Role of the cannabinoid system in modulating the reinforcing and relapse
related properties of nicotine in rats

By

Islam Gamaleddin

A thesis submitted in conformity with the requirements for the degree of Doctor of
Philosophy, Department of Pharmacology & Toxicology, University of Toronto.

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ABSTRACT

INTRODUCTION: There are several lines of evidence supporting the existence of a pivotal role of the cannabinoid system in mediating the reinforcing effects of nicotine. Characterization of the crosstalk between nicotine addiction and the cannabinoid system may have significant implications for our understanding of the neurobiological mechanisms underlying nicotine dependence.

Objectives: The current series of experiments, we investigated the effects of activating CB1 receptors, modulating CB2 receptors as well as elevating levels of the endogenous cannabinoid ligand anandamide on nicotine taking and reinstatement of nicotine seeking behaviour.

METHODS: In the first series of experiments, we investigated the effects of pretreatment with the CB receptor agonist WIN 55, 212-2 (0.1-1mg/kg), on nicotine self-administration and on the reinstatement of nicotine seeking behaviour. In the next series of experiments, we used a selective CB1 inverse agonist rimonabant (0.3mg/kg) and CB2 antagonist AM630 (5mg/kg) to delineate whether the effects observed with WIN 55, 212-2 are CB1 or CB2 mediated. Moreover, we investigated the effect of selective CB2 receptor activation (AM1241 1-10 mg/kg) and inhibition (AM630 1.25-5 mg/kg) on nicotine self-administration under fixed ratio (FR) and progressive (PR)
schedules of reinforcement and on reinstatement of nicotine seeking induced by nicotine associated cues and nicotine priming. Finally, the effects of activation of CB receptors through administration of anandamide reuptake inhibitor VDM11 (1-10 mg/kg) on nicotine self-administration and on reinstatement of nicotine seeking were investigated.

RESULTS: WIN 55,212-2 enhanced the break points for nicotine self-administration under a PR schedule of reinforcement, reinstated nicotine seeking behaviour and enhanced cue induced reinstatement of nicotine seeking. Neither activation nor blockade of CB2 receptors affected the responding of the animals for nicotine self-administration under FR or PR schedules of reinforcement or for reinstatement of nicotine seeking induced by nicotine associated cues and priming. Pretreatment with VDM11 dose dependently attenuated the reinstatement of nicotine seeking behaviour induced by nicotine associated cues and priming without affecting stable nicotine self administration.

CONCLUSION: CB1 but not CB2 receptors appear to play a pivotal role in modulating the reinforcing effects of nicotine. Inhibition of anandamide reuptake could be a potentially useful tool in modulating relapse to smoking.
ACKNOWLEDGEMENT

My greatest thanks are to God for giving me the strength, will power and faith to overcome the challenges and motivation to complete this work.

Next, I would like to thank my parents to whom I am forever indebted for everything I am and everything I will be. After becoming a father myself, I strongly appreciate your efforts to raise me on morals and principles, shaping my personality and building the foundation of my conscience, self-respect and perseverance. Thank you for your never-ending support and guidance. I am what I am today because of you.

No matter how much I try, I will not be able to sufficiently thank my wife, Riham, who has been my companion and best friend for the past fourteen years of my life. Thank you for being there for me at every stage. Thank you for putting up with me during my numerous stressful nights, endless deadlines and days of studying after work. Thank you for your never ending trust and always refreshing, sense of humor.

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I am thankful to the generous funding of the Egyptian Ministry of Higher Education, Canadian Tobacco Control Research Initiative.

