Methodological study of nicotine conditioned place preference in rats

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Abstract

It is widely believed that nicotine is the main reason for acquiring and maintaining tobacco addiction. However, animal studies suggest that nicotine is only a weak reinforcer compared to other drugs of abuse. For example, nicotine does not consistently produce a conditioned place preference (CPP), a standard measure of reward in rats. We attempted to examine the reason(s) for this discrepancy by manipulating several procedural variables in this paradigm. In addition, we hypothesized that partial monoamine oxidase (MAO) inhibition, as observed in smokers, may potentiate the rewarding effects of nicotine in the CPP paradigm. Overall, we were not able to obtain reliable nicotine CPP and none of the procedural variables tested (e.g. speed of injection, nicotine pre-treatment) proved to play an important role in acquisition of nicotine CPP. Possible reasons for the failure of our experiments and of other nicotine CPP studies are discussed.

Résumé

On le croit largement que la nicotine est la raison principale pour l'acquisition et la maintenance de la dependence du tabac. Cependant, les expériences effectuées sur des animaux suggèrent que la nicotine est seulement un renforçateur faible comparé à d'autres drogues dures. Par exemple, la nicotine ne produit pas uniformément une préférence de lieu conditionné (PLC), une mesure standard de récompense chez les rats. Nous avons essayé d'examiner la/les raison(s) de cette anomalie en manipulant plusieurs variables procédurales dans ce paradigme. En outre, nous avons présumé que l'inhibition partielle de l'oxydase de monoamine (OMA), comme observée chez les fumeurs, peut renforcer les effets récompensants de la nicotine dans le paradigme de la PLC. De façon générale, nous n'avons pas réussi à obtenir une PLC fiable par la nicotine et aucune des variables procédurales examinés (par exemple vitesse d'injection, prétraitement de nicotine) n'est apparue importante dans l'acquisition de la PLC par la nicotine. Des raisons possibles de l'échec de nos expériences et des autres études de la PLC par la nicotine sont discutées.

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List of abbreviations

6-OHDA	6-hydroxydopamine
CPP	conditioned place preference
CTA	conditioned place aversion
df	degree of freedom
FR	fixed ratio
GABA	γ-aminobutyric acid
i.p.	intraperitoneal
i.v.	intravenous
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
PR	progressive ratio
s.c.	subcutaneous
TPP	tegmental pedunculopontine
VTA	ventral tegmental area

Chapter 1: Introduction

1.1 - Tobacco smoking

According to world health organization (WHO) almost one fifth of the world population, about 1.1 billion people, smoke and four million people die annually from smoking-associated illnesses (WHO, 2002). In the U.S.A alone, where around 21% of the population smokes (CDC, 2005c), tobacco smoking is responsible for around 430,000 deaths each year (CDC, 2005b). The great difficulty in quitting smoking demonstrates how addictive tobacco smoking is. In the U.S.A 70% of smokers report that they want to quit but only about half of them try to quit annually (CDC, 2005a) and less than 10% of them successfully quit on their own (Zhou et al, 2000). Even with the help of smoking cessation therapies the rate of success is not that impressive (Hurt et al., 1997; Zhu et al., 2000; Silagy et al., 2004; Jorenby et al., 2006).

Tobacco smoke contains more than 4000 different substances (Roberts, 1988) but it is widely believed that although environmental factors also contribute to smoking, the main reason that people smoke is to obtain nicotine (Stolerman and Jarvis, 1995). Nicotine is the major psychoactive component of tobacco that plays a crucial role in tobacco dependence through its actions as a reinforcer of drug-seeking and drug-taking behaviour (Goldberg et al., 1988; Stolerman and Shoaib, 1991; Le Foll and Goldberg, 2006).

Some of the important criteria of drug addiction that are met by tobacco and support the idea that tobacco and/or nicotine are addictive are as follows:

1-Compulsive use: most smokers smoke more than 10 cigarettes on daily basis and about 60% of them light up within 30 minutes of waking up (Heatherton et al., 1991). In

addition, many smokers who have smoking-related diseases such as lung cancer and laryngectomy still continue to smoke (Davison and Duffy, 1982; Himbury and West; 1985).

2-Withdrawal syndrome: when tobacco smoking is stopped, withdrawal symptoms such as irritability, difficulty concentrating, insomnia and weight gain occur (Hughes et al., 1992). Nicotine is able to reduce some but not all of these withdrawal symptoms (Hughes et al., 1992).

3- Nicotine can serve as a positive reinforcer: many animal species including humans self-administer nicotine (for reviews see Balfour, 2002; Le Foll and Goldberg, 2006). This will be discussed further in upcoming sections.

4-Brain reward system: nicotine, like other addictive drugs is able to increase dopamine release in the ventral striatum of humans (Brody et al., 2004) and animals (Benwell and Balfour, 1992; Nisell et al., 1994). This will also be discussed further in coming sections.

Most of this evidence has led to the general belief that tobacco addiction and nicotine addiction are roughly equivalent; therefore most research has been focused on nicotine. However, two separate sets of observations call into question the importance of nicotine in maintaining tobacco smoking. The first set of findings to be discussed later in this chapter, indicate that nicotine has at best only weak rewarding properties when administered to animals. The second line of evidence concerns the poor effectiveness of nicotine replacement therapy in smoking cessation. In addition to counselling and behavioural interventions, pharmacotherapies are available for smoking cessation. The two FDA-approved pharmacotherapies for smoking cessation are nicotine replacement therapy (NRT, i.e. nicotine patch, gum, nasal spray, inhaler, and lozenge) and sustained

release bupropion. Although these therapies almost double the rate of long-term (12 months) abstinence compared to placebo, many smokers do not respond to these therapies (for reviews see George and O'Malley, 2004; Mitrouska et al., 2006). Moreover, although nicotine replacement therapies reduce withdrawal symptoms, they can not relieve them completely (Jarvis et al., 1982; Hughes et al., 1984; for review see Karnath, 2002). The low success rate of nicotine replacement therapies may be due to the difference in pharmacokinetics of nicotine delivery by nicotine replacement therapies compared to tobacco smoke; or it may suggest that smoking may be more than nicotine addiction. I will try to explore this issue in subsequent sections.

1.2 - How are the rewarding effects of drugs studied?

Reward can be defined in several ways; two of these definitions are important for understanding the two most common methods of measuring reward. According to one definition, the stimuli that are rewarding are able to elicit approach behaviour and maintain contact (Schneirla, 1959). The other definition is that rewarding stimuli have the capacity to increase the probability of responses that precede them to be repeated; this is usually referred to as 'reinforcement' (Skinner, 1938).

Rewarding stimuli influence behaviour, so how they affect behaviour can be a measure of reward. Two major methods serve to assess the rewarding properties of drugs in animals: the self-administration and conditioned place preference (CPP) paradigms. These will be discussed in more detail in later sections. The self-administration paradigm measures reward, defined as reinforcement. In this paradigm, delivery of the drug is dependent on

an operant response. If the drug is rewarding, the preceding response should be repeated (reinforced). In contrast, the CPP relies on the capacity of conditioned stimuli, which have gained their rewarding properties through repeated association with the drug (conditioning), to elicit approach responses or maintain contact.

1.2.1 - Self-administration paradigm

The most common form of the self-administration paradigm is *intravenous* self-administration. In this paradigm, an operant response such as a nose poke or lever press leads to intravenous drug infusions via a catheter implanted in the jugular vein. The pharmacological actions of drugs of abuse tend to result in repetition of the operant response to obtain more drug (reinforcement). It is worth mentioning that the lever, due to its close temporal and repetitive pairing with the drug injection, may become a conditioned reward, which could elicit approach behaviour.

Many researchers have employed this paradigm to study the behavioural and neurobiological basis of drug reinforcement. It has been possible to study the reinforcing properties of most drugs of abuse by means of this technique (for reviews see Roberts and Goeders, 1989; Gardner, 2000).

Two schedules of reinforcement have been commonly used in the self-administration paradigm. Under the fixed-ratio (FR) schedule, a fixed number of responses will result in drug infusion but under the progressive-ratio (PR) schedule, the number of operant responses required to obtain a drug infusion is increased after each infusion (Hodos, 1961) until the animal fails to meet the requirements necessary for a drug infusion

(breaking point). The breaking point is thought to reflect the reinforcing effectiveness of the drug.

The major disadvantage of the intravenous self-administration paradigm is that the animal's behaviour may be affected by arousing/sedating effects of drugs. This is avoided in the CPP paradigm where animals are tested in a drug-free state.

1.2.2 – Conditioned place preference (CPP) paradigm

CPP procedure is an alternative to the self-administration paradigm for assessing the rewarding properties of drugs of abuse in animals (Carr et al., 1989). This paradigm of associative learning and responding has been utilized by many researchers (for reviews see Carr et al., 1989; Tzschentke, 1998).

In this paradigm, one compartment of the apparatus is repeatedly paired with the administration of the drug, and it is assumed that the rewarding properties of the drug become associated with distinctive environmental cues present in the drug-paired compartment through Pavlovian conditioning (Bardo and Bevins, 2000). These distinctive environmental cues (conditioned stimuli) can eventually elicit approach behaviour and maintain contact when the animal has free access to both the drug-paired and vehicle-paired compartment in a drug-free state (Tzschentke, 1998). The time spent in the drug-paired compartment is a measure of reward or drug-seeking behaviour (Carr et al., 1989).

There are at least two compartments in each CPP apparatus, with distinctive visual and/or tactile cues. Based on the animal's initial preference for the distinct compartments, there are two types of CPP apparatus: biased vs. unbiased. If naïve untrained animals show a

spontaneous preference for one compartment (with its distinct cues), the apparatus is termed 'biased'; conversely, the apparatus is defined as 'unbiased' when the animals show no preference for one compartment over the other (Bardo and Bevins, 2000).

There are also two ways to assign the animals to the compartment to be paired with the drug in the CPP paradigm. If this compartment is based on each animal's initial individual preference, it is called a 'biased' design. In contrast, if the assignment of the compartment to be paired with the drug is done randomly without regard to initial compartment bias, it is termed the 'unbiased' design (Carr et al., 1989).

The use of 'biased' assignment has been criticized because it can produce problems in interpreting the results of the CPP procedure. For example when animals receive the drug on the non-preferred side (the usual case), and if the non-preferred side presumably causes anxiety and stress, a CPP could develop due to a stress-alleviating, rather than a rewarding effect of the drug (Schenk et al., 1985; Carr et al., 1989). This may be particularly relevant for nicotine because animal studies suggest that nicotine may exert anxiolytic and stress-alleviating effects (Morrison, 1974; Balfour, 1991; Brioni et al., 1993); therefore in a biased CPP paradigm when nicotine is paired with the non-preferred compartment, the increase in the time spent in this compartment on the test day could be due to anxiolytic or stress-alleviating rather than the rewarding properties of nicotine.

Almost all drugs of abuse that are self-administered by animals can also produce CPP (for review see Bardo and Bevins, 2000). These drugs include opiates (Mucha and Iversen, 1984; Dworkin et al., 1988), amphetamine (Yokel and Wise, 1976; Spyraki et al., 1982), cocaine (Spyraki et al., 1982; Caine and Koob, 1994) and nicotine (Fudala and Iwamoto, 1986; Corrigall and Coen, 1989).

The CPP paradigm has some advantages and disadvantages. Some of its major advantages are as follows:

1-It can measure both rewarding and aversive properties of a drug (Carr et al., 1989).

2-Testing is done in a drug-free state. This is important for drugs that effect locomotor activity such as cocaine and amphetamine (Carr et al., 1989). In contrast, in the self-administration paradigm, the behavioural activating ('arousing') effects of the drug may affect operant response rate, leading to interpretational difficulties.

3-The drug dose is controlled (Carr et al., 1989). In the self-administration paradigm, although the dose is controlled, the number of responses that lead to drug infusion and therefore the final amount of infused drug is not usually controlled. In the CPP paradigm, the drug is administered by the experimenter and therefore the dose is controlled precisely and all animals receive the same dose.

4-It is adaptable to a variety of laboratory animals (Bardo and Bevins, 2000). For example, intravenous self-administration can be done in mice but it is technically difficult, whereas CPP can easily be carried out in this species.

5-It has considerable use in probing the neuronal circuits involved in drug reward (Bardo and Bevins, 2000).

6- It requires minimal motor output. In this paradigm, reward is measured by the *proportion* of time spent in each compartment; hence the magnitude of CPP is unlikely to be effected by motor impairments that might have resulted from previous manipulations (e.g. surgery).

The major disadvantages of this paradigm are:

1-A dose-response curve is difficult to obtain (Carr et al., 1989) for two reasons. First, in the self-administration paradigm, several doses of the drug can be tested in a single

animal whereas in the CPP paradigm different groups of animals should be used for assessing the rewarding properties of different doses. Second, in the CPP paradigm the relation between magnitude of the place preference and the dose is not simple; place preference is almost an all or nothing outcome.

2-Negative results are difficult to interpret (Carr et al., 1989). CPP is very dependent on experimental conditions such as the apparatus; thus a negative result may be due to a problem with the apparatus or the experimental design.

3-CPP currently lacks face validity as an experimental protocol of drug reward in humans (Bardo and Bevins, 2000), since it is not known whether human subjects develop place preference to drugs.

1.3 - Dopamine and reward

It is widely believed that the mesolimbic dopaminergic system is critically involved in mediating the reinforcing effects of natural rewards, such as food, and drugs of abuse (for reviews see Di Chiara et al., 1993; Di Chiara, 1995; Spanagel and Weiss 1999; Wise 2000). A major component of this reward circuit is the dopaminergic projection from the ventral tegmental area (VTA), which contains dopaminergic cell bodies, to the terminal fields in the basal forebrain, in particular nucleus accumbens (NAc) (Roberts et al., 1977; Lyness et al., 1979; Mogenson et al., 1979).

Microdialysis, lesion and pharmacological manipulation studies support the role of dopamine and the dopaminergic projection from the VTA to NAC in mediating the rewarding effects of most drugs of abuse. Some important pieces of evidence from each

type of study are as follows:

1.3.1 - Microdialysis studies

Various classes of abused drugs, including psychostimulants, opioids and nicotine, while acting on different primary molecular targets, enhance dopamine neurotransmission within the NAc (Imperato et al., 1986; Di Chiara and Imperato, 1988; Hurd et al., 1989; Hemby et al., 1995), especially in the NAc shell subregion (Pontieri et al., 1995; Pontieri et al., 1996).

1.3.2 - Lesion studies

6-OHDA lesions of dopamine terminals in the NAc decrease or abolish cocaine and amphetamine self-administration (Roberts et al., 1977; Roberts et al., 1980; Lyness et al., 1979; Pettit et al., 1984; Cain and Koob, 1994) and amphetamine-induced CPP (Spyraki et al., 1982). In addition, it has been shown that 6-OHDA lesions of dopamine terminals in the NAc, decrease nicotine self-administration (Corrigall et al., 1992).

The effect of lesions of NAc on cocaine-induced CPP appears to be dependent on route of administration; while i.v. cocaine-induced CPP is reduced by 6-OHDA lesions of the shell subregion of the NAc (Sellings et al., 2006), lesions of NAc have no effect on i.p. cocaine-induced CPP (Spyraki et al., 1982; Sellings et al., 2006). It seems like that the peripheral local anaesthetic properties of cocaine may produce CPP even when its central rewarding properties are abolished (Spyraki et al., 1982).

The role of the dopaminergic projection from the VTA to NAc in mediating the

rewarding effects of opiates is more complex. Studies demonstrate that lesions of the NAc do not disrupt morphine or heroin self-administration (Pettit et al., 1984; Dworkin et al., 1988) or morphine-induced CPP (Sellings and Clarke, 2003) but do abolish heroin-induced CPP (Spyraki et al., 1983). It has been suggested that the role of dopamine in mediating the rewarding effects of opiates is dependent on the motivational state of the animal; in drug naïve animals dopamine is not responsible for the rewarding effects of opiates while in drug-dependent animals the rewarding effects are dopamine-mediated (Laviolette et al., 2002). This hypothesis clarifies findings with opiate-induced CPP but leaves the apparent dopamine-independence of i.v. self-administration unexplained.

