced reinstatement of nicotine seeking, observed in the current study, are due to its action on dopamine receptors in adjacent structures, including the CeA. Further
studies exploring the effect of SB 277011-A infusion into the CeA will be of interest to better elucidate the role of DRD₃ in this specific area.

Importantly, the current study found no effect of intra-BLA infusion of SB 277011-A on operant behaviour as assessed, in a separate experiment, by food taking under FR5 schedule of reinforcement. Infusion of SB 277011-A (1 μg / 0.5μl / side), or vehicle, into the BLA resulted in no change in the number of lever pressing (active or inactive) or, consequently, in the number of reinforcements (food pellets) earned, as compared to baseline FR5 responding (Figure 3.7). The fact that the rats were able to respond in an operant system for food (without noticeable change) under the same treatment conditions, which completely abolished responding for nicotine-associated cues, emphasizes the exclusion of general non-specific behavioural effects of the drug (SB 277011-A) or vehicle, such as disruption in locomotion, attention or interest in reward. Moreover, these results support the hypothesis that the blockade of cue-induced reinstatement of nicotine seeking, shown above, is rather specific to blocking stimulus-reward associations and the ability of conditioned stimuli to induce relapse. Consequently, the DRD₃ in the BLA appear to be involved mainly in cue-associations and processes of relapse rather than primary reinforcement and reward processes.

In summary, the current experiments show a clear and strong inhibition of cue-induced reinstatement of nicotine-seeking behaviour following the bilateral infusion of the selective DRD₃ antagonist, SB 277011-A, into the BLA. The present findings support the established role of the BLA in stimulus-reward associations, enhance the understanding of the role of DRD₃ in the BLA in cue-induced reinstatement of nicotine seeking, and suggest a strong potential for the use of selective DRD₃ ligands in the prevention of relapse to smoking.
4.1.2.2 DRD₃ Antagonism in the NAcc Has No Effect on Cue-induced Reinstatement of Nicotine-seeking Behaviour

In contrast to the above findings (Section 4.1.2.1), the present experiment showed that intra-NAcc infusions of SB 277011-A had no effect on cue-induced reinstatement of nicotine-seeking behaviour (Figure 3.4). Reintroduction of the nicotine-associated cues during reinstatement testing, resulted in significant reinstatement of active lever responding in rats treated with SB 277011-A (0.01 to 1 μg / 0.5 μl / side) to a level that was significantly higher than their baseline level of lever pressing during extinction, and that was not significantly different from their level of responding when treated with the vehicle. Therefore, blocking the DRD₃ in the NAcc is without effect on cue-induced reinstatement of nicotine seeking, suggesting a lack of role for the NAcc DRD₃ in this aspect.

The current data are consistent with a recent study that found no effect of blocking the DRD₃ in the NAcc shell, by microinfusions of SB 277011-A (at doses up to 4 μg / 0.3 μl / side), on cocaine seeking that is maintained by cocaine-associated cues under a second order schedule of reinforcement (Di Ciano, 2008). This suggests that the DRD₃ in the NAcc may not be involved in cue-association processes. The DRD₃ in the NAcc, however, have been shown to have a role in stress-induced reinstatement of cocaine seeking (Xi, Gilbert et al., 2004), which was blocked by SB 277011-A infusions into the NAcc. The DRD₃ in the NAcc, therefore, seem to be involved in some, but not all, aspects of nicotine seeking and relapse.

Several considerations implicate the NAcc as a key structure in the drug-reward circuitry. Anatomically, the NAcc comprises a part of the ventral striatum (Heimer, Alheid et al., 1997), and acts as a junction / interface between cortical (allo- and periallo-cortical) and limbic structures (Kelley, Domesick et al., 1982; Mogenson, Jones et al., 1980). Among the
important innervations of the NAcc, which are of special interest in the current study, are: a) the
dopaminergic afferents from the VTA (especially to the shell) (Deutch and Cameron, 1992), and
b) the rich innervations from the BLA (Carlsen and Heimer, 1988). In addition, the NAcc is one
of the structures exhibiting very high densities of DRD3 expression in the brain (Diaz, Lévesque
et al., 1995).

The NAcc has an established role as an important neural substrate for drug seeking, drug
reinforcement and rewarding effects of drugs of abuse (Corrigall, Franklin et al., 1992; Pontieri,
Tanda et al., 1996). Previous studies have implicated the dopaminergic input to the NAcc in the
process underlying drug taking and self-administration, where the administration of 6-
hydroxydopamine, a dopaminergic neurotoxin, in the NAcc decreased cocaine self-
administration (Roberts, Koob et al., 1980), and NAcc DRD1 blockade attenuated the
reinforcing effects of cocaine as shown by an increase in cocaine self-administration under FR
schedules of reinforcements (Caine, Heinrichs et al., 1995; McGregor and Roberts, 1993).
Moreover, the infusion of a DRD1 or a DRD2-like antagonist (but not a DRD3 or a DRD4
selective antagonist) in the NAcc has been shown to decrease the reinforcing properties of
cocaine (Bari and Pierce, 2005). Reinstatement models have shown that the inactivation of the
NAcc inhibits cocaine-induced reinstatement of cocaine-seeking behaviour (McFarland 2001),
and that selective DRD3 antagonism by local infusions of SB 277011-A into the NAcc inhibits
stress-induced reinstatement of cocaine-seeking behaviour (Xi, Gilbert et al., 2004).