Finally yet importantly, I would like to dedicate this thesis to my lovely sons Hany and Ahmed. Thank you for being my motivators. You have taught me to very effectively use my time between your soccer games and swimming lessons. It is because of you that I push myself to my maximum. May god bless you; grant you health, wisdom, goodness of heart and character.
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<td>AM404</td>
<td>N-(4-hydroxyphenyl)-arachidonamide</td>
</tr>
<tr>
<td>AM630</td>
<td>1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindoled</td>
</tr>
<tr>
<td>AEA</td>
<td>anandamide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>2AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
</tr>
<tr>
<td>BNST</td>
<td>bed of nucleus stria terminalis</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPA</td>
<td>conditioned place aversion</td>
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<tr>
<td>CPP</td>
<td>conditioned place preference</td>
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<tr>
<td>CTA</td>
<td>conditioned taste aversion</td>
</tr>
<tr>
<td>C- terminus</td>
<td>carboxyl terminus</td>
</tr>
<tr>
<td>CRF</td>
<td>continuous reinforcement</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin relaeasing hormone</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FR</td>
<td>fixed ratio,</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic pituitary adrenal axis</td>
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<tr>
<td>IM</td>
<td>intramuscular</td>
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<td>IP</td>
<td>intraperitoneal</td>
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IV     intravenous
IVSA   intravenous self-administration
LTD    long term depression
LTP    long term potentiation
MEC    mecamylamine
nAChR  nicotinic acetyl choline receptor
N- terminus amino terminus
NRT    nicotine replacement therapy
OEA    oleoylethanolamide
PEA    palmitoylethanolamide
PPAR   peroxisome proliferator-activated receptor
PR     progressive ratio
PVN    parventricular nucleus
SA     self-administration
SC     subcutaneous
THC    tetrahydrocannabinol
TRVP   transient receptor vanilloid potential
TO     time out
URB597 cyclohexyl carbamic acid 3’-carbamoyl-3-yl ester
VDM11  (5Z, 8Z, 11Z, 14Z)-N-(4-hydroxy-2-methylphenyl)-5, 8,11,14-eicosatetraenamide
VTA    ventral tegmental area
WHO    world health organization
WIN 55,212-2 R)-(+-)[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone
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INTRODUCTION

Section 1: DEFINITION AND THEORIES OF DRUG ADDICTION

Drug addiction is a chronic relapsing disorder that is characterized by (1) compulsion to take and seek the substance, (2) loss of control in limiting intake, and (3) development of negative emotional symptoms (e.g. anxiety, dysphoria and irritability) when access to the abused substance is restricted. This is distinct from occasional but limited use of an abusable drug (Koob and Le Moal 1997). Drugs of addiction are hypothesized to produce long-term neuroadaptive changes that persist despite detoxification. These changes, along with the psychological and social difficulties encountered by a former patient, put him at an increased risk of relapse especially within a year of abstinence (O’Brien and McLellan 1996; Hubbard, Balment et al. 1997; Finney and Moos 1992).

It is hypothesized that the mesolimbic dopaminergic system through its cortical inputs (glutamatergic afferents) and nucleus accumbens output (γ-aminobutyric acid [GABA] ergic efferents) comprises a major portion of the circuitry through which pleasures start to shape the habits of animals (Wise 2002). The role this circuit plays in nicotine reward is discussed in more detail below (See section 2.5).

Other theories include the prefrontal cortex/ventral striatal hypothesis of addiction, which proposes that certain brain regions involved in inhibitory response control, like the frontal cortex, become dysfunctional due to long-term exposure to drugs. This dysfunction later leads to an inability to inhibit unconditioned or conditioned responses elicited by drugs (Jentsch and Taylor 1999).
Frontal cortex dysfunction has been postulated to impair judgment, blunt affect and lead to poor insight, poor motivation and attention deficits (Parsons 1987a; Sullivan, Rosenbloom et al. 2000a). Several lines of evidence have shown that abnormalities in the frontal lobe and cerebellum are particularly relevant to substance abuse, and especially alcohol abuse due to impairment of cognitive function (Harper and Kril 1989; Kubota, Nakazaki et al. 2001).

Brain imaging studies have played an important role in revealing the different circuits involved in drug addiction. Volkow and colleagues have proposed that there are four circuits being disrupted in drug addiction: (1) Reward mediated by the nucleus accumbens and ventral pallidum, (2) Motivation/drive, mediated by the orbitofrontal cortex and subcallostal cortex, (3) Memory and learning, localized to the amygdala and hippocampus, and (4) Control, mediated through the prefrontal cortex (Volkow, Fowler et al. 2003).