1.3.3 - Pharmacological manipulations

Some dopamine agonists such as apomorphine and peribedil support self-administration (Baxter et al., 1974; Yokel and Wise, 1978) and induce CPP (Spyraki et al., 1982). Studies using dopamine antagonists indicate that dopamine receptors (D1 and D2) are involved in mediating the reinforcing effects of cocaine in the self-administration paradigm (Ettenberg et al., 1982; Roberts et al., 1989; Hubner and Moreton, 1991; Corrigall and Coen, 1991; Maldonado et al., 1993).

The effect of dopaminergic antagonists on cocaine induced CPP seems to be dependent on the route of administration of the drug (Spyraki et al., 1987); in particular, CPP produced by i.v. but not i.p. cocaine is inhibited by dopamine antagonist (Spyraki et al., 1987; Baker et al., 1998). Dopamine antagonists can also reduce or block both amphetamine and nicotine self-administration (Yokel and Wise, 1976; Corrigall and

Coen, 1991) and CPP (Spyraki et al., 1982; Acquas et al., 1989; Hiroi and White, 1991; Le Foll et al., 2005; Spina et al., 2006).

Heroin- and morphine-induced CPP are blocked by dopamine antagonists (Schwartz and Marchok, 1974; Bozarth and Wise, 1981; Spyraki et al., 1983) but opiate self-administration is not disrupted by dopamine antagonists (Ettenberg et al., 1982). Possible explanation for this controversy was discussed above.

1.4 - How does nicotine exert its rewarding effects?

Studies in rats show that mesolimbic dopamine neurons express nicotinic acetylcholine receptors on their cell bodies and/or dendrites in the VTA as well as on mesolimbic afferents to the NAc (Clarke and Pert, 1985). Acutely administered nicotine acts directly on the nAChRs on dopamine cells in the VTA to increase the firing rate (Mereu et al., 1987; Calabresi et al., 1989) and burst firing of these dopamine neurons (Grenhoff et al., 1986; Erhardt et al., 2002; Schilstrom et al., 2003), which leads to increased levels of extracellular dopamine in the NAc (Benwell and Balfour, 1992; Nisell et al., 1994), especially the shell subregion (Pontieri et al., 1996; Nisell et al., 1997).

This increased dopamine activity in the VTA may be essential to the reinforcing effects of nicotine, as studies show that blocking dopamine activity in the NAc with antagonists or lesions attenuates the reinforcing effects of nicotine as shown by reduced self-administration (Corrigall and Coen, 1991; Corrigall et al., 1992).

It has been shown that i.v. self-administration of nicotine in rats is attenuated by blockade of nicotinic receptors in the VTA (Corrigall et al., 1994), suggesting that activation of

nAChRs in the VTA is also necessary for the reinforcing effects of nicotine in this behavioural paradigm.

Nicotine-induced CPP is also disrupted by blockade of nicotinic and dopaminergic receptors. Mecamylamine, a centrally-active nicotinic receptor antagonist (crosses the blood brain barrier) but not hexamethonium, a peripherally-active nicotinic receptor antagonist (does not cross the blood brain barrier), blocks nicotine CPP (Fudala et al., 1985). This suggests that the rewarding effects of nicotine are mediated by central rather than peripheral receptors.

Systemically administered D1 antagonists block the acquisition of nicotine CPP (Acquas et al., 1989). In addition, D1 antagonists infused into the shell but not the core subregion of the NAc, block the acquisition of nicotine CPP while having no effect on its expression (Spina et al., 2006). Systemically administered D3 partial agonists and D3 antagonists block the expression of nicotine CPP (Le Foll et al., 2005).

However, it is worth mentioning that it also has been reported that lesions of tegmental pedunculopontine (TPP) block nicotine-induced CPP (Laviolette et al., 2002). Thus, nicotine can produce rewarding effects that depend on the mesolimbic dopaminergic system but this does not rule out involvement of other brain mechanisms in mediating the rewarding effects of nicotine.

1.5 - Nicotinic acetylcholine receptors (nAChRs)

Nicotinic receptors are pentameric ligand-gated ion channels that conduct cations when

activated (for review see Unwin, 2003). These receptors can exist in different functional states. They are mainly closed (resting) in the absence of agonist, are briefly open when exposed to agonist and are desensitized (inactive) when agonist is present in a high concentration or for a long period of time. The functional state of nicotinic receptors depends on many factors including the nAChR subtype, the agonist concentration and the rate of agonist application (Changeux et al., 1984).

At least six α (α2-α7) and three β (β2-β4) nAChR subunits are expressed in mammalian neurons (Klink et al., 2001). These subunits assemble to form multiple nAChR subtypes. While α7 subunits generate homooligomeric nicotinic receptors, other subunits generate heterooligomeric nicotinic receptors (Klink et al., 2001). These multiple nAChR subtypes have different pharmacological and biophysical properties, such as nicotine sensitivity and rate of desensitization (Le Novere et al., 2002).

1.5.1 - Nicotinic receptors on dopaminergic neurons in the VTA

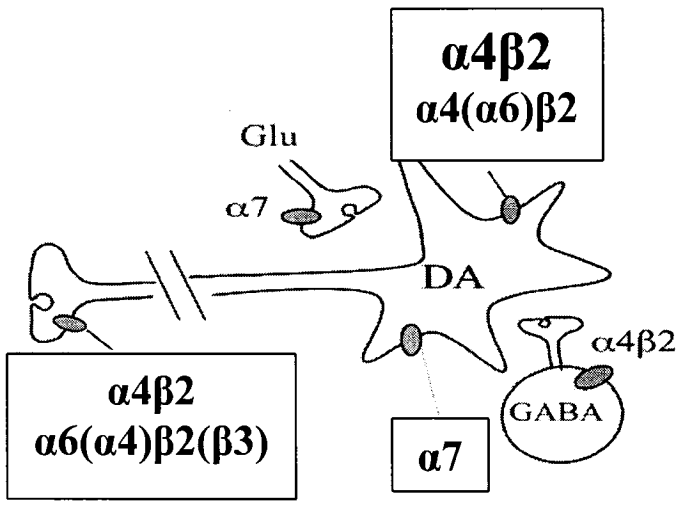
As shown by autoradiography and *in situ* hybridization, both dopaminergic and non-dopaminergic neurons in the VTA express nAChRs (Wada et al., 1989; Marks et al., 1992; Clarke, 1993). Dopaminergic neurons in the VTA express three main heteromeric nAChRs: α4β2, α6β2 and α4α6β2. All three subtypes are present on the dopaminergic neurons terminal fields, while the α4β2 subtype is the predominant subtype present on dopaminergic cell bodies (Champtiaux et al, 2003). In addition to the heteromeric nAChRs, dopaminergic neurons in the VTA also express the homomeric α7 nAChR subtype (Wu et al., 2004).

In vivo microdialysis studies in rats demonstrate that a single nicotine exposure increases the level of dopamine in the NAc for more than an hour (Imperato et al., 1986; Di Chiara and Imperato, 1988; Nisell et al., 1994; Schilstrom et al., 1998). However, in the presence of smoking-relevant concentrations of nicotine, nAChRs on the VTA dopaminergic neurons desensitize rapidly (Pidoplichko et al., 1997; Dani et al., 2000). Therefore, additional mechanisms should contribute to the prolonged increase in dopamine levels while most of the nAChRs on the VTA dopaminergic neurons are desensitized.

1.5.2 - Nicotinic receptors on non-dopaminergic neurons in the VTA

nAChRs are also expressed on the GABAergic inputs to the VTA dopaminergic neurons. These nAChRs are pharmacologically similar to the nAChRs expressed by VTA dopaminergic neurons and are likely to contain α4 and β2 subunits (Pidoplichko et al., 1997; Charpantier et al., 1998). In addition, glutamatergic afferents projecting to the VTA possess α7 nAChRs (Mansvelder and McGehee, 2000; Schilstrom et al., 2003; Jones and Wonnacott, 2004). As mentioned before, different nAChR subtypes have different activation and desensitization properties (Pidoplichko et al., 1997; Le Novere et al., 2002). In particular, the α7 and α4β2 nAChR subtypes differ considerably in this regard (Fenster et al., 1997; Corringer et al., 1998). The β2-containing nAChRs have a high affinity for nicotine and slow activation and desensitization kinetics. Therefore, exposure to concentrations of nicotine obtained from smoking, desensitizes them rapidly (Wooltorton et al., 2003). In contrast, the α7 nAChRs have rapid activation and desensitization profiles (Alkondon et al., 1992; Castro and Albuquerque, 1995; Gray et

al., 1996). Although these receptors desensitize rapidly in the presence of high concentrations of nicotine (500 μM), they have a low affinity for nicotine and therefore in the presence of low concentrations of nicotine obtained from smoking (100-500 nM; Henningfield et al., 1993) are not substantially desensitized (Wooltorton et al., 2003).



Adapted from Champtiaux et al., 2003

Nicotine at concentrations similar to those achieved by smokers activates the nAChRs in the VTA. Activation of β2-containing nAChRs residing on the dopaminergic and GABAergic neurons results in a transient increase in dopamine and GABA release. After a few minutes of exposure to these concentrations of nicotine, these nAChRs desensitize (Wooltorton et al., 2003) and therefore dopamine and GABA release are inhibited. Dopaminergic neurons are under tonic inhibitory control by GABA and inhibition of GABA release results in an indirect increase in dopamine release (Ikemoto et al., 1997). Moreover, the α7 nAChRs residing on dopaminergic neurons and glutamatergic terminals are activated and stay activated by the same concentrations of nicotine (Wooltorton et al.,

2003).. The activation of α7 nAChRs on the glutamatergic terminals results in increased glutamate release in the VTA (Schilstrom et al., 2000). Glutamate activates the NMDA receptors residing on VTA dopaminergic neurons (Seutin et al., 1990) and indirectly increases dopamine release (Schilstrom et al., 1998a, b). Overall, the removal of inhibition by GABA and activation by glutamate of the VTA dopaminergic neurons results in prolonged dopamine release by concentrations of nicotine similar to those obtained by smokers. This may explain the prolonged increase in dopamine levels seen in *in vivo* microdialysis studies after a single nicotine exposure (Imperato et al., 1986; Di Chiara and Imperato, 1988; Nisell et al., 1994; Schilstrom et al., 1998).

However, although *in vivo* microdialysis and some behavioural studies suggest that the α7 nAChRs play an important role in the nicotine-induced dopamine and glutamate release in the VTA (Schilstrom et al., 1998; Schilstrom et al., 2000) and the rewarding effects of nicotine (Laviolette and van der Kooy, 2003), studies with genetically modified mice do not support this notion. These studies have demonstrated that while β2-null mice no longer responded to or self-administered nicotine (Picciotto et al., 1998; Epping-Jordan et al., 1999) or showed nicotine CPP (Walters et al., 2006), α7-null mice still were able to acquire nicotine discrimination (Stolerman et al., 2004) and CPP (Walters et al., 2006). Therefore, these studies have concluded that the β2 subunit is pivotal for mediating the rewarding properties of nicotine whereas the α7 subunit is not important. A possible explanation for this discrepancy may be the compensation mechanisms that take place in knock-out mice.

1.6 - Is nicotine rewarding?

1.6.1 - Intravenous nicotine self-administration in animals

Nicotine is intravenously self-administered by several animal species, including rats (Corrigall and Coen, 1989; Donny et al., 1995; Rose and Corrigall, 1997), mice (Martellotta et al., 1995; Rasmussen and Swedberg, 1998) and squirrel monkeys (Goldberg et al., 1981).

Several aspects of nicotine self-administration in rodents demonstrate that nicotine is reinforcing. First, when nicotine is replaced with saline, responding extinguishes (Corrigall and Coen, 1989; Donny et al., 1995; Shoaib et al., 1997). Second, the active (i.e. drug-paired) lever is preferred over the inactive lever (Corrigall and Coen, 1989; Donny et al., 1995). Third, responding varies as a function of dose (within a limited dose range) (Corrigall and Coen, 1989; Donny et al., 1995; Shoaib et al., 1997), and fourth, non-contingent delivery of the drug alone does not maintain operant responding (Donny et al., 1998).

However the rates of responding and drug infusion for nicotine in the self-administration paradigm are modest (for review see Le Foll and Goldberg, 2006) and the conditions under which self-administration occurs are limited (Lang et al., 1977; Henningfield and Goldberg, 1983; Donny et al., 1998). For example, it has been reported that feeding schedules influence nicotine self-administration; rats that are weight restricted or acutely food deprived show higher rates of nicotine self-administration (Lang et al., 1977; Donny et al., 1998). Although nicotine is self-administered by rats under both fixed-ratio (Corrigall and Coen, 1989; Shoaib and Stolerman, 1999; Caggiula et al., 2001) and

progressive-ratio schedules (Donny et al., 1999), under the progressive-ratio schedules nicotine is only a weak reinforcer compared to other drugs of abuse such as cocaine (Donny et al., 1998; Caggiula et al., 2001, 2002). Moreover, in a two lever self-administration choice study, rats almost always (>80%) chose i.v. cocaine over i.v. nicotine (Manzardo et al., 2002). This suggests that the reinforcing effects of nicotine, by itself, are weaker than those of other drugs of abuse such as psychostimulants and opioids (Caggiula et al., 2001).

It has been suggested that the presence of conditioned environmental stimuli are crucial for nicotine to serve as an effective reinforcer (Goldberg et al., 1981; Donny et al., 1998; Caggiula et al., 2001, 2002). Evidence for this notion comes mainly from experiments in which a signal light was paired with nicotine infusions. The combination of light and nicotine delivery maintained higher rates of lever pressing than either light or nicotine infusion alone (Caggiula et al., 2001, 2002).

1.6.2 - Intravenous nicotine self-administration in humans

One of the principal arguments in support of the presumed reinforcing effects of nicotine is that human smokers do self-administer nicotine intravenously. There are four human i.v. nicotine self-administration studies with different results (Henningfield and Goldberg, 1983; Henningfield et al., 1983; Rose et al., 2003; Harvey et al., 2004). The two earlier studies by Henningfield and his colleagues only provide weak evidence that nicotine is intravenously self-administered by abstinent smokers because of the problems in subject selection, experimental design and interpretation of the results. First of all, participants

did not have a choice between nicotine and saline infusions within the same session. Although they were presented with two levers, pressing on one lever had no consequence while pressing on the other lever resulted in nicotine or saline infusions depending on the session; therefore pressing for saline or nicotine infusion was recorded in alternating sessions, not concurrently in one session and there was no direct comparison between pressing for nicotine or saline in the same session. Moreover, a total of ten subjects were used in both studies and seven out of ten had a history of abuse of illicit drugs such as opioids, stimulants and sedatives. There is evidence that in such participants, drug-related stimuli such as sight of a needle or even saline injection tend to be reinforcing (Wen and Ho, 1982; Powell, 1995); therefore the results obtained from these participants can not be completely trusted. In the other subjects without history of illicit drug abuse the rates of nicotine self-administration were *lower* than rates of saline self-administration (Henningfield and Goldberg, 1983). In order to claim strongly that a drug is self-administered, it has to be self-administered more than the vehicle and in these two studies, the participants did not self-administer nicotine significantly more than saline. Therefore these studies do not prove that smokers self-administer pure nicotine. The only argument by the authors supporting the reinforcing effect of intravenous nicotine in smokers is that while participants administered both nicotine and saline, nicotine injections occurred in a regular pattern (Henningfield et al., 1983) which on its own it is not enough to conclude that intravenous nicotine is reinforcing in smokers.

In the study by Rose et al. (2003), it was reported that smokers self-administered i.v. nicotine after overnight abstinence from smoking and that pre-treatment with the nicotinic antagonist, mecamylamine increased the rate of i.v. nicotine self-administration. Although this study has the advantage of offering puff-sized bolus doses of nicotine based

on each smokers smoking manner, it has two main problems. First, it does not provide any information about history of illicit drug abuse for the subjects, which as mentioned before may influence the results of the study. Second, subjects do not have access to a second lever which results in saline infusions; therefore rates of responding for nicotine vs. saline can not be compared to see whether subjects self-administered nicotine more than saline.

Finally, in another more recent study, smokers were reported to self-administer more nicotine than saline (Harvey et al., 2004). This was the case especially under a progressive-ratio schedule. In this study, concurrent nicotine and saline injections were available to participants in each session. However, as in the earlier studies (Henningfeild and Goldberg, 1983; Henningfield et al., 1983), this study was also compromised by the inclusion of subjects with history of drug abuse. Most participants had previously used illegal drugs such as psychostimulants, opiates and hallucinogens.