However, the involvement of the NAcc in conditioned reinforcement is more complex.
Such complexity is a result of the complex anatomical structure of the NAcc. The NAcc is
differentiated into two subregions, namely; the shell and the core. This differentiation is not only
based on anatomical differences, but also, on pharmacological grounds. Whereas the NAcc core
is considered to be more connected to the striatum, the NAcc shell seems to be leaning more towards the limbic system (Deutch and Cameron, 1992) incorporating striatal neurons as well as neurons of the extended amygdala (Heimer, Alheid et al., 1997). Functionally, the two subregions have been shown to have differential effects on stimulus-reward associations. Selective excitotoxic lesions of the core, but not the shell, disrupted pavlovian conditioning, as well as conditioned reinforcement (Everitt, Parkinson et al., 1999). Moreover, inactivation of the NAcc core, but not the NAcc shell, has abolished cue-induced reinstatement of cocaine seeking (Fuchs, Evans et al., 2004). On the other hand, deactivation of the shell and not the core, attenuated amphetamine-induced potentiation of conditioned reinforcement (Everitt, Parkinson et al., 1999). Therefore, it can be concluded that the NAcc core plays a more important role in cue-association processes than the NAcc shell.

As mentioned above, in 2004, Fuchs and co-workers found a decrease in cue-induced reinstatement of cocaine-seeking behaviour following the inactivation of the NAcc core (Fuchs, Evans et al., 2004). The fact that such an effect was not observed in the current study could be attributed to the difference in the pharmacological agents used. Whereas a GABA-agonist was used to deactivate the NAcc in the above study, the current experiment targeted specifically and exclusively the DRD3 in the NAcc. It is worth mentioning, however, that a previous study, where the undifferentiated NAcc was deactivated, also using a different pharmacological agent, failed to show an effect on cue-induced reinstatement (Grimm and See, 2000).

The current experiment did not target each of the NAcc subregions (shell vs. core) individually. It is possible that a different outcome would have been obtained had this been the case. However, this is unlikely because in the current experiment the responding of the rats was rather uniform, without remarkable variations, even though the intra-NAcc placement of the
cannulae, and the site of infusion of the SB 277011-A, were either bordering the NAcc core and NAcc shell or rather uniformly distributed between the two subregions (Figure 3.9). Nevertheless, it cannot be absolutely excluded that the lack of an effect, of intra-NAcc DRD3 blockade on cue-induced reinstatement of nicotine seeking, demonstrated in the present study is due to targeting the nucleus accumbens as a whole rather than individually targeting the DRD3 in the NAcc shell or the NAcc core. Further experiments are warranted to investigate such a possibility.

Interestingly, it has been suggested that the amygdala is involved in the regulation of the dopaminergic innervations of the NAcc. While the projections from the CeA to the VTA suggest a mechanism as to how dopaminergic lesions of the CeA are reflected on dopaminergic levels in the NAcc (Louilot, Simon et al., 1985), a recent study has shown that the BLA plays a role in the modulation of NAcc dopamine signalling, without an effect on VTA-evoked stimulation of dopamine release in the NAcc (Jones, Day et al., 2010a). Thus, it can be suggested that the effect of the BLA on controlling stimulus-reward associations is mediated through an interaction with the NAcc. However, in the current study, differential effects, on cue-induced reinstatement of nicotine seeking, were found upon blocking the DRD3 in each of these areas individually, where such blockade in the BLA completely abolished the response while no effect was seen as a result of the same blockade in the NAcc. In other words, in the presence of an intact BLA, DRD3 blockade in the NAcc per se is not enough to attenuate cue-induced reinstatement. The case in the current study is different from the above studies in that, the present blockade is specific to a single dopamine receptor, the DRD3. It is possible that blocking the DRD3, the dopamine receptor exhibiting the highest binding affinity to dopamine (Sokoloff, Giros et al., 1990), which leaves the other dopamine receptors in the NAcc (DRD2) free for the dopamine to bind, plays a role in the reinstatement of the behaviour by the reintroduction of the
cues. Nonetheless, it can be concluded, based on the present findings, that the DRD$_3$ in the NAcc are not mediators of cue-induced reinstatement processes.

In summary, the current study found no effect of DRD$_3$ antagonism within the NAcc on cue-induced reinstatement. These results are in line with the existing body of evidence suggesting a role for the NAcc in aspects of drug-seeking behaviour other than cue-induced relapse. According to the above results, the NAcc, also, serves as a neuroanatomical control excluding that the action of SB 277011-A is extending beyond the sites it is administered. More importantly, the present findings emphasise the regional selectivity of DRD$_3$ in controlling cue-induced reinstatement of nicotine-seeking behaviour.