Neurobiological theories involving relapse to drug seeking have been almost exclusively derived from animal studies. It is hypothesized that the reinstatement of drug seeking through exposure to the drug itself, drug related cues or stress produce distinct perceptions yet they all produce a related interoceptive state (Kalivas and McFarland 2003a). So it could be further hypothesized that these distinct perceptions converge into one common pathway which is proposed to be dopaminergic neurons from the anterior cingulate to the core of nucleus accumbens (Kalivas and McFarland 2003a). Several lines of evidence have proposed a pivotal role of dopaminergic and glutamatergic neurons in the ventral tegmental area, nucleus accumbens and ventral pallidum in drug induced reinstatement (Grimm and See 2000). Activation of these midbrain DA neurons through local infusion of morphine (Di Chiara and North 1992) or NMDA (Karreman, Westerink et al. 1996) infusions reinstates heroin and
cocaine seeking (Stewart 1984; Vorel, Liu et al. 2001). Furthermore, local infusions of amphetamine, dopamine and cocaine into the terminal regions of the nucleus accumbens (NAc) or the medial prefrontal cortex (mPFC) also reinstate drug seeking (Stewart and Vezina 1988; Cornish and Kalivas 2000; McFarland and Kalivas 2001; Park, Bari et al. 2002). In contrast, reversible inactivation of the dopaminergic neurons in the ventral tegmental tegmental area (VTA) by GABAergic agonists attenuates cocaine-induced reinstatement (McFarland and Kalivas 2001). Parts of the medial prefrontal cortex (notably the anterior cingulate/prelimbic cortices) and amygdala (rostral basolateral amygdala) appear to be particularly involved in cue-induced reinstatement (Kalivas and McFarland 2003a; Kantak, Black et al. 2002b; Shaham, Shalev et al. 2003).

Studies in rats with a history of cocaine self-administration point to critical roles for D1-like receptors in cue-induced reinstatement. Systemic injections of D1-like receptor antagonists attenuate reinstatement induced by discrete (Alleweireldt, Weber et al. 2002), discriminative (Ciccocioppo, Sanna et al. 2001) and contextual (Crombag, Grimm et al. 2002) cues previously associated with cocaine. Furthermore, intra-basolateral amygdala injections of a D1-like, but not D2-like, receptor antagonist block discrete cue induced reinstatement of cocaine seeking (See, Kruzich et al. 2001). In addition, exposure to discriminative cues following periods of withdrawal induces DA release and Fos immunoreactivity in the basolateral amygdala (BLA) (Neisewander, Baker et al. 2000; Weiss, Maldonado-Vlaar et al. 2000), and D1-like receptor antagonists block the effect of these cues on Fos-induction (Ciccocioppo, Sanna et al. 2001). There is also evidence from lesion studies that the BLA is involved in reinstatement of cocaine (Meil and See 1997) and heroin (Fuchs and See 2002) seeking by discrete cues. In addition, it
has been found that inactivation of the central nucleus of the amygdale (CeA) and the medial prefrontal cortex (mPFC) by infusions of tetrodotoxin (TTX) can attenuate reinstatement of cocaine seeking by discrete cues (See 2002). Stress induced reinstatement studies on the neuronal mechanisms underlying footshock-induced reinstatement have identified two neurotransmitter systems and two brain structures that are critically involved in footshock stress-induced, but not in drug-induced, reinstatement, namely, brain corticotopin releasing factor (CRF) and noradrenaline (NA) systems and the CeA and the bed nucleus of the stria terminalis (BNST) (Erb, Shaham et al. 2001b; Shaham, Erb et al. 2000a). Selective CRF1 and non-selective CRF receptor antagonists attenuate footshock-induced reinstatement in heroin-, cocaine- and alcohol-trained rats (Shaham, Adamson et al. 1997; Erb, Shaham et al. 1998; Le, Harding, et al. 2000). These receptor antagonists, but not a CRF2 receptor antagonist, also attenuate footshock-induced reactivation of morphine and cocaine CPP (Lu, Liu et al. 2001, 2000a). In addition, α2-adrenoceptor agonists, which decrease nucleus accumbens (NA) cell firing and release, attenuate footshock-induced reinstatement of heroin, cocaine and speedball (a heroin cocaine combination) seeking (Erb, Hitchcott et al. 2000a; Highfield, Yap et al. 2001; Shaham, Erb et al. 2000b) (Shaham, Shalev et al. 2003a).