In conclusion, it is obvious that although these studies provide some evidence that intravenous nicotine is reinforcing in smokers under specific conditions (history of drug abuse, progressive–ratio schedule), better controlled and better designed studies are needed to show whether *typical* human smokers self-administer pure nicotine in the absence of other components of tobacco smoke, voluntarily or not.

1.6.3 - Nicotine CPP

In CPP studies in rats, nicotine in a dose range of 0.1-1.2 mg/kg either produces CPP (Fudala et al., 1985; Fudala and Iwamoto, 1986; ; Horan et al., 1997; Dewey et al., 1999;

Horan et al., 2001; Le Foll et al., 2005; Le Foll and Goldberg, 2005a), conditioned place aversion (Jorenby et al., 1990; Horan et al., 1997; Laviolette and van der Kooy, 2003) or no effect (Clarke and Fibiger, 1987; Jorenby et al., 1990; Shoaib et al., 1994; Vastola et al., 2002; Laviolette and van der Kooy, 2003).

Reported results of nicotine CPP studies suggest that nicotine dose and the experimental design (biased vs. unbiased) strongly influence the outcome of nicotine CPP (for review see Le Foll and Goldberg, 2005a). It has been proposed that biased designs are more effective than unbiased designs for inducing nicotine CPP (Calcagnetti and Schechter, 1994) and published results support this idea. Although Gardner's group have been able to obtain nicotine CPP using an unbiased design (Horan et al., 1997; Dewey et al., 1999; Horan et al., 2001; Ashby et al., 2002), most published studies that have used the unbiased design were not able to induce CPP (Clarke and Fibiger, 1987; Shoaib et al., 1994; Laviolette and van der Kooy, 2003), while most published studies that have employed the biased design report CPP (Fudala et al., 1985; Fudala and Iwamoto, 1986; Le Foll and Goldberg, 2004; Le Foll et al., 2004). A few studies that have compared the biased and unbiased designs directly, report that significant nicotine CPP is only induced when nicotine is paired with the non-preferred side (Acquas et al., 1989; Carboni et al., 1989; Calcagnetti and Schechter, 1994; Le Foll and Goldberg, 2005a).

1.6.4 - Additional factors affecting nicotine CPP

1.6.4.1 - Age

Rats are more sensitive to the rewarding and anxiolytic properties of nicotine in their adolescence (Vastola et al., 2002; Belluzzi et al., 2004; Torrella et al., 2004; Shram et al., 2006). In one study employing a biased design, nicotine (0.6 mg/kg s.c.) induced significant CPP in adolescent rats (P28) but not in adult rats (P58) (Vastola et al., 2002). In another study 0.6 mg/kg s.c. nicotine did not produce a significant CPP in either adolescent (P30) or adult (P60) rats in a biased design. However, nicotine increased the time spent in the initially non-preferred compartment in adolescent but not in adult rats (Torrella et al., 2004). This suggests that adolescent rats may respond more to the anxiolytic effects of nicotine. Similarly, a third study reported that nicotine (0.8 mg/kg s.c.) also produced CPP in periadolescent (P21) but not in adult (P53) rats in an unbiased design (Shram et al., 2006). Finally, nicotine (0.5 mg/kg s.c.) produced significant CPP in rats in their early adolescence (P28) in an unbiased design even after a single pairing. However, the same dose of nicotine failed to induce CPP in rats in their late adolescence (P38) or adulthood (P90) even after four pairings (Belluzzi et al., 2004).

Unfortunately, adolescent rats are not a suitable model for studies employing stereotaxic surgery. Since this was a long-term goal of ours, we decided not to use adolescent rats.

1.6.4.2 - Strain

It has been reported that nicotine (0.4 and 0.6 mg/kg s.c.) after four or five pairings, produces CPP in Lewis but not in Fischer 344 rats (Horan et al., 1997; Philibin et al., 2005). In addition, Fischer 344 rats showed a conditioned place aversion after ten pairings while Lewis rats still showed a significant CPP (Horan et al., 1997). These data suggest

that a genetic component could perhaps be involved in nicotinic cholinergic and/or dopaminergic receptor function.

1.6.4.3 - Nicotine pre-treatment

It has been shown that pre-treatment with nicotine in the home cage facilitates nicotine-induced CPP (Shoaib et al., 1994; Forget et al., 2005). Shoaib et al. reported that nicotine (0.6 mg/kg s.c.) failed in inducing CPP in rats in an unbiased design whereas following pre-treatment with nicotine (0.4 mg/kg s.c) for 7 days, s.c. nicotine at doses of 0.4-0.8 mg/kg was able to induce a significant CPP in the same design.

Following nicotine (same dose as CPP) pre-treatment for three days, nicotine (0.06 and 0.125 mg/kg s.c) produced CPP in rats in an unbiased design (Forget et al., 2005). All other nicotine CPP studies report that nicotine can induce CPP in the dose range of 0.1-1.2 mg/kg s.c. (Le Foll and Goldberg, 2005a). To my knowledge, the study by Forget and his colleagues is the only report of a rewarding effect for nicotine, as determined by CPP, at a dose lower than 0.1 mg/kg s.c.; this suggests that nicotine pre-treatment may shift the dose-response curve for the acute rewarding effects of nicotine to the left, such that lower doses become rewarding.

1.7 - Nicotine plus?

Numerous reports indicate that in animals, nicotine is able to support self-administration and induce CPP; both of these phenomena only occur under limited conditions

(Henningfield and Goldberg, 1983; Acquas et al., 1989; Calcagnetti and Schechter, 1994; Donny et al., 1998; Le Foll and Goldberg, 2005b) suggesting that nicotine is a weak reinforcer in animals. Moreover, some sets of observations in smokers suggest that nicotine is not the only reason for maintaining smoking behaviour. Nicotine replacement therapies have achieved only limited success in helping smoking cessation (Balfour and Fagerstrom, 1996); denicotinized cigarettes acutely reduce craving and withdrawal symptoms of abstinent smokers (Butschky et al., 1995; Rose et al., 2000) and although pure nicotine reduces craving to some extent, it does not produce a significant satisfaction or reward in abstinent smokers (Rose et al., 2000).

Taken together, these findings suggest that compared to other drugs of abuse, nicotine acts as a weak reinforcer in both animals and humans; therefore it is likely that tobacco addiction relies critically on additional factors. Non-nicotine factors may play an important role in supporting nicotine self-administration in both animals and humans.

1.7.1 - Role of conditioned reinforcers

Animal studies demonstrate that although nicotine supports operant behaviour in the absence of nonpharmacological stimuli (Caggiula et al., 2002; Donny et al., 2003), combining nicotine delivery with a nonpharmacological stimulus such as a light facilitates responding for nicotine (Caggiula et al., 2002; Caggiula et al., 2002; Chaudhri et al., 2005); removing these nonpharmacological stimuli that were paired with nicotine self-administration reduces responding (Goldberg et al., 1981). In addition, these nonpharmacological stimuli can maintain self-administration in the absence of nicotine

(Donny et al., 1999, 2000).

The repeated pairing between nicotine and nonpharmacological stimuli during self-administration enhances the reinforcing efficacy of these stimuli; through Pavlovian conditioning these non-drug stimuli become conditioned reinforcers and subsequently gain control over the responding behaviour. For example, presentation of these nonpharmacological stimuli is able to reinstate responding after extinction of responding induced by removal of nicotine (Caggiula et al., 2001, 2002; Cohen et al., 2005) implying that they are as important as nicotine in reinstating the operant behaviour. The control over behaviour by nonpharmacological stimuli only occurs if delivery of nonpharmacological stimuli and nicotine are contingent (Caggiula et al., 2002b), implying that these stimuli are conditioned reinforcers.

1.7.2 - Reinforcement enhancement

Recently it has been shown that nicotine is also able to enhance responding for a reinforcing nonpharmacological stimulus (Donny et al., 2003; Chaudhri et al., 2006). Studies have shown that both contingent and noncontingent nicotine can enhance responding for an unconditioned stimulus such as light and conditioned reinforcers (Donny et al., 2003; Olausson et al., 2004; Chaudhri et al., 2006; Chaudhri et al., 2006). The fact that noncontingent nicotine can enhance the reinforcing properties of both unconditioned and conditioned stimuli suggests that nicotine may exert a rather general reinforcement-enhancing effect.

In tobacco smoking, the nonpharmacological stimuli such as taste and smell of cigarette smoke can also exert powerful control over smoking behaviour; through literally thousands of previous pairings of these stimuli with effects of nicotine, the primary reinforcer, they become conditioned reinforcers (Rose et al., 1985; Rose and Levin, 1991). In support of the latter hypothesis, as mentioned before, denicotinized cigarettes, which offer some of the sensory stimuli associated with smoking, are able to reduce craving and withdrawal symptoms of abstinent smokers to an important extent (Butschky et al., 1995; Rose et al., 2000). It seems as if tobacco addiction reflects an interaction between psychopharmacological properties of nicotine and many other stimuli experienced by smokers when they inhale tobacco smoke. Both nicotine and sensory stimuli associated with smoking are necessary for complete reduction of craving (Rose et al., 2000).

1.7.3 - Non-nicotine components of tobacco smoke

Other than nicotine and its metabolites, there are more than 4000 chemical substances in tobacco (Roberts, 1988). Some chemical components have psychopharmacological effects and may contribute to tobacco addiction in humans. Tobacco smoke contains acetaldehyde (Bates et al., 1999). It has been shown that under specific conditions, acetaldehyde is intravenously self-administered by rats (Myers et al., 1982). Intracerebrally administered acetaldehyde also supports self-administration and induces CPP (for review see Amit and Smith, 1985) and therefore acetaldehyde is reinforcing on its own; it is worth mentioning that doses of acetaldehyde that were used in these studies

are much higher than the doses obtained from tobacco smoke. A more recent study reports that a combination of nicotine and acetaldehyde in doses similar to those obtained from tobacco smoke, is self-administered by juvenile rats more than is either nicotine or acetaldehyde alone (Belluzzi et al., 2005). This finding raises the possibility that in smokers, nicotine and acetaldehyde exert additive or synergistic reinforcing effects.

Another chemical component in tobacco smoke inhibits monoamine oxidase (MAO) (Fowler et al., 1996a, b). MAO is responsible for the metabolism of monoamine neurotransmitters such as dopamine, serotonin and noradrenaline. Hence, the inhibition of this enzyme may contribute to the reinforcing properties of tobacco, as discussed in the next section.

1.8 - Smoking and MAO inhibition

MAO is an enzyme located in mitochondrial membrane of neuronal and non-neuronal cells in the brain and in peripheral organs. It catalyzes the oxidative deamination of amines from both endogenous and exogenous sources; thereby its activity influences the concentration of neurotransmitter amines as well as many xenobiotics (Singer and Ramsay, 1995; Richards et al., 1998).

MAO occurs as two subtypes, MAO-A and MAO-B; these subtypes have different inhibitor and substrate specificities (Johnston, 1968; Goridis and Neff, 1971). MAO-A preferentially oxidizes norepinephrine and serotonin and is selectively inhibited by clorgyline (Johnston, 1968; Neff and Goridis, 1972) while MAO-B preferentially breaks down benzylamine and the trace amine phenethylamine and is selectively inhibited by L-deprenyl (Hall et al., 1969; Knoll and Magyar, 1972; Yang and Neff, 1973). Both *in vivo*

and *in vitro* studies have shown that both forms can oxidize dopamine (Yang and Neff, 1974; Green et al., 1977; O'Carroll et al., 1983; Youdim and Riederer, 1993). It is worth mentioning that some *in vivo* studies in rats have reported that, MAO-A is the main subtype responsible for deamination of dopamine in the striatum and the MAO-B subtype only plays a role when MAO-A is inhibited (Waldmeier et al., 1976; Butcher et al., 1990). Both animal and human studies have shown that cigarette smoke inhibits MAO activity. Cigarette smoke and extracts of cigarette smoke inhibit MAO-A and B both *in vivo* (Carr and Rowell, 1990) and *in vitro* (Yu and Boulton, 1987; Carr and Basham, 1991), and saliva from smokers inhibits both MAO-A and B (Yu and Boulton, 1987) in rodents.

Cigarette smokers have reduced platelet MAO-B (Oreland et al., 1981); the level of the enzyme returns to normal 4 weeks after smoking cessation (Norman et al., 1987; Rose et al., 2001). Tobacco exposure also inhibits MAO-A as shown by decreased levels of plasma catecholamine metabolites (Berlin et al., 1995). PET imaging studies also show reduced brain MAO-A and B levels in smokers relative to non-smokers and former smokers (Fowler et al., 1996a, b). Human brain MAO-A and B down-regulation in smokers is partial, with an average reduction of 30% for MAO-A and an average reduction of 40% for MAO-B being observed (Fowler et al., 1996a, b). The fact that there is no difference in MAO levels in former smokers compared to non-smokers, suggests that MAO inhibition is due to tobacco smoking rather than a genetic difference. The synthesis rate of human MAO-B is slow, with a half-life of about 40 days; therefore if inhibition is irreversible *in vivo*, then the effects of cigarette smoking would presumably be cumulative (Fowler et al., 1994).

The mechanism of MAO inhibition by cigarette smoke is not known. It is known that

nicotine in smoking-relevant concentrations does not inhibit MAO (Oreland et al., 1981; Carr and Basham, 1991; Yong and Perry, 1986). A few compounds such as 2,3,6-trimethyl-benzoquinone, 2-naphthylamine and β-carboline alkaloids such as harman and norharman that are present in tobacco smoke, inhibit MAO *in vitro* (Khalil et al., 2000; Hauptmann and Shih, 2001; Herriaz and Chaparro, 2005). It also has been proposed that cyanomethylation (brought about by formaldehyde and cyanide in smoke) of the reactive amino groups in the MAO protein may reduce its catalytic activity (Boulton et al., 1988) but which of these compounds or mechanisms is responsible for inhibition of MAO by tobacco smoke *in vivo,* is still not clear.

MAO-A and B are involved in the breakdown of dopamine. As dopamine has been implicated in mediating the reinforcing properties of drugs of abuse, it has been proposed that MAO inhibition by cigarette smoke may enhance nicotine-induced dopamine transmission and therefore contribute to the addictiveness of tobacco smoking. Based on this hypothesis, the reversible MAO-A inhibitor, moclobemide (Berlin et al., 1995a,b, 2001) and combination of nicotine and L-deprenyl, an irreversible MAO-B inhibitor, (George et al., 2003) are being studied as smoking cessation treatments in smokers.

A few animal studies have demonstrated that MAO inhibitors potentiate the reinforcing effects of nicotine in rodents. MAO inhibitors dramatically increased the motivation to self-administer nicotine, as shown by PR schedule of reinforcement. The increase in motivation to self-administer nicotine was more prominent in rats selected for high responsiveness to novelty than in rats with low responsiveness to novelty (Guillem et al., 2005). It has also been shown that, whereas naïve rats did not readily self-administer

nicotine (10 μg/kg/injection) under a FR1 schedule, when pre-treated with tranylcypromine (3 mg/kg), an irreversible MAO-A and MAO-B inhibitor, a robust self-administration of nicotine occurred (Villegier et al., 2005). Although this evidence suggests that MAO inhibitors enhance the reinforcing properties of nicotine in rodents, it is worth mentioning that in none of these studies was MAO activity actually measured and therefore it is not clear what level of MAO inhibition was achieved and whether the extent of inhibition had any relevance to the partial inhibition of MAO induced by smoking in humans. Therefore further studies with defined levels of MAO inhibition would be a better model for determining the role of MAO inhibition in smoking.

1.9 – Statement of purpose

Studies of nicotine CPP have provided every possible outcome: significant CPP, no CPP and even CPA have been reported by different groups (for review see Le Foll and Goldberg, 2005a). By manipulating different procedural variables, we wanted to identify the most important factors and hence establish conditions that would result in reliable nicotine CPP. The ultimate goal of this work was to be able to test whether partial MAO inhibition (as found in smokers) increased nicotine's rewarding effect in this behavioural paradigm.

Chapter 2 - Assessing the factors affecting nicotine CPP

2.1 - Overview of experiments

Experiment 1 was done to test whether our CPP apparatus was unbiased, in the sense that there was no pre-existing preference between two sensory stimuli that would be paired with drug and saline (see section 1.2.2). In our experiments, these stimuli were two different floor tiles (mesh and bar).