4.1.2.3 DRD$_3$ Antagonism in the LHb Attenuates Cue-induced Reinstatement of Nicotine-seeking Behaviour

Another interesting and novel finding of the current study is that intra-LHb infusions of SB 277011-A attenuate cue-induced reinstatement of nicotine-seeking behaviour (Figure 3.5). In the present experiment, the reintroduction of the cue light previously associated with nicotine significantly reinstated nicotine seeking behaviour in the rats upon treatment with the vehicle only (in comparison to their baseline responding during extinction). However, when the rats were treated with SB 277011-A (0.01-1 µg/ 0.5 µl / side), the reinstatement was significantly reduced as compared to the vehicle treated group. It is important to note that, although the attenuation of reinstatement in this group of rats was clear and statistically significant, the reinstatement was not completely abolished, as was the case upon the antagonism of the DRD$_3$ in the BLA. These findings indicate an important role for the LHb DRD$_3$ in the regulation of cue-induced relapse to nicotine seeking.
The LHb is emerging as a neural site potentially involved in neuropsychiatric disorders including depression, schizophrenia and drug addiction (Ellison, 1994; Lecourtier, Neijt et al., 2004; Morris, Smith et al., 1999; Sartorius, Kiening et al., 2010). This region, previously categorized as the motor subregion of the habenular complex (Herkenham and Nauta, 1977), has been recently functionally reconsidered, and found to be additionally involved in several processes including response to stress (Chastrette, Pfaff et al., 1991), learning (Lecourtier and Kelly, 2007) and reward (Morissette and Boye, 2008; Vachon and Miliareissis, 1992).

Anatomically, the habenular complex comprises a part of the epithalamus (together with the pineal body). The habenular complex is divided into two subregions; i.e. the medial (MHb) and the lateral (LHb) habenular nuclei. In turn, the LHb is further divided into medial and lateral subdivisions (Andres, von During et al., 1999). The connections of the LHb play an important role in the range of functions mediated by this structure. Afferents into the LHb arise mainly from the limbic regions and the basal ganglia (Geisler and Trimble, 2008; Herkenham and Nauta, 1977). Among the important afferents innervating the LHb is the strong input it receives from the ventral pallidum (Groenewegen, Berendse et al., 1993), which is in turn densely innervated by the NAcc (Zahm, 1999). Importantly, the main efferents of the LHb are directed towards the monoaminergic neurons-containing nuclei, namely: the dorsal and median raphe (serotonergic neurons), lateral and dorsal tegmental nucleus (cholinergic neurons) and the VTA (dopaminergic neurons) (Herkenham and Nauta, 1979), all of which have been implicated in mechanisms underlying psychiatric disorders and especially drug addiction. Through this circuit of connections, the LHb can be considered to be acting as a converging point for the complex information coming from the cerebral cortex, through the limbic and basal ganglia, and headed to the monoaminergic nuclei in the brainstem. Thus, the connections of the LHb offer an explanation for the role
suggested to be played by this structure in different psychological disorders, including drug addiction, schizophrenia and depression.

In particular, the relationship between the LHb and the dopaminergic neurons in the VTA is of special interest in the current study. Interestingly, it has been shown that some dopaminergic neurons in the VTA project back to the LHb (Gruber, Kahl et al., 2007). In order to understand the significance of such connections in the context of the current study, it is important to briefly refer to the role of the dopamine neurons in such a context. As previously discussed in Chapter 1, the dopamine neurons are involved in mediating reward signals and reward prediction errors (Schultz, 1998). More specifically, the dopamine neurons have been suggested to translate errors in prediction, and to be firing to promote behaviours that predict reward and discourage behaviours that have negative consequences (Morris, Nevet et al., 2006; Schultz, Apicella et al., 1993). In addition, they are involved in processing information about novel environmental changes affecting motor behaviour (Redgrave and Gurney, 2006).

Studies have shown that stimulation of the LHb inhibits dopaminergic neurons in the VTA as well as the in substantia nigra (Christoph, Leonzio et al., 1986), an inhibition which is followed by delayed excitation (Ji and Shepard, 2007). Moreover, phasic inhibition of the LHb resulted in decreased extracellular dopamine levels in the NAcc (Lecourtier, Defrancesco et al., 2008). This effect has been hypothesized to be mediated through stimulation of GABA interneurons in the VTA (Ji and Shepard, 2007). A recent study has also shown that glutamatergic axons from the LHb terminate on GABAergic neurons in the VTA as well as (although to a lesser degree) on dopaminergic neurons (Brinschwitz, Dittgen et al., 2010). These connections offer an explanation to the tonic inhibition exhibited by the LHb on the dopamine neurones.
In primates, the response of the LHb was found to be opposing and preceding that of the VTA dopamine neurons following the presentation of to reward-predictive or non-predictive stimuli, and weak stimulation of the LHb resulted in the inhibition of the dopamine neurons (Matsumoto and Hikosaka, 2007). In addition, recent studies have shown that electrolytic lesions of the habenula attenuated brain stimulation reward (Morissette and Boye, 2008). In the light of drugs of abuse, very few studies have investigated the effect of modulating the functions of the LHb on drug taking and/or reinstatement (Ellison, 2002). In 2005, Zhang and colleagues demonstrated that the reintroduction of heroin-associated cues, which reinstated extinguished heroin-seeking behaviour, also increased cFos immunoreactivity in the LHb, a sign of neuronal activation. Furthermore, a recent study has found that deep brain stimulation (DBS), but not lesioning, of the LHb decreased cocaine self-administration and promoted extinction behaviour (Friedman, Lax et al., 2010). Additionally, LHb DBS was shown to attenuate cocaine-induced reinstatement of cocaine-seeking behaviour without affecting sucrose taking, or locomotor activity (Friedman, Lax et al., 2010). These data suggest a role for the LHb in drug conditioning and reinstatement processes.