SECTION 2: SMOKING

SECTION 2.1 INCIDENCES OF SMOKING WORLDWIDE AND IN CANADA

Tobacco use and cigarette smoking are the most prevalent and persistent forms of drug taking in the modern age. Tobacco smoking continues to be a worldwide major health problem and is the leading preventable cause of death in the world (WHO/WPRO 2006). In Canada, the incidence of
smoking has dropped steadily since the 1990’s. The Canadian Tobacco Use Monitoring Survey by Health Canada has reported that the percentage of current smokers above 15 years of age in Canada has dropped from 25% (~6 million people) in 1999 to 20% (~5 million people) in 2005 (CDC 2007). However, since 2007 this percentage has been stable and has not declined (CTUMS 2009). In contrast, the situation in developing countries is even more critical and they are considered to be in the initial stages of a tobacco epidemic. For example, it estimated that 67% of men in a China are smokers. Data indicate that the number of smokers globally is 1.3 billion people, 1 billion men and 300 million women (Shafey, Dolwick et al. 2003). Although developed countries are relatively more successful than developing countries in controlling the rates of smoking, yet, the recent stability in the numbers of smokers in developed countries requires deeper analysis and reassessment of the current prevention and treatment modalities and necessitates the development of novel and more efficacious treatment modalities.

SECTION 2.2 CONSTITUENTS OF TOBACCO SMOKE

Cigarettes contain more than 4000 chemicals. There are at least 43 known carcinogens in tobacco smoke as reported by the (CTUMS 2009), including heavy metals (e.g. chromium, cadmium) chemicals (e.g. benzene, vinyl chloride), polyaromatic hydrocarbons (e.g. benzo[a]pyrene) and nitrosamines (e.g. NNK, N’-nitrosonornicotine (NNN). Cigarettes also contain other harmful compounds, such as phenols, that cause lung damage, and catechols that cause high blood pressure. There are approximately 600 additives identified in cigarette smoke some of which are toxic and others which are benign flavourings (NCCDP 2004; IPRC 2005).
It has been proposed that ammonia may increase the reinforcing effects of cigarettes (Henningfield, Pankow et al. 2004). This assumption is based on the fact that ammonia transforms protonated nicotine to free-base nicotine, which crosses the blood brain barrier easier than protonated nicotine. Surprisingly, no study has looked at the effect of ammoniation on blood nicotine levels (Armitage, Dixon et al. 2004). Despite the presence of several substances in tobacco that could potentially contribute to its addictive properties, there is agreement that nicotine is the main component in tobacco that is responsible for addiction (Balfour 1994; Stolerman and Olufsen 2001; Stolerman and Jarvis 1995a).

Section 2.3 HEALTH CONSEQUENCES OF SMOKING

In 1998, more than 30,000 men and 17,000 women died from active and passive smoking in Canada (Illing and Kaiserman 1995). Apart from mortality, smoking also results in several morbidities. In 2004, the U.S surgeon general reported that there is a strong link between smoking and ten different types of cancers (e.g., lung, kidney, bladder and leukemia) and four cardiovascular diseases (e.g., atherosclerosis, coronary heart disease). Additionally, smoking has been also linked to seven respiratory related illnesses (e.g., pneumonia and chronic obstructive pulmonary disease), four types of obstetric problems (e.g., intrauterine fetal death, stillbirths and reduced fertility), and four other health problems (e.g. cataracts, esophageal reflux and peptic ulcer). The economic cost of smoking is daunting. It has been estimated by the US Center for Disease Control and Prevention that $ 157 billion dollars were spent on the direct and indirect costs of smoking from 1995 to 1999 (CDC 2007).
SECTION 2.4 PHARMACOLOGICAL TREATMENTS FOR SMOKING CESSION

Currently there are three pharmacotherapies approved by the FDA and Health Canada for smoking cessation: nicotine replacement therapy (NRT), bupropion (Zyban, Wellbutrin) and varenicline (Champix). NRT is available in five formulations: nicotine gum, patch, spray, inhaler and lozenge. NRT has shown to be effective in controlling the symptoms of withdrawal, however, it has limited efficacy in attenuating the reinforcing effects of nicotine (Moolchan, Robinson et al. 2005; Cepeda-Benito, Reynoso et al. 2004b).

Bupropion (which was originally used as an antidepressant) has shown to be efficacious in decreasing the reinforcing effects of nicotine, as well as in alleviating its withdrawal symptoms. However, bupropion has several side effects that should be taken into account, such as increased risk of seizures (Mooney and Sofuoglu 2006).