Experiment 2 was performed to establish whether novelty- or nicotine-associated locomotor activity can predict nicotine CPP. There is considerable individual variability in the response to addictive properties of drugs and there is evidence that animals susceptible to drug-seeking can be identified on the basis of their response to environmental or pharmacological challenges (Piazza et al., 1989). It has been shown that novelty- or drug-induced locomotor activity can predict the propensity of the animals to acquire amphetamine, cocaine and nicotine intravenous self-administration (Piazza et al., 1989; Mantch et al., 2001; Suto et al., 2001). However, the ability of novelty- or drug-induced locomotor activity to predict drug-induced CPP is controversial. It has been reported that novelty-induced locomotor activity can predict amphetamine CPP (Klebaur and Bardo, 1998) but it also has been reported that novelty seeking can not predict cocaine and amphetamine CPP (Erb and Parker, 1993; Gong et al., 1996). The ability of novelty- or nicotine-induced activity to predict nicotine CPP has not been studied.

In **Experiment 3**, we asked whether there is any difference in giving the i.v. nicotine dose as a single bolus or as multiple divided doses.

In **Experiment 4**, two questions were examined. The first was whether nicotine CPP could be enhanced by subchronic pre-treatment with nicotine in the home cage as reported by Shoaib et al. (1994). The second question was whether nicotine CPP is dependent on the speed of i.v. nicotine infusion. There is evidence that for cocaine, the speed of injection influences the reinforcing properties of the drug in human and nonhuman primates (Balster and Schuster, 1973; Kato et al., 1987; Panlilio et al., 1998; Abreu et al., 2001; Woolverton and Wang, 2004).

Experiment 5 was done to determine whether a deliberately *biased* design results in i.v. nicotine CPP and whether the duration of conditioning is important. Most groups that report significant nicotine CPP use the biased design (for review see Le Foll and Goldberg, 2005a). Our apparatus is unbiased in that rats as a group have no general preference for mesh or bar. However, each individual animal does have a slight initial preference for one compartment over the other. In the biased design, the assignment of animals to the compartments is based on their individual initial preference for the compartment and the drug is usually paired with the non-preferred compartment.

There is evidence that for drugs that exert their effects very fast, such as intracranial cocaine, shorter conditioning sessions result in larger CPP than longer conditioning sessions (Ikemoto and Donahue, 2005). Since the rewarding effects of i.v. nicotine should appear quickly, shorter (i.e. 5 minute) conditioning sessions were tested in this experiment.

Experiment 6 was carried out to see whether the two floor stimuli (i.e. mesh and bar) are equally conditionable to nicotine. By this stage we had started to suspect that there was a significant difference in the magnitude of CPP when nicotine was paired with one of the stimuli vs. the other.

In **Experiment 7** we tried to replicate the results of Gardner's group that report s.c. nicotine CPP using an unbiased design. As mentioned in section 1.6.3, this group is the only group that has reported significant nicotine CPP in adult rats employing an unbiased design (Horan et al., 1997; Dewey et al., 1999; Horan et al., 2001; Ashby et al., 2002).

2.2 - General methods

2.2.1 - Subjects

Male Long-Evans rats (Charles River, St. Constant, Quebec, Canada), weighing 280-330 g at the beginning of each experiment, were maintained on a 12/12 h light-dark cycle. They had *ad libitum* access to water and food except during the behavioural testing. They were housed in groups of two or three in Plexiglas cages and were left to acclimatize to the animal colony, with controlled temperature and humidity, for 2-3 days and then handled once daily for 2-3 days before each experiment. All experiments were approved by the McGill Faculty of Medicine Animal Care Committee in accordance with Canadian Council on Animal Care guidelines.

2.2.2 - Locomotor activity testing

Twelve locomotor activity cages (42 long ×25 wide ×31 cm high) were used in Experiment 2. These cages were made of clear Plexiglas and were equipped with 2 parallel infrared photobeams, 34 cm apart. Each photobeam interruption was registered by a computer program and locomotor activity was measured by the number of photobeam interruptions.

Rats were left to habituate to the locomotor activity room (in their home cages) for 30 min before locomotor screening.

2.2.3 - Intravenous catheterization

This procedure was used in Experiment 2, 3, 4, 5 and 6. Rats were anesthetised with a mixture of ketamine hydrochloride (80 mg/kg i.p.) and xylazine hydrochloride (16 mg/kg i.p.). Their head and the area around their left shoulder was shaved and wiped with 70% ethanol. A small incision was made in the scalp and close to the left shoulder, and the left jugular vein was cleared of connective tissue. A chronic indwelling silastic catheter (0.51 mm I.D. and 0.94 mm O.D., Fisher Scientific, Montreal, Quebec) was then inserted into the vein. Using surgical silk sutures, the catheter was tied to the vein to keep it in place. The other end of the catheter was led subcutaneously to the scalp where it was connected to a 22 gauge cannula with a plastic connector (Model number C313G-5UP or C313FL-5UP, Plastics One, Roanoke, VA). This connector was fixed on the skull with small stainless steel skull screws (Lomir, Notre-Dame-de-L'Ile Perrot, Quebec) and dental cement (Stoelting, Wood Dale, IL). To keep the catheter patent, 0.1 ml of heparinized

0.9% saline was injected immediately after surgery and then every 2-3 days until the end of the experiment. Rats were given the analgesic dipyrone (100 mg/kg s.c.) at the end of surgery and left to recover for 7-10 days before behaviour testing.

2.2.4 - Conditioned Place Preference (CPP)

Standard CPP procedure: This method is adapted from the method of Vezina and Stewart (1987). Eight or twelve CPP cages (58 cm long x 29cm wide x 53.0 cm high) were used. There was no wall dividing the cage into two compartments. These cages were placed on linoleum flooring and the bottom of each cage was covered with sawdust. Two square tiles (28.5 cm x 28.5 cm x 5.5 cm high) fit into the bottom of each cage. The floor tiles served as conditioned tactile stimuli and were of two types: mesh (steel grid with squares of 1 x 1 cm) and bar (12 stainless steel bars of 1.2 cm diameter separated by 1.5 cm edge to edge).

All behavioural testing was performed in a room lit with two Kodak GBX-2 safelight filters (Vistek, Toronto, Ontario, Canada) providing far-red illumination (wavelength >650 nm) to minimize visual cues. The rats were monitored by a closed circuit television video camera (Panasonic) linked to a commercial tracking system (EthoVision v3.0, Noldus Information Technology, Leesburg, VA).

The *standard CPP procedure* that was used in most experiments consisted of three phases occurring on 8 consecutive days: pre-exposure, conditioning and test. In all three phases rats were habituated to the room for 10 min before being placed in the CPP cages. In the pre-exposure phase (day 1), rats were placed in the CPP cages for 20 min with sawdust

replacing the usual floor tiles. This phase served to habituate the rats to the CPP cages.

In the conditioning phase (days 2-7), two mesh tiles and two bar tiles were placed at the bottom of the CPP cages on alternate days. The conditioning phase comprised of 3 pairings with nicotine alternating with 3 pairings with saline. During conditioning, half of the rats received nicotine on mesh and saline on bar; and the other half received nicotine on bar and saline on mesh. The conditioning session duration was 15 min.

In the test phase (day 8), one mesh tile and one bar tile were placed in the bottom of each CPP cage. The test duration was 10 min and the time spent on each tile (one previously paired with nicotine, the other previously paired with saline) was measured. Note that animals were tested in a drug-free state.

Any variations in this *standard CPP procedure* are noted in individual experiments.

2.2.5 - Drugs

The drugs used were as follow: (-)-nicotine hydrogen tartrate salt (Sigma-Aldrich, Oakville, Ontario); dipyrone (Vetoquinol, Quebec, Quebec); ketamine HCl (Vetalar, Vetrepharm, London, Ontario); xylazine HCl (Anased, Novopharm, Toronto, Ontario). All other chemicals were obtained from Fisher Scientific (Montreal, Quebec).

Nicotine was dissolved in sterile saline and pH was adjusted to 7.2 ± 0.1 with 0.05 N NaOH. In Experiments 2, 3, 4, 5 and 6 the i.v. dose of nicotine (base) was 0.015 mg/kg. The i.v. dose of 0.015 mg/kg was used since it resembles the dose of nicotine that a smoker obtains from smoking one cigarette, and since in previous unpublished studies in our lab it resulted in significant CPP (Sellings et al., in preparation). In Experiment 7, s.c.

nicotine doses of 0.1 and 0.4 mg/kg (base) were used. These doses are on the ascending limb of the dose-response curve of rewarding effects of nicotine and result in plasma nicotine concentrations similar to smokers.

2.2.6 - Data analysis

Systat version 10.2 commercial software (Systat, Evanston, IL, U.S.A) was used for all statistical analyses. In all experiments, one sample t-tests were used to compare time spent on the tile paired with the drug and 300 s. Since the CPP procedure was counterbalanced (i.e. half the rats received drug on mesh, half on bar tile), an absence of CPP would correspond to an expected drug-paired time of 300 s (i.e. 50% of the test session duration). Where the experimental design predicts a chance value of 300 s, this has been shown on graphs by a horizontal line. To examine the effect of nicotine-paired tile on the magnitude of CPP, two-way analysis of variance (two-way ANOVA) and post-hoc two sample t-tests were used. Since most of the results were negative, unprotected t-tests (no Bonferroni correction) were used. A p value of less that 0.05 was considered significant.

2.3 - Procedures and Results

2.3.1 - Experiment 1: Spontaneous preference for mesh vs. bar.

Rats (n=8) received s.c. saline (1 ml/kg) during the conditioning phase of the *standard CPP procedure* (section 2.2.4) on both mesh and bar tiles. During the test phase, for greater confidence, three tests were carried out on consecutive days, and the mean time spent on each tile was determined for each rat.

Although there appeared to be a slight preference for the mesh tile, this preference was not statistically significant (time on mesh vs. 300 s: $t = 0.50$, $df = 7$, $p = 0.63$; Figure 1). Since there was no clear preference for mesh or bar, in subsequent experiments time spent on drug-paired tile that exceeded 300 s was regarded as CPP.

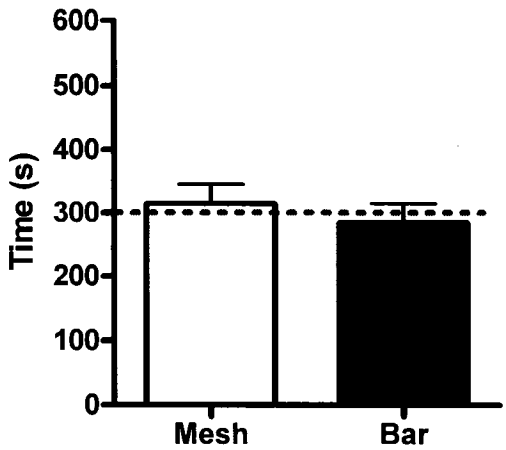


Figure 1

2.3.2 - Experiment 2: Predicting the acquisition of nicotine CPP by novelty-associated or nicotine-associated locomotor activity.

Locomotor activity screening. Rats (n=16) were placed in the locomotor activity cages and their novelty-associated locomotor activity was measured for 60 min. They were then removed from the cages, injected s.c. with 0.6 mg/kg nicotine and placed back into the locomotor cages. Their nicotine-associated locomotor activity was then measured for 60 min. Based on their locomotor activity scores, rats were divided into groups. Rats with scores higher than the median score were labelled high responders (HR) and those with scores lower than the median score were labelled low responders (LR). This categorization was done separately for novelty and nicotine test data.

CPP. During the conditioning phase of the *standard CPP procedure*, i.v. catheterized rats (n=16) received 0.015 mg/kg nicotine i.v., injected over 10 s. The test phase comprised three tests given on consecutive days, and time spent on each tile was the mean of the three tests.

When the rats were considered as a single group (i.e. n=16), there was a small but significant overall CPP ($t = 2.76$, $df = 15$, $^*p < 0.05$; Figure 2A). Note that since equal numbers of subjects received the drug on bar as mesh, the chance value of time spent on the drug-paired tile is 300 s out of 600 s, i.e. 50%. This is indicated here and elsewhere by a solid horizontal line. At this point we did not realize that there was a difference in the magnitude of CPP when nicotine was paired with mesh vs. bar. However, subsequent reanalysis by two sample t-test revealed that there was a significant difference in the magnitude of CPP when nicotine was paired with the mesh tile vs. the bar tile ($t = 2.84$, $df = 14$, $^*p < 0.05$; Figure 2B). Since there appears to be no *spontaneous* preference for

mesh over bar as shown by **Experiment 1** (section 2.3.1), this suggests that nicotine CPP may only occur when the drug is paired with mesh.

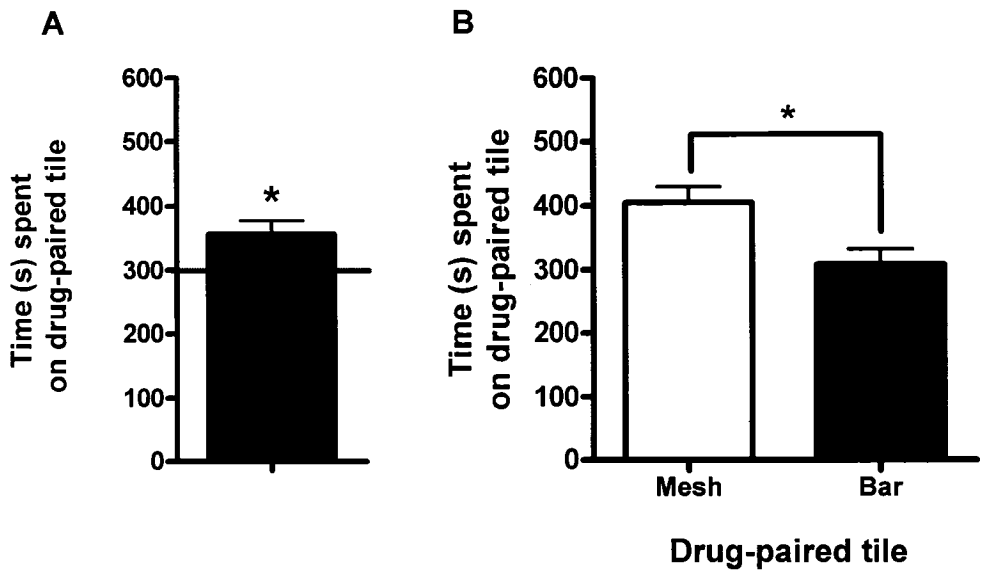


Figure 2

'Low novelty responders' showed a significant CPP ($t = 4.73$, $df = 7$, $^{***}p < 0.005$; Figure 3A) while the 'High novelty responders' did not show a CPP ($t = 0.41$, $df = 7$, $p = 0.69$, Figure 3A). Reanalysis of the data based on the nicotine-paired tile using two-way ANOVA revealed that there was a significant main effect of novelty-associated locomotor activity [$F(1, 12) = 5.12$, $^{*}p < 0.05$] and nicotine-paired tile [$F(1, 12) = 6.23$, $^{*}p < 0.05$] on the magnitude of CPP, but no significant interaction between these two factors [$F(1, 12) = 0.26$, $p = 0.62$. Post-hoc two sample t-tests revealed that there was no significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar for either the low novelty responders ($t = 1.5$, $df = 6$, $p = 0.18$; Figure 3B) or high novelty responders ($t = 9.8$, $df = 6$, $p = 0.09$; Figure 3B).