Taken together, the findings of the current study add to a growing body of evidence supporting a role for the LHb-dopaminergic interaction in reward signalling, reward prediction and drug conditioning. Indeed, the current study is the first to acknowledge and investigate the role of DRD3 in the LHb on drug-seeking behaviour, suggesting an involvement of these receptors in the role played by the LHb in cue-induced reinstatement of nicotine-seeking behaviour.

It is important to mention that, a few studies have investigated the role of the MHb in nicotine taking and withdrawal. Particularly, the nicotinic acetylcholine receptors (nAChRs) in
the MHb have been explored for a potential role in these processes. Functional nAChR subunit α4 have been demonstrated in the MHb (Fonck, Nashmi et al., 2009). It has also been suggested that the MHb is involved in mediating the role of α2 and α5 subunits of the nicotinic acetylcholine receptors in nicotine withdrawal (Salas, Sturm et al., 2009). Moreover, the MHb nAChRs subunit α5 was suggested to be implicated in nicotine-taking behaviour, where knocking down the MHb α5 subunit modulated brain-stimulation reward thresholds by blocking the inhibitory effects of the higher doses of nicotine on brain reward (Fowler, Lu et al., 2010).

Since the MHb and the LHb are both relatively small structures, it could be suggested that the microinfusions of SB 277011-A could have extended beyond the LHb to the MHb. Although microinfusion of the dye at the infusion sites revealed localization of the infusion to the LHb, it remains possible that some of the infused drug could have diffused into the MHb as well. However, due to the lack of DRD3 in the MHb, and the selectivity of the DRD3 antagonist used, it is not likely that the effect shown in the current study was mediated through the MHb as well as the LHb.

The current study can be considered an addition to the increasing surge of interest in the LHb in field of drug addiction in general. The current findings indicate a strong potential for the LHb DRD3 in cue-induced reinstatement of drug seeking, and serve as a basis upon which further studies can be directed exploring the role of the LHb DRD3 in the mechanisms underlying drug-seeking behaviour.
4.1.3 Cue Light per se is Unable to Maintain Active Lever Pressing Under a Fixed Ratio Schedule

Another finding of the current study is that light stimuli had no motivational properties of their own, under the present conditions. This was evidenced by the same level of responding observed on the active and inactive levers after a few days of training (Figure 3.11). These results are quite different from what has been reported by (Caggiula, Donny et al., 2002b) who found a relatively higher level of responding on the active lever in animals pressing for saline and cue-lights only. However, this discrepancy may be attributed to the difference in the strain of animals used (Sprague–Dawley as opposed to the Long–Evans strain used in the present study) or to the variability in the presentation and duration of the cue-light and/or houselight extinctions in both studies. Further investigation is needed to outline the exact role of cue presentation in the process of acquisition and reinstatement of drug-seeking behaviour. It has been proposed that environmental stimuli are important contributors of nicotine self-administration (Caggiula, Donny et al., 2002a). It could, therefore, have been expected that blocking the DRD3 could produce a decrease of nicotine self-administration, as removal of environmental cues could produce such a decrease in responding (Caggiula, Donny et al., 2001). The lack of such an effect could be due to a different role of cues between different strains or due to different experimental conditions. Further studies are required to delineate this. It is interesting to note that in a recent study performed in naive squirrel monkeys, the responding of nicotine-experienced animals was directed more towards delivery of nicotine than towards presentation of the associated brief light stimuli (Le Foll, Wertheim et al., 2007). Further, after repeated exposure to cycles of saline substitution, nicotine-associated stimuli, by themselves, appeared unable to maintain significant self-administration behaviour under FR and PR
schedules (Le Foll, Wertheim et al., 2007). Those studies suggest that nicotine delivery may be more important than cue presentation in maintaining self-administration behaviour.
4.2 Conclusions and Summary

The current study is the first report demonstrating the role of the DRD$_3$ in cue-induced reinstatement of nicotine-seeking behaviour systemically as well as locally.

The current study demonstrated that systemic administration of a selective DRD$_3$ antagonist (SB 277011-A) results in significant attenuation of cue-induced reinstatement of nicotine-seeking behaviour. This attenuation does not appear to be due to non-specific effects. Furthermore, DRD$_3$ antagonism does not affect nicotine taking. The effect observed in the current study appears to be specifically mediated through the DRD$_3$ and not the DRD$_2$.

The current study also pointed out, using microinfusions of the same DRD$_3$ antagonist into discrete brain areas, that intra BLA and intra LHb antagonism of the DRD$_3$ resulted in a clear reduction in cue-induced reinstatement of nicotine seeking. These findings are consistent with an established role for the BLA in cue-association processes and an emerging understanding of the role of the LHb in the mechanisms underlying reward processing. Moreover, no such effect was observed as a result of the DRD$_3$ antagonism into the NAcc. This is further supported by the existing body of evidence implicating the NAcc in drug reinforcement and reward rather than stimulus-reward associations.

The main findings of the current study can be summarized as:


2. Antagonism of the DRD$_3$ that are located in the BLA or the LHb results in a reduction in cue-induced reinstatement of nicotine seeking.