Varenicline is an $\alpha_4\beta_2$ nicotinic receptor partial agonist that is loosely based on the structure of cytisine. One of the initial characteristics of cytisine that led to the development of varenicline was its partial agonist activity at nicotinic receptors (Coe, Brooks et al. 2005a). Varenicline is a potent partial agonist at $\alpha_4\beta_2$ receptors and is less potent at $\alpha_3\beta_4$ receptors. It acts as a partial agonist at $\alpha_3\beta_2$ and $\alpha_6$-containing receptors and a potent full agonist at $\alpha_7$ receptors. It is highly accepted that dopamine release in the striatum is partially regulated through the involvement of presynaptic $\alpha_4$- and $\alpha_6$-containing receptors (Salminen, Murphy et al. 2004). To summarize, partial agonism of $\alpha_4\beta_2$ receptors is the most acceptable potential mechanism of action of varenicline (Coe, Brooks et al. 2005a). However, the potent high efficacy activation of $\alpha_7$ receptors may possibly play a role in the effect of varenicline in smoking cessation (Mihalak,
Carroll et al. 2006). Despite its promising effects as a smoking cessation agent, varenicline use has been associated with increasing the risk of suicidality and depression as well as increased risk of coronary heart disease (Harrison-Woolrych 2009; Singh, Loke et al. 2011).

Most of the attempts to quit smoking whether self directed or treatment aided are reportedly followed by relapse despite the availability of therapeutic agents for smoking cessation (Hughes, Gulliver et al. 1992; Hunt, Barnett et al. 1971). In other words, the currently available therapies fall short by either being less specific or being less effective than hoped for. In order to develop novel therapeutic agents that are more specific and more efficacious, we need to have an extensive understanding of the neurobiological mechanisms underlying nicotine dependence and relapse to nicotine seeking behaviour. This requires the integration of different research approaches and utilization of different preclinical and genetic models as well as clinical trials.

In the current body of work, we used the nicotine intravenous self administration/reinstatement model in rodents to evaluate the respective role of CB1, CB2 receptors and endogenous cannabinoid ligands on modulation of nicotine intake, magnitude of reinforcement produced by nicotine and reinstatement of nicotine seeking behaviour induced by nicotine priming and nicotine associated cues. In fact, there are several factors that can trigger relapse to smoking after a period of abstinence, including re-exposure to the environmental stimuli previously associated with smoking (drug-associated cues) or re-exposure to nicotine (Brandon, Tiffany et al. 1990; Kassel, Stroud et al. 2003; O'Brien, Childress et al. 1992; Wikler 1973).
SECTION 3 ANIMAL MODELS OF NICOTINE ADDICTION

Much of the recent progress in the understanding of the neurobiological mechanisms underlying addiction has been derived from the different animals models of drug addiction. Animal models provide the means to assess the neurobiological and behavioural aspects and processes underlying addiction. These factors cannot not be readily tested in humans due to ethical reasons and confounding factors e.g. history of drug abuse. Under controlled conditions, animal models can be employed to examine the processes involved in acquisition, maintenance, extinction and relapse to drug taking behaviours. Moreover, these models can be used to test the influence of environmental, behavioural, developmental and neurobiological factors contributing to individual differences in vulnerability to drug taking (Koob 2000).

Although there is no animal model that fully mimics the human condition, yet the available models allow the study of specific aspects of drug addiction. Such aspects can be studied by models of positive and negative reinforcement, models of addiction cycles and staging and models of the actual symptomatology of addictions as framed by the psychiatric disease classification (Koob 1998). Thus, animal models have both construct and predictive validity and provide valuable tools to let us better understand complex psychiatric and mental disorders. These models further allow the utility of different pharmacological tools and therapeutic agents that could have a potential therapeutic benefit for the treatment of these complex disorders (Ebel, 1961; Willner, 1984).

Reinstatement models have been mainly developed to measure the ability of different triggering factors to reinstate a previously extinguished drug seeking behaviour in animals after a
period of abstinence. These models have been used to determine the neurobiological mechanisms underlying the process of relapse. Over time, animal models of relapse have shown a strong predictive validity despite the presence of some procedural differences between them and the actual relapse phenomenon in humans. This validity is demonstrated by the correlation between outcomes of different studies assessing factors that provoke relapse to drug seeking after a period of abstinence (Breiter, Gollub et al. 1997; Shiffman, Paty et al. 1996; Sinha 2001) (also see Shaham, Shalev et al. 2003 for a review of reinstatement models).