CPP data vs. novelty-associated locomotor activity

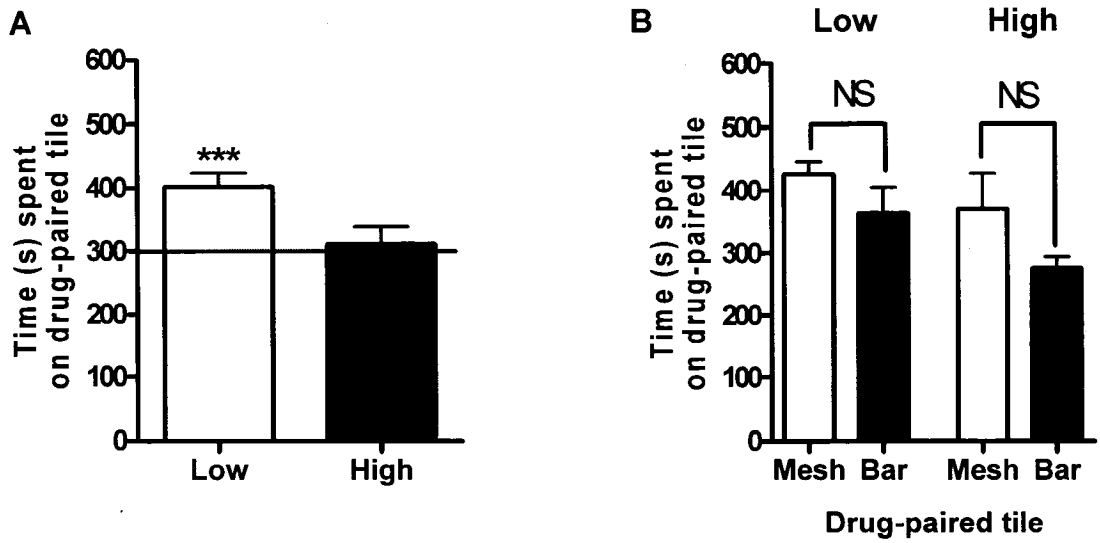


Figure 3

'Low nicotine responders' showed a small but significant CPP ($t = 2.4$, $df = 8$, $^*p < 0.05$; Figure 4A) but 'High nicotine responders' did not show a CPP ($t = 1.4$, $df = 6$, $p = 0.21$; Figure 4A). Again, when we reanalyzed the data based on the nicotine-paired tile, two-way ANOVA revealed that there was significant main effect of nicotine-paired tile [$F(1, 12) = 7.4$, $^*p < 0.05$) on the magnitude of CPP, but no significant effect of nicotine-associated locomotor activity [$F(1, 12) = 0.5$, $p = 0.49$] and no significant interaction [$F(1, 12) = 0.0$, $p = 0.96$]. Post-hoc two sample t-tests revealed that there was a significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar for the low nicotine responders ($t = 2.38$, $df = 7$, $^*p < 0.05$; Figure 4B) but not for the high nicotine responders ($t = 1.56$, $df = 5$, $p = 0.18$; Figure 4B). It is worth mentioning that there was no correlation between novelty-associated and nicotine-associated locomotor activity.

CPP data vs. nicotine-associated locomotor activity

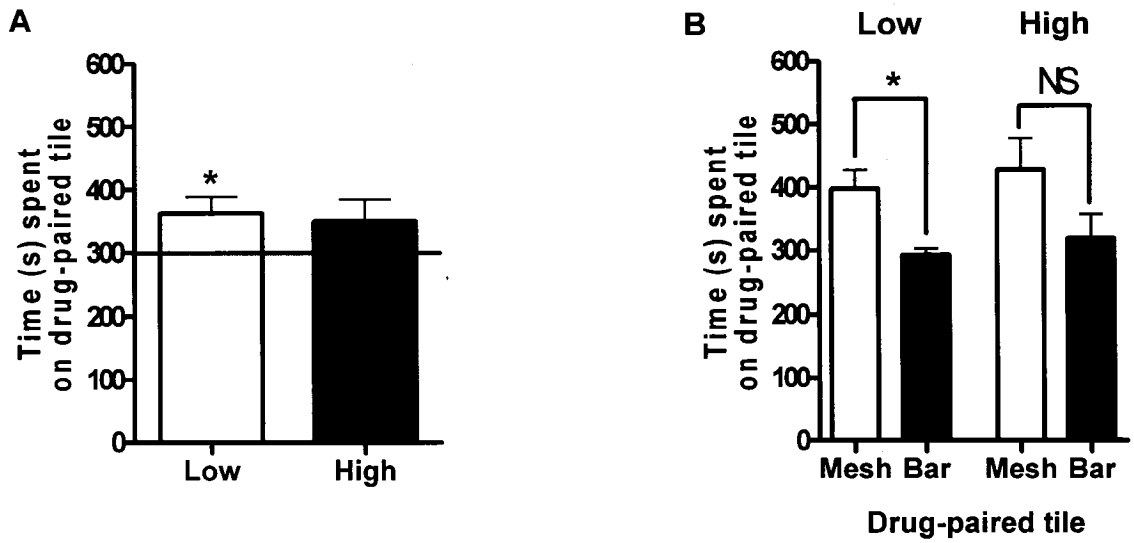


Figure 4

2.3.3 - Experiment 3: Do divided infusions of i.v. nicotine produce a larger CPP than a single nicotine bolus?

During the conditioning phase of the *standard CPP procedure*, half (n=7) of the i.v. catheterized rats received 0.015 mg/kg nicotine i.v. as a bolus, injected over 10 s. The other half received the same total dose of nicotine administered as ten 12 s infusions at 1 minute intervals. Drug infusions were delivered by using Razel Model R-E syringe pumps (Razel scientific instruments, Stamford, CT, U.S.A) in conjunction with a three channel electronic timer (Lab controller and timer-Traceable, Sciencelab.com, Houston, Texas, U.S.A). Rats were tested on three consecutive days and time spent on each tile was the mean of the three tests.

Neither of the groups (bolus or multiple divided infusions) showed a significant CPP (bolus: $t = 1.56$, $df = 6$, $p = 0.17$; divided: $t = 0.56$, $df = 7$, $p = 0.59$; Figure 5A). Reanalysis of the data by two-way ANOVA showed that there was a significant main effect of infusion schedule [$F(1, 11) = 5.21$, $^{*}p < 0.05$] and a very significant main effect of nicotine-paired tile [$F(1, 11) = 29.07$, $^{***}p < 0.005$) on the magnitude of CPP, but no significant interaction [$F(1, 11) = 0.35$, $p = 0.56$]. Post-hoc two sample t-tests revealed that there was a significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar, for both the bolus ($t = 5.01$, $df = 5$, $^{***}p < 0.005$) and the multiple divided dose group ($t = 3.12$, $df = 6$, $^{*}p < 0.05$; Figure 5B).

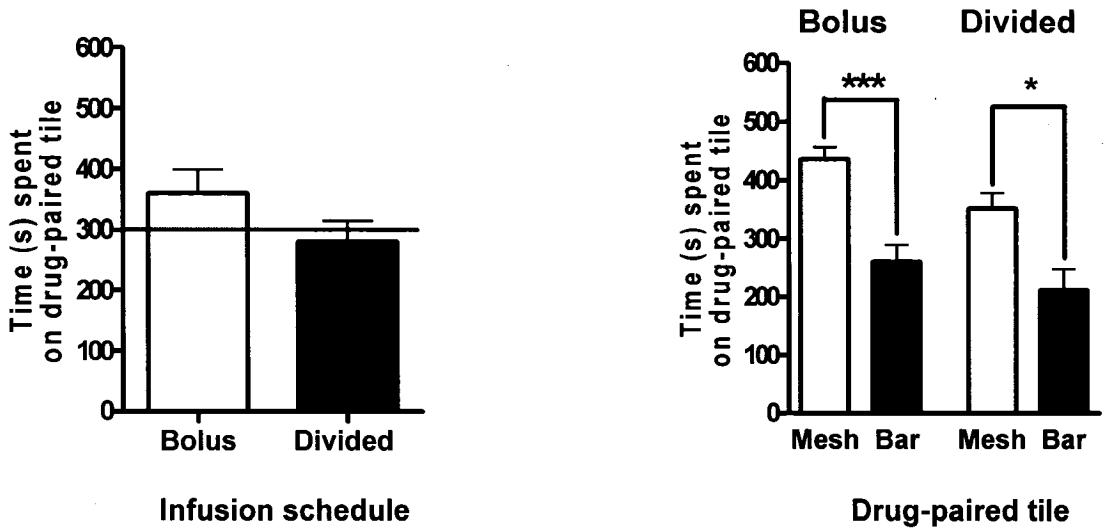


Figure 5

2.3.4 - Experiment 4: Does home cage nicotine pre-treatment facilitate CPP? Does speed of i.v. nicotine infusion affect the magnitude of CPP?

Pre-treatment. Rats (n=32) were catheterized and randomly allocated to two groups. Between 4-6 days later, one group received 0.4 mg/kg nicotine s.c. twice a day (6 hours apart) for three consecutive days and the other group received saline 1 ml/kg s.c. in the same manner. Behavioural procedures started the following day.

CPP. During the conditioning phase of the *standard CPP procedure,* nicotine was administered in a dose of 0.015 mg/kg (i.v.). Half of the rats in each group (n=8) received the drug as a 10 s bolus. The other half received it as a 30 s bolus. Rats were tested for three consecutive days as before.

As shown in Figure 6A, none of the groups showed significant CPP (saline pre-treatment and 10 s: $t = 0.87$, $df = 7$, $p = 0.41$; saline pre-treatment and 30 s: $t = 1.57$, $df = 7$, $p = 0.16$; nicotine pre-treatment and 10 s: $t = 0.46$, $df = 6$, $p = 0.66$; nicotine pre-treatment and 30 s: $t = 1.08$, $df = 7$, $p = 0.31$). Upon reanalysis by two-way ANOVA, it emerged that there was a very significant main effect of nicotine-paired tile [$F(1, 23) = 14.73$, ***$p < 0.005$) on the magnitude of CPP. There was no significant main effect of nicotine pre-treatment [$F(1, 23) = 0.04$, $p = 0.84$] or infusion speed [$F(1, 23) = 0.71$, $p = 0.41$] and none of the interactions were significant [nicotine-paired tile × nicotine pre-treatment: $F(1, 23) = 1.19$, $p = 0.29$; nicotine-paired tile × infusion speed: $F(1, 23) = 1.03$, $p = 0.32$; infusion speed × nicotine pre-treatment: $F(1, 23) = 0.29$, $p = 0.59$; nicotine-paired tile × infusion speed × nicotine pre-treatment: $F(1, 23) = 0.08$, $p = 0.78$].

As shown in Figure 6B, post-hoc two sample t-tests revealed a significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar, but only in the saline pre-treated rats that received nicotine in 30 s ($t = 5.57$, $df = 6$, $^{***}p < 0.005$).

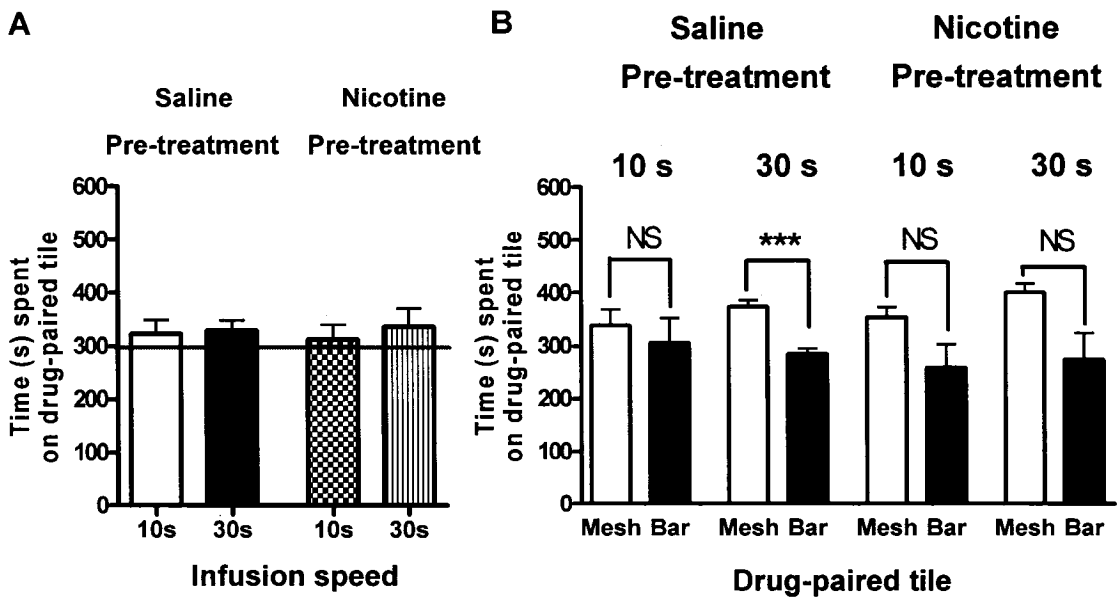


Figure 6

2.3.5 - Experiment 5: Does a biased design result in significant i.v. nicotine CPP? Does the duration of conditioning sessions affect the magnitude of i.v. nicotine CPP?

Experiment 1 (section 2.3.1) seemed to reveal small individual preference for one floor texture or the other. In order to establish the individual initial preference of each rat for the mesh vs. bar floor texture, the pre-exposure phase of the *standard CPP procedure* was

modified as follows. One mesh tile and one bar tile were placed in each of the CPP cages and time spent on each tile was measured in three pre-exposure sessions occurring in three consecutive days. In the conditioning phase, nicotine was always paired with the initially less preferred tile. During conditioning rats (n=20) received nicotine 0.015 mg/kg i.v., given as a 10 s bolus. For half of the rats (n=10) conditioning sessions were 15 min long, and for the other half, they were 5 min long. Experience from previous experiments suggested that the time spent on drug-paired tile did not differ significantly across three consecutive test days. For this reason, all subsequent experiments employed only a single test day. On test day, time spent on each tile was measured and the difference between time spent on each tile on the test day and baseline was calculated.

Neither group showed a significant CPP (5 min conditioning: $t = 1.94$, $df = 10$, $p = 0.08$; 15 min conditioning: $t = 0.60$, $df = 10$, $p = 0.56$; Figure 7). Hence in this experiment, we were not able to obtain significant CPP with a biased design; and the duration of conditioning did not make a significant difference.

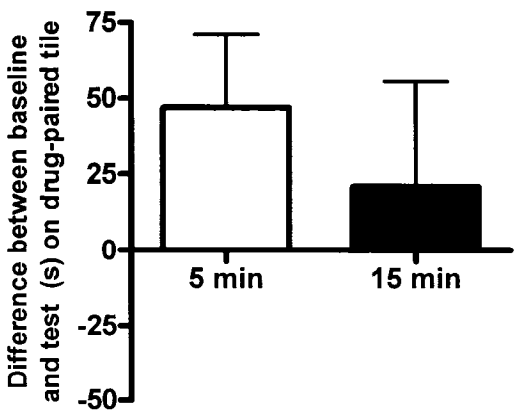


Figure 7

2.3.6 - Experiment 6: Is the mesh tile more nicotine-conditionable than the bar tile?

In the conditioning phase of the *standard CPP procedure,* half of the rats (nicotine-saline group; n=16) received nicotine 0.015 mg/kg and saline 1 ml/kg i.v. as a 10 s bolus on alternate days on different floor tiles and the remainder (saline-saline group; n=16) received saline 1 ml/kg i.v. as a 10 s bolus on both floor tiles. In this experiment the conditioning phase consisted of 4 pairings (instead of 3) each with nicotine and saline.

For the control (i.e. saline-saline) group, one floor texture was randomly assigned as the 'drug-paired' tile for each individual rat. Rats in the control group showed no significant CPP ($t = 0.73$, $df = 14$, $p = 0.47$; Figure 8A), as expected. Nicotine did not produce a significant CPP ($t = 0.60$, $df = 15$, $p = 0.56$; Figure 8A). Two-way ANOVA revealed a significant main effect of nicotine-paired tile on the magnitude of CPP [$F(1, 27) = 9.49$, $^{**}p < 0.01$] but no main effect of drug [$F(1, 27) = 0.00$, $p = 0.97$] and no interaction between these two factors [$F(1, 27) = 2.39$, $p = 0.13$]. A Post-hoc two sample t-test showed that there was a significant difference in the magnitude of time spent on drug-paired tile when nicotine was paired with mesh vs. bar ($t = 3.46$, $df = 14$, $^{***}p < 0.005$; Figure 8B). These data suggest that as suspected, the mesh tile is more conditionable with nicotine.