3. Antagonism of the DRD$_3$ that are located in the NAcc has no effect on cue-induced reinstatement of nicotine seeking.
Taken together, the above results suggest a rather selective role for the DRD₃ in the effect of nicotine-associated cues and their importance as key factors in relapse to nicotine seeking, that is not non-specifically mediated through all brain areas expressing the DRD₃, but selective to the BLA and LHb. This, may serve as an explanation as to why the DRD₃ appear to be involved in some aspects of nicotine-seeking behaviour and not the others.

The current study supports a body of evidence implicating the DRD₃ in processes underlying relapse to smoking, and substance use in general. Furthermore, the current findings broaden and expand the basic understanding of the mechanisms and areas through which this receptor exerts its role in the development of stimulus-reward associations which later on lead to relapse in abstinent smokers.

Extrapolation of the current data to humans, suggests a strong potential for the DRD₃ as a target and for DRD₃ selective antagonists as therapeutic agents for the prevention of relapse to smoking.
4.3 Future recommendations

4.3.1 DRD₃ Antagonism and Stress-induced Reinstatement of Nicotine-seeking Behaviour

Stress is an important trigger of relapse to drugs of abuse, including nicotine, in abstinent human users (Sinha, 2001). Blocking the DRD₃ using SB 277011-A, significantly reduced stress-induced reinstatement of cocaine-seeking behaviour. Interestingly, animal models have not yet been used to explore the role of DRD₃ in stress-induced reinstatement of nicotine seeking. Such a factor cannot be ignored when studying the potential of selective DRD₃ ligands as therapeutic agents for the prevention of relapse to smoking. Such information will indeed be needed before the current findings could be extrapolated to humans. Furthermore, the investigation of the neural substrates underlying such a role, if proven, would be of high importance in elucidating the neurobiological circuitry of stress in regards of the involvement of the DRD₃.

4.3.2 Investigating the Effect of Modulating the Function of the LHb DRD₃ on Other Aspects of Nicotine-seeking Behaviour

The findings presented in the current study represent only a starting point highlighting the role of the LHb DRD₃ in cue-induced reinstatement of nicotine seeking. Further studies are needed to elucidate the complete role of these receptors in this specific area in other aspects of nicotine seeking, such as stress induced reinstatement and nicotine induced reinstatement of nicotine seeking.
4.3.3  **Effect of a DRD\textsubscript{3} Agonist on Nicotine Taking and Reinstatement**

The effects obtained by the selective DRD\textsubscript{3} antagonist shown in the current study suggest a strong potential for this category of ligands, as therapeutic agents for the prevention of relapse to smoking. However, before such results could be extrapolated to humans, a missing piece of information should be fulfilled; i.e., the investigation of the effect of stimulating the DRD\textsubscript{3} on different aspects of nicotine taking and nicotine seeking behaviour.

4.3.4  **Investigating the Effect of Intracranial Administration of DRD\textsubscript{3} Selective Antagonists on Nicotine-priming-induced Reinstatement of Nicotine-seeking Behaviour.**

DRD\textsubscript{3} antagonism by SB 277011-A has been shown to block nicotine-induced reinstatement of nicotine-seeking behaviour, in rats. However, the exact mechanisms underlying this effect in the brain remain unknown. Targeting specific areas in the brain, with specific DRD\textsubscript{3} antagonist, is needed to assess the mechanisms underlying nicotine priming-induced reinstatement and the role of intracranial DRD\textsubscript{3} in such mechanisms.

4.3.5  **Use of DRD\textsubscript{3} Knock-out Mice to Confirm the Selectivity of SB 277011-A**

Although the SB 277011-A is used as a highly selective DRD\textsubscript{3} antagonist, such selectivity can only be absolutely proven if the above findings fail to be replicated in DRD\textsubscript{3} knock-out mice.
4.3.6 Replication of the Current Study Using Other Selective DRD₃ Ligands to Confirm the Results

One of the limitations of the current study is the use of only one drug as an example of DRD₃ antagonists. Future experiments should be performed using other novel DRD₃ selective ligands such as the S-22 and R-22 compounds (Newman, Grundt et al., 2009), to confirm the results obtained in the current study, and also to expand the variety of ligands that can be used in clinical studies at a later stage.
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APPENDICES
APPENDIX I

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Alan Frazer, Ph.D.
Professor and Chairman
Department of Pharmacology
UTHSCSA
7703 Floyd Curl Drive - MSC 7764
San Antonio, TX 78229-3900
frazer@uthscsa.edu
210-567-4205
210-567-4300 (FAX)

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MSc / PhD Student
Translational Addiction Research Laboratory
Centre for Addiction and Mental Health
University of Toronto
33 Russell Street, Toronto, ON, Canada M5S 2S1
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APPENDIX II

Standard Operating Procedure for Intravenous Catheterization Surgery
Standard Operating Procedure for Intravenous Catheterization Surgery

PURPOSE:
Insertion of a catheter, in the rat’s right jugular vein, for delivery of drug/s in self-administration sessions.

LAB. LOCATION: T618

INSTRUMENTS:
1. 2 syringes (1cc) fitted with blunted 22 gauge needle,
2. Small iris Scissors,
3. 2 pairs of forceps (1 straight, 1 curved),
4. Needle driver/holder,
5. Trocar (4 inch length of PE 190),
6. Catheter (please see SOP for Catheter Construction for catheter specifications),
7. Micro scissors,
8. Cyano acrylic glue (KRAZY Glue),
9. Suture needles (2-3).

All instruments should be autoclaved before the beginning of the surgeries (at the start of each day of surgery). Subsequently, in between the surgeries, the instruments can be sterilized by immersion in a cold sterilant (for e.g. Virox or Accel CS 20) for 20 minutes. The instruments must be cleaned thoroughly of blood residues before being placed in the sterilant.

Everyday, at the end of the surgical procedures, the instruments should be cleaned, sonicated and left to dry then stored safely (please see SOP for Surgical Suite Disinfection for details).

YOU WILL ALSO NEED:
1. 2 beakers (250 cc each) of the cold sterilant.
2. 1 beaker (250 cc) of saline,
3. 1 small vial of saline (10-20 cc).

All glassware should be autoclaved and the packs should be freshly opened on the day of surgery.

SURGICAL PACKS:
Each surgical pack consists of the following:
- Surgical Draping, folded appropriately: 1 big square sheet (bottom liner), 1 smaller square sheet (for moving the rat around), and 1 rectangular sheet folded length-wise in 2 (for the catheter).
- Cotton-tipped Applicators (2-3)
- Gauze
- Suture thread (silk 4.0) around 45 cm (may vary by user) divided in 2.
Surgical packs should be autoclaved ahead of time. Opened packs should be discarded or re-autoclaved before use.

**PREPARATION:**

**A) Catheter and surgical area preparation:**
- Catheters are flushed with the cold sterilant, checked for patency; pressure tested for leaks and for length of the insertion tip (not longer than 30mm). Catheters should be immersed in the cold sterilant for 20 minutes.
- Turn the glass bead sterilizer on, and keep it ready for use in case of immediate need of sterilization during the surgery.
- Place a clean bench pad liner on the clean surgical table
- Open a catheter pack and place the bottom liner draping on the bench pad liner, then the smaller square sheet of draping on top of that one. Draping sheets should be handled only using the sterile forceps.

**B) Rat Preparation:**
- Rats should be assessed on the day before the surgery to ensure their state of health can endure the surgical stressor.
- Rats are brought up to the surgical suite, weighed and the weight recorded on the surgical data sheet before the procedure is started. It is recommended that the rat weighs at least 300 grams.
- Adequacy of anesthesia can be confirmed by pinching the distal portion of the tail or the toes of the hind limb and noting absence of a withdrawal reflex.
- Shave the incision areas: 1- the right ventral region of the neck and 2- the dorsal area between the scapulae.
- Administer the preoperative medications as follows:
  - Marcaine (1.25%) subcutaneously along the incision line (0.1 ml/site).
  - Ketoprofen (Anafen) subcutaneously. Please see SOP for preparation and dose.
  - Ringer lactate can be given for rehydration following the formula 10ml/kg/hr. For surgeries < 45min. it’s up to the user to decide whether or not rehydration is needed.
- Apply eye ointment (Lacrilube) bilaterally to avoid corneal dryness.
- Clean the incision areas with betadine scrub (scrub 3 times in a circular motion starting from the inside to the outside), rinse with 95% alcohol and wipe in the same way, then apply Betadine on the top.
- Move the rat to the surgical table and place on the prepared draping in dorsal recumbency.

**C) Surgeon Preparation:**
- Hands should be washed thoroughly.
- Sterile gloves should be opened and donned at the surgical table, immediately prior to the beginning of the surgery. Care should be taken to minimize touching non-sterile surfaces including the body of the rat. Handling the rat thereafter should be done using the surgical draping.
SURGICAL PROCEDURE:

- Make an oblique incision of the skin in the ventral region of the right side of the neck. Clear the superficial muscle layer by blunt dissection.
- Locate the jugular vein and using finely serrated forceps, strip it of all fascia. Run a locating suture beneath the vein to facilitate later retrieval.
- Place the rat in ventral recumbence and make a transverse incision between the scapulae. By blunt dissection, clear a subcutaneous pocket in the tissue surrounding the incision. It must be large enough to accommodate both the mesh assembly and the excess catheter tubing.
- Insert the needle driver into the dorsal incision and direct it behind the right forearm and towards the ventral incision. In order to make this “tunnel” as superficial as possible, the tips of the driver should be pointed upwards during this process. Punch through the connective tissue to emerge at the ventral incision. Open the jaws of the driver and securely grab the trocar. Relock the jaws and pull the trocar to the dorsal incision by gently twisting the needle driver. Once the trocar has been “threaded” through both incisions, the catheter may be passed through the trocar. It must be fed in a dorsal-ventral direction; i.e. the insertion tip is introduced into the trocar from the dorsal end. Once a suitable length of catheter is visible at the ventral site, the trocar may be removed simply by pulling it out through the ventral incision. This procedure, while cumbersome, ensures that there is no stress exerted on the catheter, thereby protecting its integrity.
- Orient the catheter such that the silastic runs medial to the PE and all tubing will ultimately lie flat. Attach a 1 cc syringe with a 22 blunted needle to the catheter and flush with sterile saline. Ensure that there are no bubbles in the line.
- Lift the jugular and using micro-scissors, make an incision 2/3 of the way through the vein. Release the vein.
- Grasp the silastic tip of the catheter and insert into the jugular incision. The tubing should feed in freely, all the way to the heat shrink. It may be necessary to adjust the amount of PE tubing present at the incision site; pulling on the scapular end may do this. Once inserted, the entire assembly should lie flat and neat.
- Tie off the catheter to the jugular with two sutures, one at each end of the heat shrink connection. It is vital that the suture does not slip off onto the silastic, as it will occlude the tubing.
- Anchor the PE 10 tubing to deep muscle with a single suture. The heat shrink is then secured to underlying tissue by a single drop of Krazy glue on its underside.
- Verify the patency of the catheter by drawing back on the syringe and obtaining blood in the PE tubing. Flush back into the rat with a small amount of saline (0.1 ml)
- Close the superficial muscle layer with 1-2 sutures. This serves as additional protection should the rat scratch at his incision. Close the skin with interrupted sutures.
- Turn the rat over and disconnect the syringe from the catheter. Cap the catheter with a filled silastic plug.
- Feed the excess PE 20 tubing into the subcutaneous pocket in a looping fashion. It is quite normal for it to encircle the incision. Insert the mesh assembly and work with it until it lies flat, centered between the scapulae, and superficial to all tubing.
• Suture the skin around the nylon bolt, making sure neither to purse the skin nor catch the mesh in the sutures.
• Move the rat to the recovery area for immediate post-surgical care.