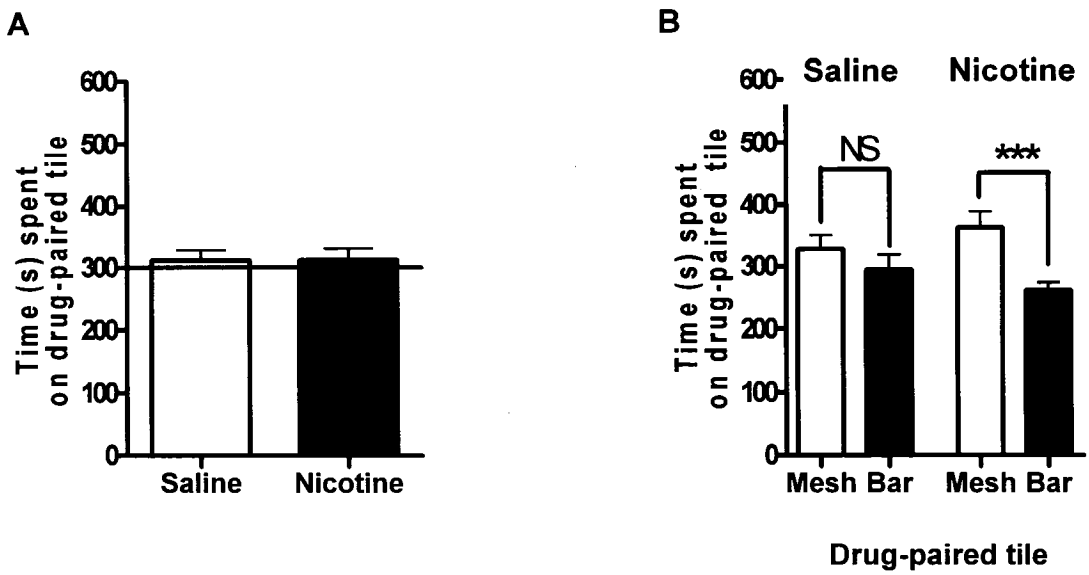


Figure 8

2.3.7 - Experiment 7: Does more extended conditioning produce robust nicotine CPP?

Gardner's group has reported nicotine CPP using an unbiased design (section 1.6.3), and this experiment was an attempt to replicate their findings by copying their method as exactly as possible. The entire CPP procedure lasted for 20 days. The pre-exposure phase consisted of three daily habituation sessions, each of 15 min duration. During this phase, the CPP boxes as usual contained no tiles but sawdust instead. The conditioning phase lasted 16 days and consisted of 8 pairings each with nicotine and saline. The conditioning session duration was 30 min. During conditioning, half of the rats (n=8) received nicotine 0.1 mg/kg and saline 1 ml/kg s.c. on alternate days and the other half (n=8) received nicotine 0.4 mg/kg and saline 1 ml/kg s.c. on alternate days.

There was no significant CPP for either dose of nicotine tested (0.1 mg/kg: t = 1.35, df = 7, p = 0.22; 0.4 mg/kg: t = 1.56, df = 7, p = 0.16; Figure 9A). However, reanalysis with two-way ANOVA showed that there was a significant main effect of nicotine dose [F (1, 12) = 6.96, *p < 0.05] and nicotine-paired tile [F (1, 12) = 10.6, **p < 0.01] on the magnitude of CPP with no significant interaction [F (1, 12) = 1.85, p = 0.2). Post-hoc two sample t-tests revealed that there was a significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar in the rats that received the 0.1 mg/kg nicotine dose (t = 3.3, df = 6, *p < 0.05; Figure 9B) but not in the rats that received the 0.4 mg/kg nicotine dose (t = 1.32, df = 6, p = 0.23; Figure 9B).

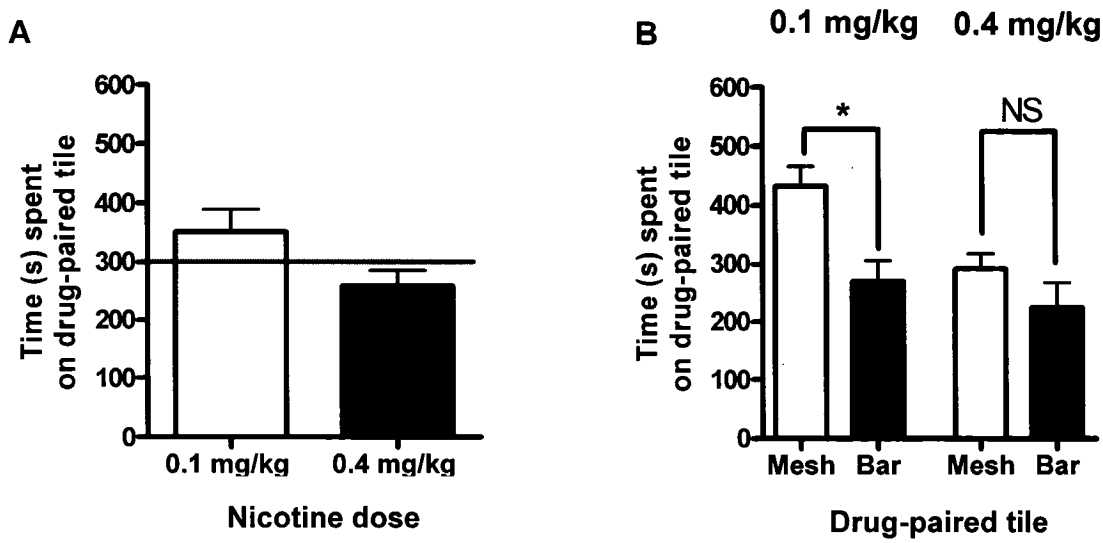


Figure 9

2.4 – Discussion

In this series of experiments, different procedural variables were changed in order to try to achieve reliable nicotine CPP. One of the main changes from the common nicotine CPP procedure employed by most researchers was the route of nicotine administration. It is believed that the more rapidly drugs of abuse reach the brain the greater their potential for addiction (for review see Samaha and Robinson, 2005). In particular, it has been shown that rapid delivery of nicotine promotes behavioural sensitization and alters its neurobiological impact in rats (Samaha et al., 2005). Despite this evidence, in all published nicotine CPP studies, nicotine was administered subcutaneously which results in a slower delivery of nicotine to the brain compared to smoking. After a puff of smoke or a bolus intravenous nicotine injection, peak arterial nicotine concentrations are reached in approximately 20 s (Rose et al., 1999), whereas after subcutaneous nicotine injection, the peak arterial nicotine concentrations are observed after approximately 25 min in humans (Le Houezec et al., 1993); this slower rise in arterial nicotine concentration after s.c. nicotine injection results in slower delivery of nicotine to the brain compared to smoking and i.v. nicotine injection. In our experiments (Experiment 2-6), in order to emulate the rapid delivery of nicotine by smoking, nicotine was administered intravenously. Only in Experiment 7 was nicotine administered s.c. and this was done in order to try to replicate the findings of Gardner's group.

In our attempt to predict nicotine CPP from novelty- or drug-associated locomotor activity, our results contradict the commonly held view that 'high responders' to novelty are more sensitive to the rewarding effects of drugs of abuse in general. However, a closer look at the literature suggests that this relationship may only hold for i.v. self-

administration studies. Thus, it has been reported that 'high responders' to novelty acquire amphetamine, cocaine and nicotine self-administration more readily than 'low responders' to novelty (Piazza et al., 1989; Mantsch et al., 2001; Suto et al., 2001). In contrast, the ability of novelty- or drug-associated locomotor activity to predict drug-induced CPP is controversial, with both positive and negative results (Erb and Parker, 1993; Gong et al., 1996; Klebaur and Bardo, 1998). The positive results show a higher magnitude of CPP for novelty 'high responders'. In our experiment, both 'low responders' to novelty and 'low responders' to nicotine showed a higher magnitude of CPP compared to 'high responders'. This may be either due to the specific properties of nicotine or the locomotor screening procedure, as discussed next.

Nicotine has both arousing and sedating effects in humans (for review see Robinson and Pritchard, 1992); it also tends to increase arousal levels in rats when initial behavioural rates are low, while decreasing arousal levels when initially high (Rosecrans, 1995). It also has been shown that nicotine tends to decrease dopamine release in the NAc of rats when baseline dopamine concentrations were > 5 nM, while the opposite occurred when dopamine baseline levels were < 5 nM (Johnson et al., 2000). In addition, the activation of mesolimbic dopaminergic system appears to mediate the locomotor stimulant effects of nicotine in rats (Clarke et al., 1988). These data, taken together, suggest that nicotine may modulate the level of behavioural arousal by either facilitating or inhibiting dopamine release in naïve subjects. Finally, it also has been shown that rats that are 'high responders' to novelty have higher dopaminergic activity in the NAc compared to 'low responders' (Piazza et al., 1991). Thus, it is possible that nicotine increases dopamine levels in the NAc in the novelty 'low responders', and therefore produces significant CPP in these rats, whereas it decreases dopamine levels in the NAc in novelty 'high

responders' and therefore it is not rewarding to this group of rats. However, since it has been shown that 6-OHDA lesions of the core subregion of the NAc abolish the locomotor stimulant effects of *amphetamine* without affecting its rewarding effects in the CPP paradigm (Sellings and Clarke, 2003), it is likely that the dopaminergic mechanisms underlying locomotor activity may not be the same as mechanisms underlying CPP (or i.v. self-administration).

Another possible explanation for the discrepancy between our results from Experiment 2 and results from other studies that have examined the relationship between novelty-associated locomotor activity and sensitivity to rewarding effects of drugs of abuse, is that our novelty-associated locomotor scores were not a true measure of novelty-associated locomotor activity. Our 42 cm long locomotor cages are equipped with two parallel infrared photobeams which are 34 cm apart and although not likely, it is possible that the rats were locomoting in the middle of the test cage without breaking either beam (or breaking only one); this would result in misleading locomotor scores and therefore false categorization of the rats into 'high responders' and 'low responders'.

In Experiments 3-7, no significant CPP was observed no matter which variables were manipulated; i.e. infusion schedule, speed of infusion, nicotine pre-treatment, experimental design, duration of conditioning sessions, number of conditioning sessions, route of administration and nicotine dose. It appears as if none of these factors had any effect on the magnitude of nicotine CPP. Some of these factors have been tested before and were shown to lead to significant CPP. First, Shoaib et al. (1994) reported that nicotine pre-treatment was necessary in order to obtain significant nicotine CPP in an unbiased design. Second, Gardners' group have been able to observe s.c. nicotine CPP with extended number of conditioning sessions in an unbiased design. Finally, Le Foll

and Goldberg (2005) have shown that nicotine in a biased design produces significant CPP.

There may be a few explanations for the absence of significant nicotine CPP in our experiments. One possible explanation is that our apparatus is not sensitive enough to demonstrate nicotine CPP. The two compartments of our apparatus are only distinct in terms of tactile cues. Although previous research both in our lab (Sellings and Clarke, 2003; Sellings et al., 2006) and other labs (Vezina and Stewart, 1987) has shown that it is possible to acquire morphine, amphetamine, cocaine and sometimes even nicotine CPP with only one stimulus modality as cue, usually the two compartments of a CPP apparatus have distinct visual *and* tactile cues (for review see Carr et al., 1989). Furthermore, it has been reported that using multiple stimulus modalities as cues results in stronger conditioning (Mucha et al., 1982). Therefore, since nicotine is a weak primary reinforcer compared to other drugs of abuse, it may be particularly important to have more than one distinct stimulus modality as cue to obtain significant nicotine CPP.

Another possible explanation is that since nicotine has both rewarding and aversive effects (Jorenby et al., 1990; Rose and Corrigall, 1997), its aversive effects mask its rewarding effects and therefore prevent the emergence of a CPP.

Finally, it is possible that nicotine is not rewarding in the CPP paradigm. This may be the reason for the failure of many nicotine CPP studies employing the unbiased design in our lab and many other labs (Clarke and Fibiger, 1987; Shoaib et al., 1994; Laviolette and van der Kooy, 2003). As mentioned before (see section 1.6.3), most studies that report a significant nicotine CPP use the biased design; in this design it is possible that the anxiolytic properties of nicotine are measured rather than its rewarding effects (see section 1.2.2). However, we should have been able to see at least the anxiolytic effects of

nicotine in Experiment 5 where we employed the biased design but we did not. Since, in the biased design the baseline preference of each rat has to be determined in order to know which compartment to pair with the drug, rats are exposed to the distinct conditioning cues before the conditioning phase. Occurrence of latent inhibition as a result of extensive baseline testing may be the reason for not acquiring nicotine CPP in a biased design.

An interesting finding of this series of experiments was that the mesh tile was more nicotine-conditionable than the bar tile (see section 2.3.6). In all experiments, when the data were reanalyzed based on the nicotine-paired tile, it was revealed that when nicotine was paired with the mesh tile, rats spent more time on the drug-paired tile compared to when it was paired with the bar tile. Since rats show a slight spontaneous preference for the mesh tile (see section 2.3.1), it is possible that nicotine is making this slightly rewarding stimuli more rewarding and this may be the reason for getting higher scores for time spent on the drug-paired tile when nicotine is paired with the mesh tile. This would accord with findings from Donny et al. (see section 1.7.1) which indicate that nicotine may increase the rewarding properties of slightly rewarding nonpharmacological stimuli.

Chapter 3 - MAO inhibition and nicotine CPP

Since tobacco smoke partially inhibits MAO-A and MAO-B activity and dopamine is metabolized by both subtypes, this inhibition should lead to higher dopamine levels (see section 1.8). We hypothesized that graded inhibition of MAO-A and MAO-B activity should produce graded increases in the magnitude of nicotine CPP. In these experiments, MAO-A and MAO-B were inhibited by administering a combination of MAO inhibitor (MAOI) drugs. Five experiments were performed.

3.1 - Overview of experiments

Experiment 8- to determine the time course of the MAO assay.

Experiment 9- to determine the Vmax and Km of the MAO assay.

Experiment 10- to establish the dose-response curve of MAO inhibition with a combination of MAO-A and MAO-B selective inhibitors.

Experiment 11- to determine whether different degrees of MAO inhibition facilitates s.c nicotine CPP in a graded manner.

Experiment 12- to determine whether MAO inhibition to different degrees facilitates i.v. nicotine CPP in a graded manner.

3.2 – Methods

3.2.1 - Subjects

See section 2.2.1.

3.2.2 - Intravenous catheterization

See section 2.2.3. This procedure was used in **Experiment 12**.

3.2.3 - Conditioned Place Preference (CPP)

See section 2.2.4.

3.2.4 - Radiochemical MAO assay

This method is adapted from that of Lyles and Callingham (1982), taking into account technical advice from Dr. Andrew Holt (University of Alberta, Edmonton).

Rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and decapitated. Brains were rapidly removed and the hippocampus, striatum (i.e. caudate-putamen) and cerebral cortex were dissected, weighed, frozen in -40°C isopentane and stored in a -80°C freezer.

Tissue homogenates were prepared as follows: thawed brain regions were homogenized in an appropriate volume (80 ml per gram tissue) of ice cold 0.2 M potassium phosphate buffer (pH 7.8) using a Polytron (Brinkmann, Rexdale, Ontario) homogenizer for 20 s. The tissue suspension was centrifuged at 1600 rpm at 4°C for 30 s in a Beckman model J-6B centrifuge. The supernatant was used for the protein and MAO assay.

Protein assay. BioRad dye reagent concentrate (BioRad Labratories, Montreal, Quebec) was added to 5 different concentrations of bovine serum albumin (Sigma-Aldrich, Oakville, Ontario) standards (1, 2, 5, 7 and 10 μg/ml) and samples (triplicates). Absorbance was measured at 595 nm using a Shimadzu UV160U spectrophotometer. Protein concentrations of samples were calculated by reference to the standard concentration curve.

MAO assay. Tissue samples were analyzed in triplicate. Appropriate radiolabelled substrates were added to the samples. For MAO-A, the substrate was [^{14}C]5-HT 250 μM (~2×Km) and for MAO-B the substrate was [^{14}C]PEA 15 μM (~2×Km) (the Km for each substrate was determined in **Experiment 9**). The substrate and samples were incubated at 37°C for 10 min (the optimal incubation time was determined in **Experiment 8**). The incubation time permits the MAO enzymes in the sample to react with their specific radiolabelled substrates and form radiolabelled aldehydes. The reaction was stopped with the addition of 3M HCl. The radiolabelled aldehydes were extracted into an ethyl acetate/toluene mixture (1:1 v/v), vortexed briefly and centrifuged at 1000-2000 rpm at 4°C for 30 s in a Beckman model J-6B centrifuge. 700 μl of the organic phase was added to 3 ml of Ready Safe scintillation cocktail (Beckman Coulter, U.S.A). Radioactivity was

counted by a Wallac 1410 liquid scintillation counter.