IMMEDIATE POST-OPERATIVE CARE:
• Upon completion of the surgery, rats should be moved to the recovery area, and placed on a thermostatically controlled heating blanket (to avoid anaesthetic-induced hypothermia).
• Rats should NEVER be left unattended during the recovery period.
• Topical antiseptics could be applied to the surgical wounds. For example, Cicatrin® cutaneous powder.
• Rats should be monitored closely to ensure post-operative analgesia.
• Breathing, heart beats, warmth and colour of the paws should be assessed regularly.
Upon full recovery from anaesthesia, rats should be moved to a bedded cage lined with paper towels (to avoid inhalation of bedding) until transported to their respective home cage.
APPENDIX III

Standard Operating Procedure for Intracranial Cannulae Implantation Surgery
Standard Operating Procedure for Intracranial Cannulation Surgery

PURPOSE:

Insertion of guide cannulae into discrete areas in the rat’s brain, for guiding the insertion of mincroinjectors for the delivery of drugs intracranially.

LAB. LOCATION: T618

INSTRUMENTS:

1. Stereotaxic frame mounted with cannula holders (especially if using Plastics One cannulae) and containing ear bars,
2. Scalpel and sterile scalpel blades,
3. 4 bulldog clamps for retraction of the scalp,
4. 1-2 Guide cannulae (to be inserted),
5. 1 pair of forceps (preferably curved),
6. Skull mounting screws,
7. Screwdriver,
8. Tap drill,
9. Dental drill equipped with a drill bit,
10. Dental acrylic powder and jet acrylic fluid as a solvent,
11. Spatula,

All instruments should be autoclaved before the beginning of the surgeries (at the start of each day of surgery). Subsequently, in between the surgeries, the instruments can be sterilized by immersion in a cold sterilant (for e.g. Virox or Accel CS 20) for 20 minutes. Alternatively a hot (glass) bead sterilizer can be used for disinfection of instrument tips in between the surgeries. The instruments must be cleaned thoroughly of blood residues before being placed in the sterilant.

Everyday, at the end of the surgical procedures, the instruments should be cleaned, sonicated and left to dry then stored safely (please see SOP for Surgical Suite Disinfection for details).

YOU WILL ALSO NEED:

1. 2 beakers (250 cc each) of the cold sterilant.
2. 1 beaker (250 cc) of saline,
3. A calculator,
4. Data sheets with the specific insertion coordinates and,
5. A pencil

All glassware should be autoclaved and the packs should be freshly opened on the day of surgery.
SURGICAL PACKS:

Each surgical pack consists of the following:

- Surgical Draping, folded appropriately: 1 big rectangular sheet (bottom liner; between the rat and the stereotaxic), 1 smaller rectangular sheet (blanket; covers the back of the rat), and 1 small square sheet with a circular hole in the middle (head cover; optional),
- Cotton-tipped Applicators (5-6),
- Gauze,
- 1-2 obturators (dummy cannulae).

Surgical packs should be autoclaved ahead of time. Opened packs should be discarded or re-autoclaved before use.

PREPARATION:

A) Cannulae and surgical area preparation:

- Cannulae should be autoclaved ahead of time or sterilized using the hot bead sterilizer. Cannulae should not be sterilized using the cold sterilant.
- Place the cannulae into mounting holders and secure them in place using the side screws on the holder.
- The hot bead sterilizer should be kept on and ready for use in case of immediate need of sterilization during the surgery.
- Place a clean bench pad liner on the clean surgical table and place the stereotaxic on top of it.
- A “Lazy Suzan” or turning tray can be placed on top of the bench pad liner and under the stereotaxic to facilitate moving the stereotaxic device.
- Open a surgical pack and place the bottom liner draping on the stereotaxic. Draping sheets should be handled only using the sterile forceps.

B) Rat Preparation:

- Rats should be assessed on the day before the surgery to ensure their state of health can endure the surgical stressor.
- Rats are brought up to the surgical suite, weighed and the weight recorded on the surgical data sheet before the procedure is started. It is recommended that the rat weighs at least 300 grams.
- After the anaesthetic is delivered, adequacy of anaesthesia can be confirmed by pinching the distal portion of the tail or the toes of the hind limb and noting absence of a withdrawal reflex.
- Shave the incision area at the top of the skull.
- Administer the preoperative medications as follows:
  - Marcaine (1.25%) subcutaneously along the incision line (0.1 ml/site).
  - Ketoprofen (Anafen) subcutaneously. Please see SOP for preparation and dose.
  - Ringer lactate can be given for rehydration following the formula 10ml/kg/hr. For surgeries < 45min. it’s up to the user to decide whether or not rehydration is needed.
- Apply eye ointment (Lacrilube) bilaterally to avoid corneal dryness.
- Clean the incision area with betadine scrub (scrub 3 times in a circular motion starting from the inside to the outside), rinse with 95% alcohol and wipe in the same way, then apply Betadine on the top.
• Move the rat to the surgical table and place on the prepared draping on the stereotaxic. Insert the ear bars into the rat’s ear canals. Signs for successful correct insertion include blinking and/or nose pointing straight and to the midline. Tighten the ear bar enough to keep the head from moving, but taking care not to over-tighten resulting in the rupture of the rat’s ear drums.
• Place the incisors on the incisor bar and tighten it into place.
• Drape the body of the rat and the skull using the autoclaved surgical drapings.

C) Surgeon Preparation:
• Hands should be washed thoroughly.
• Sterile gloves should be opened and donned at the surgical table, immediately prior to the beginning of the surgery. Care should be taken to minimize touching non-sterile surfaces. Handling the rat thereafter should be done using the surgical draping.

SURGICAL PROCEDURE:
• Make a midline incision in the scalp using the scalpel blade. Retract the skin by pulling on the fascia using the bulldog clamps.
• Scrape off the periosteum using the scalpel blade and/or cotton-tipped applicator. Bregma and Lambda should be exposed and clear.
• Level the skull horizontally by taking the DV coordinates of Bregma and Lambda. Allow for 0.05 mm difference, otherwise change the nose piece position up or down to achieve a flat horizontal level of the skull.
• Three dimensional coordinates (AP, ML, and DV) of Bregma are noted and cannulae insertion coordinates are calculated accordingly, and marked. Set the stereotaxic arms to the new coordinates (AP and ML) and swing them out of the way temporarily.
• Use a tap drill to make 3-4 holes in the skull to insert the mounting screws using a screwdriver.
• Use the dental drill to make a hole in the skull at the sites already marked for cannulae insertion. Drilling is best performed perpendicularly to the skull. Use a thin sterile needle to puncture the dura. In case of excessive bleeding, use a cotton tipped applicator or gauze to ensure stoppage of the bleeding prior to cannulae insertion.
• Swing the stereotaxic arms back into place, taking care to align them at perfect zero (so their previous position is not altered). Carefully lower the cannulae to the set DV coordinates.
• Prepare the dental cement and carefully apply over the cannulae using the spatula, taking care to cover a good proportion of the cannulae protruding above the skull surface without cementing the cannulae to the cannulae holder.
• Wait for the cement to completely dry before attempting to move or unscrew the holders out of place.
• Place the obturators (dummy cannulae) in place.
• Gently remove the bulldog clamps, and remove the rat from the ear and incisor bars.
• Move the rat to the recovery area for immediate post surgical care.

PLEASE NOTE:
- If the insertion requires angulations, the angles should be set right at the beginning of the procedure and all the coordinates should be noted and calculated accordingly.
For some areas, especially if the insertion is at an angle, the DV coordinates are best read at the skull surface at the target insertion point.

- Screws should not be mounted on suture lines. The depth of insertion of the screws should only be halfway into the skull to avoid widening of the holes or cracking of the bone.
- If you are inserting bilateral cannulae into a structure that is close to the midline, it may be difficult to insert both cannulae at the same time. Alternatively, you can insert the cannula on one side and cement it in place. Care should be taken, in such a case, not to occlude or cover the target site on the other side with the cement. Once the cement has completely dried and the holder is removed, the other cannula can be inserted. A thin layer of cement should then be added to cover the 2 sides homogenously.

**IMMEDIATE POST-OPERATIVE CARE:**

- Upon completion of the surgery, rats should be moved to the recovery area, and placed on a thermostatically controlled heating blanket (to avoid anaesthetic-induced hypothermia).
- Rats should **NEVER** be left unattended during the recovery period.
- Topical antiseptics could be applied to the surgical wounds. For example, *Cicatrin® cutaneous powder*.
- Rats should be monitored closely to ensure post-operative analgesia.
- Breathing, heart beats, warmth and colour of the paws should be assessed regularly.

Upon full recovery from anaesthesia, rats should be moved to a bedded cage lined with paper towels (to avoid inhalation of bedding) until transported to their respective home cage.

- Following intracranial surgery, rats may need soft food to avoid painful mastication resulting from ear and incisor bars insertion (Please refer to the SOP for post operative care for details).

**ABBREVIATIONS:**

DV: Dorsal / Ventral.
AP: Anterior / Posterior.
ML: Medial / Lateral.