3.2.5 - Drugs

The drugs used were as follow: N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine HCl (clorgyline HCl), pargyline HCl (Sigma-Aldrich, Oakville, Ontario); dipyrone (Vetoquinol, Quebec, Quebec); sodium pentobarbital (Biomune, Kansas, U.S.A); [^{14}C]5-HT creatinine sulphate, [^{14}C]phenyl ethyl amine (PEA) (Amersham Biosciences, England). For the remaining drugs, see section 2.2.5. Both clorgyline HCl and pargyline HCl were dissolved in sterile saline. Nicotine was dissolved in sterile saline and its pH was adjusted to 7.2 ± 0.1 with 0.05 N NaOH. In **Experiment 11**, an s.c. nicotine dose of 0.1 mg/kg (base) was used; and in **Experiment 12**, the i.v. nicotine dose of 0.015 mg/kg (base) was used. The rationale for using these doses was discussed in section 2.2.5.

3.2.6 - Data analysis

For **Experiments 8** and **Experiment 9,** GraphPrism version 4.0 Software (GraphPadSoftware Inc, San Diego, CA, U.S.A.) was used. For **Experiments 10-12**, see section 2.2.6.

3.3 - Procedures and Results

3.3.1 - Experiment 8: Product formation as a function of incubation time.

Cerebral cortex (n=3 rats) was used for this experiment. Five different incubation times (5-25 min) were tested. Figure 10 shows the product (aldehyde) concentration plotted against incubation time. The linear part of the curve was used to determine the optimal incubation time. Therefore, an incubation time of 10 min was chosen for subsequent experiments.

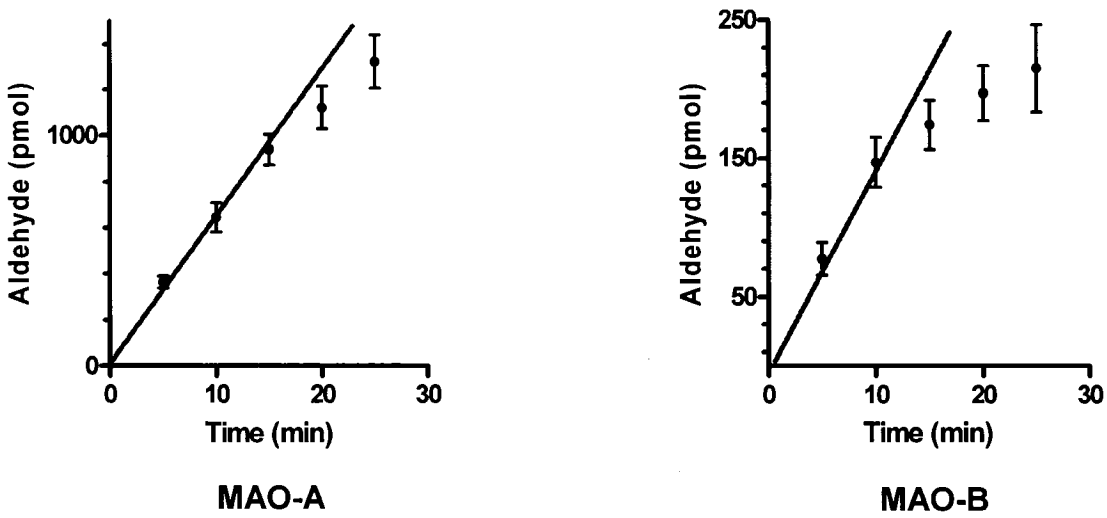


Figure 10

3.3.2 - Experiment 9: Determination of the Vmax and Km for MAO-A and MAO-B.

Cerebral cortex (n=3 rats) was used for this experiment. Ten substrate concentrations for MAO-A (15-1000 μM) and MAO-B (2- 200 μM) were used. Substrate concentration was plotted vs. enzyme velocity, and Vmax and Km were determined for each substrate

Figures 11 and 12 show the enzyme velocity (nmol product per hour) as a function of substrate concentration for MAO-A and MAO-B, respectively. The calculated Vmax for [^{14}C]5-HT as MAO-A substrate, was 807 nmol/hour (95% confidence interval: 774 to 840) and the Km was 142 μM (95% confidence interval: 125 to 160) (Figure 11). The Vmax for [^{14}C]PEA as MAO-B substrate was 130 nmol/hour (95% confidence interval: 116 to 144) and the Km was 7.9 μM (95% confidence interval: 4.3 to 11.6) (Figure 12).

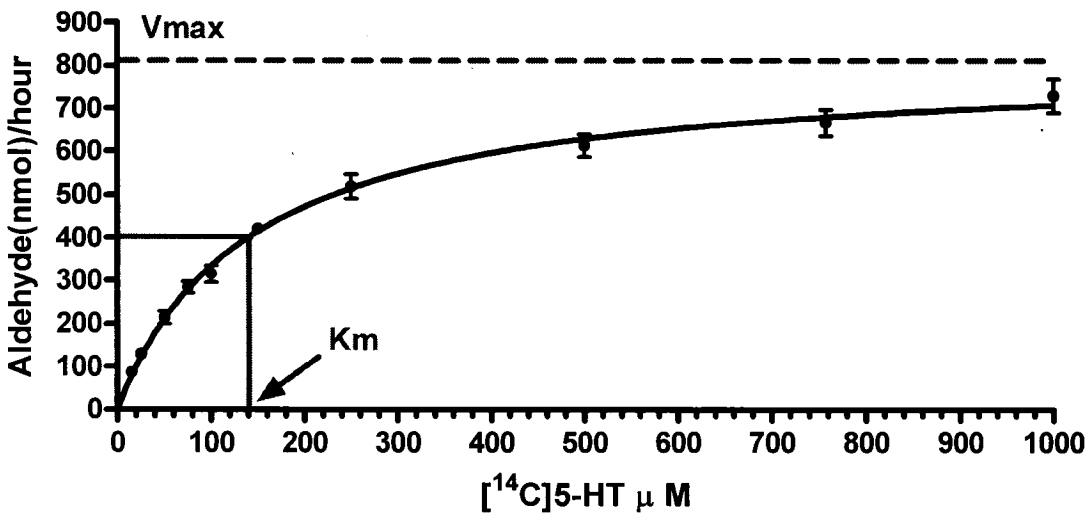


Figure 11

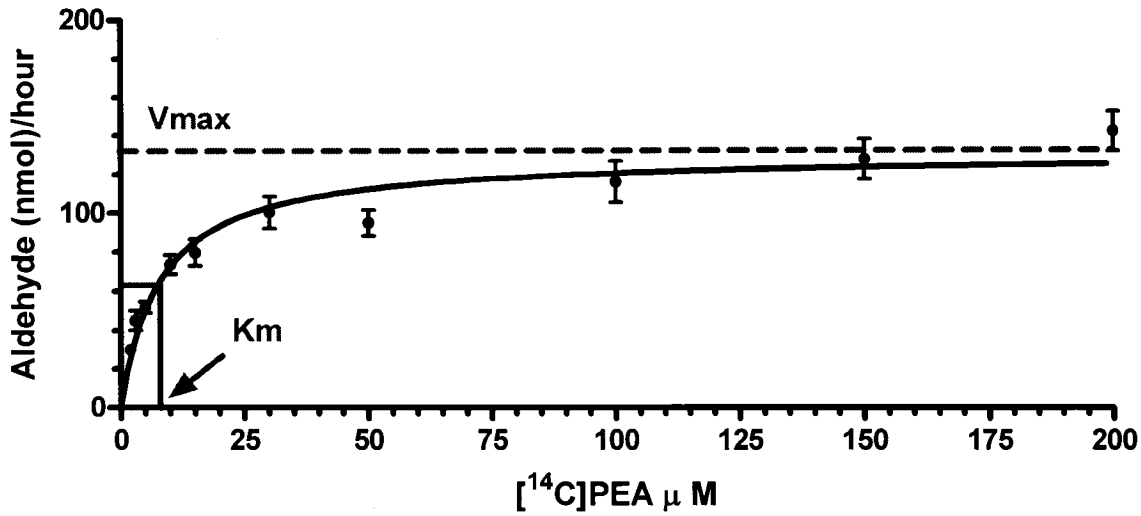


Figure 12

3.3.3 - Experiment 10: Dose-dependent MAO inhibition by a combination of different doses of a selective MAO-A inhibitor (clorgyline HCl) and a selective MAO-B inhibitor (pargyline HCl).

The aim of this experiment was to identify a dose combination of clorgyline HCl and pargyline HCl that produced a degree of MAO-A and B inhibition similar to that observed in smokers (i.e. 30-40%). Rats were randomly allocated to five groups (n=6 per group) and injected s.c. with a combination of clorgyline HCl and pargyline HCl. The doses were as follows:

Dose (mg/kg, s.c.)	Control	Low	Medium	High	Super High
Clorgyline HCl	0	0.01	0.03	0.1	1
Pargyline HCl	0	0.04	0.12	0.4	4

The rats were sacrificed 6 days later and the MAO assay was performed for 3 brain regions: hippocampus, striatum and cerebral cortex. The 6th post-treatment day was chosen since it corresponds to the mid-point of behavioural testing in later experiments.

As shown in Figure 13, MAO inhibition was dose-related in all brain regions tested. The high dose combination achieved a degree of MAO-A and MAO-B inhibition (~35% and 45% averaged from all brain regions, respectively; Figure 14) comparable to that found in smokers.

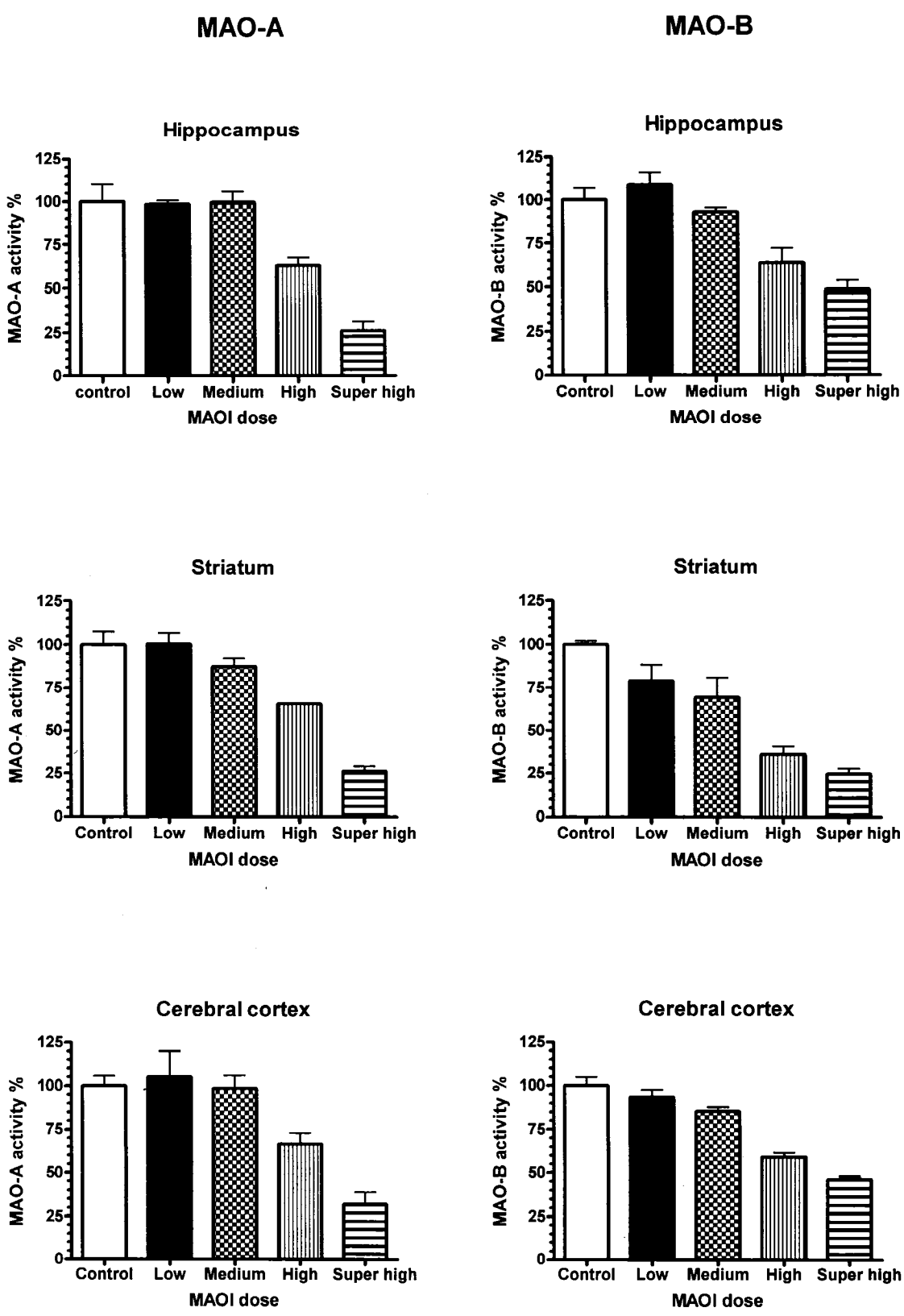

MAO-A
MAO-B
Hippocampus
MAO-A activity %
125
100
75
50
25
0
control
Low
Medium
High
Super high
MAOI dose
Hippocampus
MAO-B activity %
Control
Striatum
Striatum
Cerebral cortex
Cerebral cortex

Figure 13

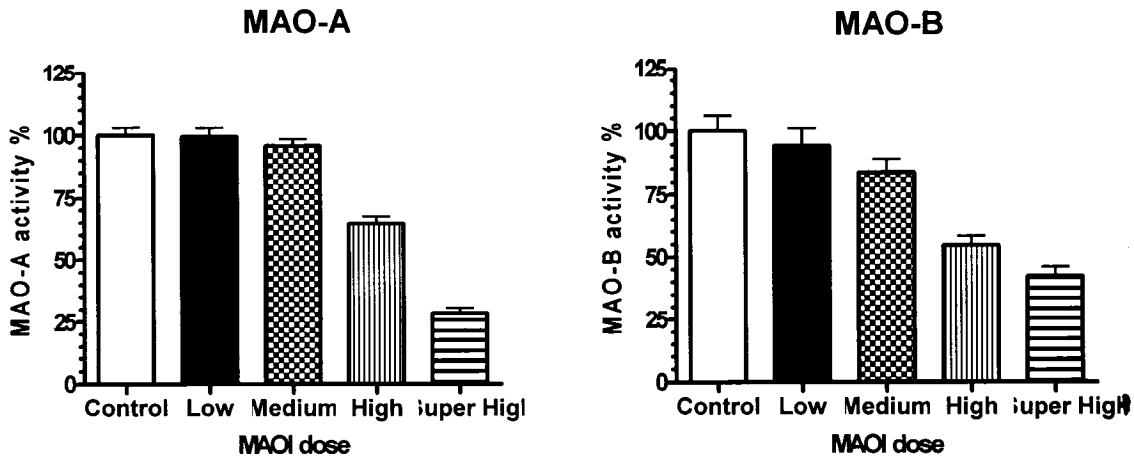


Figure 14

3.3.4 - Experiment 11: Effect of graded MAO inhibition on subcutaneous nicotine CPP.

This experiment featured two parallel groups of rats. One group underwent behavioural testing (Behaviour group) and the other group was used for MAO assay (Assay group). Both groups were subdivided into four subgroups to receive different dose combination of MAO inhibitors one day before the start of the behaviour testing, as shown below:

Dose (mg/kg, s.c.)	Control	Low	Medium	High
Clorgyline HCl	0	0.03	0.1	1
Pargyline HCl	0	0.12	0.4	4

The Behaviour group consisted of 32 rats (8 rats per dose combination) and the Assay group consisted of 20 rats (5 rats per dose combination). Rats in the Assay group were sacrificed 6 days after receiving the MAO inhibitors.

CPP. In the conditioning phase of the *Standard CPP procedure*, rats received 0.1 mg/kg nicotine s.c.

None of the groups showed significant CPP (control: $t = 0.48$, $df = 7$, $p = 0.64$; low: $t = 1.62$, $df = 7$, $p = 0.15$; medium: $t = 1.65$, $df = 7$, $p = 0.14$; high: $t = 0.05$, $df = 7$, $p = 0.96$; Figure 15A). Reanalysis of the data based on nicotine-paired tile using two-way ANOVA revealed that there was no significant main effect of the MAOI dose [$F(3, 24) = 0.55$, $p = 0.65$] or nicotine-paired tile [$F(1, 24) = 2.13$, $p = 0.16$] on the magnitude of CPP and the interaction between these factors was also non-significant [$F(3, 24) = 0.45$, $p = 0.72$). Post-hoc two sample t-tests showed that there was no significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar for any of the MAOI doses (control: $t = 1.23$, $df = 6$, $p = 0.26$; low: $t = 1.55$, $df = 6$, $p = 0.17$; medium: $t = 0.18$, $df = 3.1$, $p = 0.87$; high: $t = 0.45$, $df = 6$, $p = 0.67$; Figure 15B).

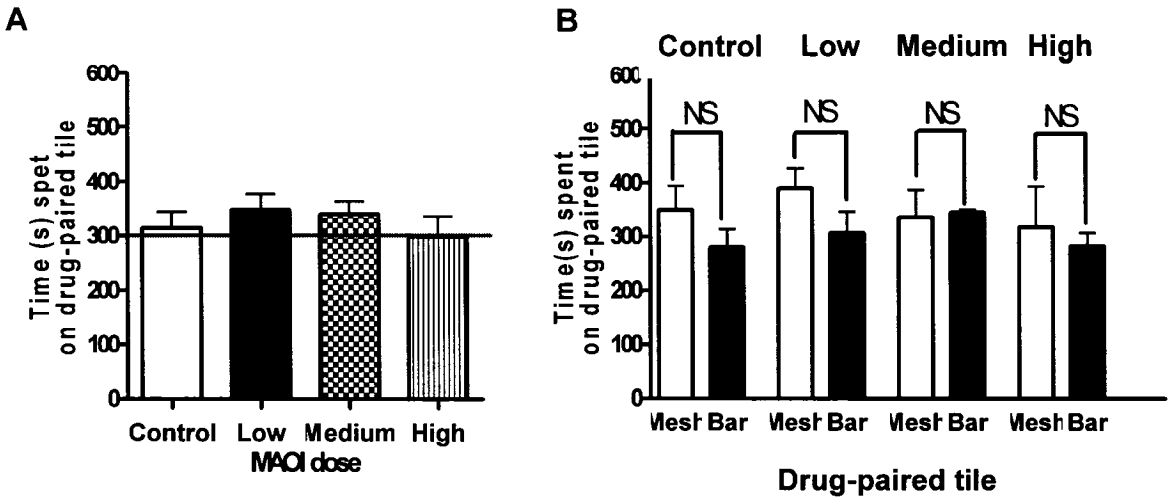


Figure 15

3.3.5 - Experiment 12: Effect of graded MAO inhibition on intravenous nicotine CPP.

This experiment was performed exactly like **Experiment 11** except for route of administration of nicotine which was i.v. instead of s.c, and dose of nicotine which was 0.015 mg/kg instead of 0.1 mg/kg.

As shown in Figure 16A, none of the groups showed significant CPP (control: $t = 0.15$, $df = 7$, $p = 0.89$; low: $t = 0.17$, $df = 6$, $p = 0.87$; medium: $t = 1.17$, $df = 6$, $p = 0.29$; high: $t = 0.51$, $df = 7$, $p = 0.62$). Two-way ANOVA showed that there was a significant main effect of nicotine-paired tile on the magnitude of CPP [$F(1, 22) = 12.62$, $p = 0.002$] but no significant main effect of MAOI dose [$F(3, 22) = 0.14$, $p = 0.93$] and no significant interaction between the MAOI dose and nicotine-paired tile [$F(3, 22) = 0.76$, $p = 0.53$).

Post-hoc two sample t-tests revealed that there was a significant difference in the magnitude of CPP when nicotine was paired with mesh vs. bar in the rats that received the low dose of MAOIs ($t = 2.88$, $df = 5$, $*p < 0.05$; Figure 16B) but not the other doses (control: $t = 2.11$, $df = 6$, $p = 0.08$; medium: $t = 0.82$, $df = 5$, $p = 0.45$; high: $t = 1.35$, $df = 6$, $p = 0.23$; Figure 16B).

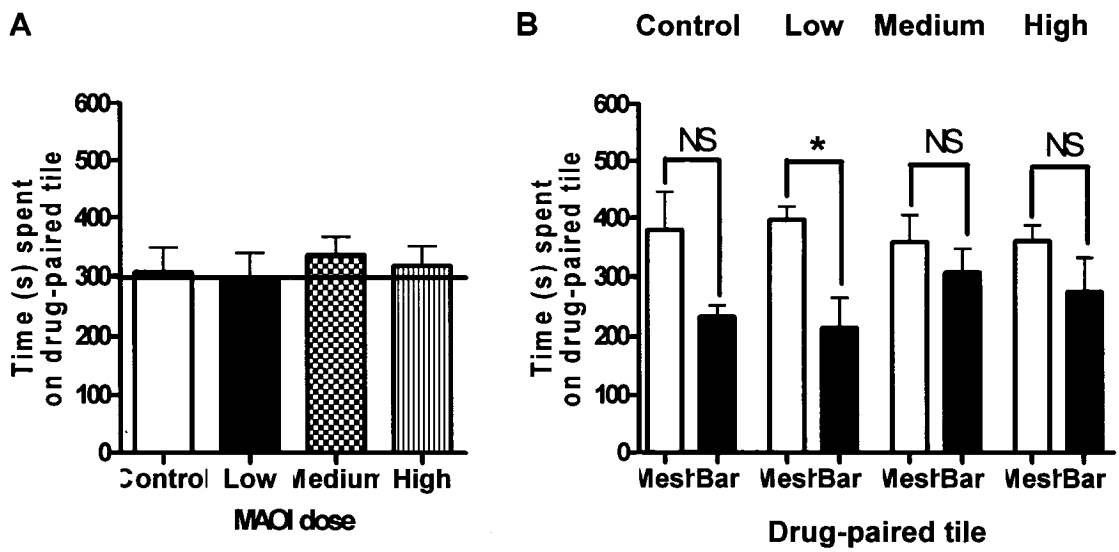


Figure 16

3.4 -Discussion

As shown in Experiments 11 and 12, no significant CPP was observed with either s.c. or i.v. nicotine after graded MAO inhibition. There are four possible explanations for the absence of nicotine CPP after MAO inhibition. First, it is possible that nicotine was not rewarding on its own; therefore there was no rewarding effect in the first place that might have been potentiated by MAO inhibition. Second, *partial* MAO inhibition may not be enough to potentiate the rewarding properties of nicotine. As mentioned before (see section 1.8), studies that have reported that MAO inhibitors increased nicotine self-administration in rats, used large doses of MAO inhibitors and they did not measure the level of inhibition achieved; therefore, it is very likely that MAOs were inhibited nearly 100% rather than partially in these studies and this is the reason for the contradiction between their results and ours. Third, it is possible that dopamine transporters play a more important role in the clearance of dopamine from the extracellular space than MAO isoforms. It has been shown that at least in the dorsal striatum, the termination of the effect of dopamine in the synapse mainly occurs via neuronal reuptake and dopamine transporter blockers are able to increase the concentration of extracellular dopamine whereas MAO-B inhibitors have no effect (Janhunen et al., 2005). To my knowledge, there are no studies comparing the role of dopamine transporters or MAO enzymes in the clearance of dopamine in the ventral striatum. However, if dopamine reuptake is also the main mechanism of dopamine clearance in the ventral striatum, it is not surprising that MAO inhibition does not facilitate nicotine CPP. Finally, MAO inhibitors are notoriously non-specific; in the sense that they are able to interact with proteins unrelated to MAO enzymes. These protein targets include other enzymes, receptors and uptake pumps.

MAO inhibitors belonging to different chemical classes interact with different types of proteins and with different potency (for review see Holt et al., 2004). Therefore, the non-MAO inhibiting effects of MAO inhibitors depend on which proteins they interact with and to what extent. Few examples of the proteins that MAO inhibitors can interact with and are of particular interest to behavioural studies evaluating rewarding properties of drugs of abuse, are as follows. First, it has been reported that some MAO inhibitors such as clorgyline, phenelzine, pargyline and tranylcypromine antagonize dopamine D2 receptors in the rat brain (Levant et al., 1996). Second, it has been shown that deprenyl and clorgyline block dopamine uptake via blocking the dopamine transporter in the rat striatum, whereas pargyline has no effect (Fang and Yu, 1994). Third, tranylcypromine is able to inhibit CYP 2A6 (Draper et al., 1997), a member of cytochrome P450 enzymes that is responsible for metabolizing nicotine (Messina et al., 1997).

It is likely that the outcome of studies that examine the effects of MAO inhibitors on reinforcing or rewarding effects of nicotine depends on the specific MAO inhibitors used. If the reported effects of MAO inhibitors (e.g. increased nicotine self-administration, nicotine-induced locomotor activity) are really due to MAO inhibition, results should generalize across different MAO inhibitors. However, Villegier et al. (2005) reported marked differences between MAO inhibitors in their ability to allow a nicotine-induced locomotor response in mice. Hence, a further possible reason why we failed to observe an enhancement of nicotine CPP may be that our MAO inhibitors lacked the necessary non-MAO action.

Chapter 4 - Nicotine and expression of amphetamine and morphine CPP

There is evidence that nicotine can not only serve as a primary reinforcer but also has the ability to make other non-nicotine salient stimuli and conditioned stimuli rewarding (Donny et al., 2003; Olausson et al., 2004; Chaudhri et al., 2006a, b; also see section 1.7.2). We therefore hypothesized that nicotine can increase the rewarding properties of conditioned stimuli (in our case tiles that were paired with amphetamine or morphine) in a CPP paradigm.

4.1 - Overview of experiment

This experiment examined whether nicotine increases the expression of CPP in rats conditioned with either amphetamine or morphine. Both amphetamine and morphine have been reported to produce significant and reliable CPPs (for reviews see Carr et al., 1989; Tzschentke, 1998). However, nicotine had not previously been tested for its effect on CPP expression.

4.2 – Methods

4.2.1 - Subjects

See section 2.2.1.

4.2.2 - Conditioned Place Preference (CPP)

Some aspects of the conditioning and test phases differed from the *standard CPP procedure* explained in section 2.2.4. During the conditioning phase, which was done over 4 consecutive days (days 2-5), rats received 2 pairings with the drug and 2 pairings with saline on alternate days. The duration of each conditioning session was 45 min. This conditioning duration was chosen based on previous work in our lab (Sellings and Clarke, 2003) and the review paper by Bardo et al. (1993). Both publications suggest this conditioning duration is suitable for obtaining amphetamine and morphine CPP. The test phase took place on two consecutive days (days 6-7); in this phase, rats received a nicotine challenge (0.2 mg/kg s.c.) on one of the test days and a saline challenge (1 ml/kg s.c.) on the other, in a counterbalanced manner. The rest of the CPP procedure was same as the *standard CPP procedure.*

4.2.3 – Drugs

The drugs used were as follow: morphine sulphate (gift from Sabex 2002 Inc., Boucherville, Quebec); D-amphetamine sulphate (Bureau of Drug Research, Ottawa, Ontario). For rest of the drugs, see section 2.2.5. Morphine sulphate and amphetamine

sulphate were dissolved in sterile saline.

4.2.4 - Data analysis

See section 2.2.6.

4.3 - Procedure and Results

In the conditioning phase, one group of rats (n=12) received morphine sulphate (4 mg/kg i.p.) and the other group (n=12) received amphetamine sulphate (1 mg/kg i.p.).

Amphetamine and morphine both produced significant CPP during saline challenge (amphetamine: $t = 2.23$, $df = 10$, $*p<0.05$; morphine: $t = 4.97$, $df = 11$, $***p < 0.005$) but not during the nicotine challenge (amphetamine: $t = 1.4$, $df = 10$, $p = 0.19$; morphine: $t = 1.92$, $df = 11$, $p = 0.08$). Paired t-tests showed that there was no significant difference in the magnitude of amphetamine or morphine CPP when rats were challenged with nicotine compared to saline (amphetamine: $t = 0.55$, $df = 10$, $p = 0.59$; morphine: $t = 2.07$, $df = 11$, $p = 0.06$; Figure 17). Hence, nicotine challenge did not significantly enhance or inhibit the expression of amphetamine or morphine CPP.

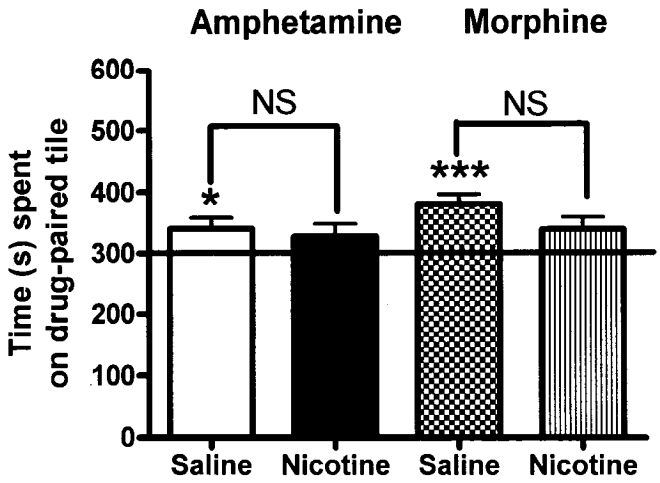

Amphetamine
Morphine
Time (s) spent on drug-paired tile
600
500
400
300
200
100
0
NS
NS
*
***
Saline
Nicotine
Saline
Nicotine

Figure 17

4.4 - Discussion

In this experiment, nicotine failed to enhance the rewarding properties of conditioned stimuli. In contrast, nicotine self-administration studies have shown that nicotine has the ability to enhance the reinforcing properties of conditioned stimuli (see section 1.7.2). The reason for this discrepancy may relate to aversive effects of acute nicotine. Aversive effects of nicotine challenge during the test phase of morphine and amphetamine CPP may have interfered with its ability to enhance the rewarding properties of conditioned stimuli. In nicotine self-administration studies, nicotine is administered repeatedly and since repeated exposure to nicotine can abolish its aversive effects (Iwamoto and Williamson, 1984), these effects presumably do not interfere with the ability of nicotine to enhance the reinforcing properties of conditioned stimuli. Pre-treating the rats with nicotine in their home cage before the CPP procedure might have diminished the aversive effects of acute nicotine challenge and thereby revealed the reinforcement enhancement ability of nicotine.

Chapter 5 – Summary and future research

In conclusion, we were not able to acquire reliable nicotine CPP. For future nicotine CPP studies, several approaches may be worth trying. First, the use of a more distinct two-compartmental or three-compartmental CPP apparatus may be helpful. Another solution may be to always pair nicotine with the mesh tile and have a control (saline-saline) group run in parallel. Finally, if in our hands nicotine CPP was masked by the drug's aversive effects, it may be possible to associate these aversive effects with a novel taste, as explained below.

As mentioned before, nicotine has both rewarding and aversive effects (Jorenby et al., 1990; Rose and Corrigall, 1997). This feature is not unique to nicotine; since other drugs of abuse such as amphetamine also have rewarding and aversive properties (Reicher and Holman, 1977). It has been shown that the aversive properties of amphetamine are readily associated with taste whereas its rewarding effects are readily associated with place (Reicher and Holman, 1977). In addition, Lett (1988) has demonstrated that when rats were permitted to associate the aversive properties of amphetamine with a novel taste, they showed a larger amphetamine CPP. The same procedure could be used for nicotine, since nicotine also produces taste aversion (Kumar et al., 1983).

To further investigate the effects of partial MAO inhibition on the rewarding properties of nicotine, it would be useful to examine the effects of partial MAO inhibition on the rate of i.v. nicotine self-administration. This would allow a comparison between the role of partial vs. complete MAO inhibition on nicotine self-administration, which in term may shed light on the role of partial MAO inhibition in acquisition and maintenance of

smoking behaviour. Moreover, employing MAO inhibitors with fewer known non-specific effects would be desirable. As mentioned before, it is still not clear which component of tobacco smoke inhibits MAOs in smokers. When further studies determine the compounds responsible for MAO inhibition in tobacco smoke, testing them in nicotine CPP and self-administration paradigm would be a good approach.

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