In the early stages of use, drugs serve as positive reinforcers that increase approach responses and maintain drug taking behaviour (Stolerman 1992). There has been much debate over the terms “reward” and “reinforcement” in drug addiction psychology literature (White 1989). A stimulus is considered rewarding if it stimulates an approach response. However, reinforcement is a process by which the probability of a behaviour increases or decreases based upon the immediate consequence or outcome of that behaviour. A reinforcer could possess the property of increasing the probability of response if the behaviour results in the delivery of rewarding stimulus (positive reinforcement) or if it abolishes an aversive or painful stimulus (negative reinforcement).

The rewarding and reinforcing effects of drugs are crucial aspects for the acquisition and maintenance of drug taking (Schenk and Partridge 1997). However, the pleasure contingent to drug use may indeed gradually fade with compulsive drug use, with craving playing a more important role in maintaining drug use and precipitating relapse (Robinson and Berridge 1993). With more prolonged use, the role of withdrawal and relapse become more prominent in maintaining drug taking.
Despite the existence of several animal models that investigate drug taking and seeking behaviour, the intravenous self-administration model and conditioned place preference (CPP) are the two most widely used paradigms. These two paradigms are reviewed in detail in the following section. For a brief description of other less commonly used paradigms, see table 1 (page 19).

SECTION 3.1. CONDITIONED PLACE PREFERENCE

The conditioned place preference paradigm is used to measure the rewarding as well as the aversive effects of drugs of abuse (Tzschentke 1998). CPP is based on the development of an association between drug taking and a specific environmental cue (conditioned stimulus) with which a drug is paired through conditioning. This conditioning can be achieved using a two compartment CPP apparatus. Drug is administered to the animal and paired to one compartment while vehicle administration is paired with the other compartment, which has different visual, textural and olfactory parameters. Following a single or multiple pairings, the drug free animal is placed between the two compartments with free access to both compartments. During the test, the time that the animal spends in the drug paired compartment is measured and provides an index for the rewarding (or aversive) effects of a drug. CPP has a relatively good compatibility with the human drug abuse liability in that drugs that are abused by humans can elicit CPP in animals. Alcohol is exceptional in that it produces conditioned place aversion (CPA) in rats (Cunningham, Ferree et al. 2003; Fidler, Bakner et al. 2004; Funk, Vohra et al. 2004) despite being readily administered under voluntary oral self administration conditions in humans. (For reviews, see Cunningham, Howard et al. 2000; Samson and Czachowski 2003)
In CPP experiments, there are two commonly used methods; biased and unbiased. In the biased condition, the drug is paired with the initially non preferred compartment due to ceiling effects that would prevent the full expression of CPP in the initially preferred compartment (Cunningham, Ferree et al. 2003). The biased procedure is limited by a higher susceptibility to false positive results since it measures the relative increase in time spent in the drug paired compartment as an indicator of CPP (Tzschentke 1998). This change in preference could be attributed to the rewarding effects of the drug or possibly due to its anxiolytic effects. A more accurate determinant of CPP is the absolute preference for the drug paired compartment i.e. spending more than 50% of the time in the drug paired compartment on the test day. On the other hand, using the unbiased method is more accurate as it eliminates the confounding effects associated with the biased method since there is no initial preference for either compartment prior to the conditioning sessions. Thus, a preference for a specific compartment is a direct measure for the rewarding (or aversive) effects of a drug. Hence, the unbiased paradigm should be the procedure of choice in CPP experiments.

The CPP paradigm has the advantage of providing a direct method for assessing the effects of a drug that are significant in controlling drug taking behaviour (Bozarth 1987). Furthermore, the CPP paradigm does not require surgery and requires less time to administer than the self administration paradigm (Bardo and Neisewander 1986; Bardo, Valone et al. 1999a; Spina, Fenu et al. 2006). Other advantages include that the CPP testing is performed under drug free condition, thus eliminating the possible motor impairing effects of certain drugs that could interfere with the animal's behaviour. CPP is also sensitive to low drug doses and the experimenter has control over the dose of drug and the cues paired with its administration (Bardo and Bevins 2000). The
disadvantages of CPP are that only one data point per animal can be generated following several conditioning sessions. Other disadvantages of CPP are its low face validity (Olmstead 2006) and that animals have no control over their drug intake, which could produce different results as compared to the self administration paradigm in which drug administration is active (Dworkin, Mirkis et al. 1995; Jacobs, Smit et al. 2003).

The conditioned place preference has been useful in providing information on the interaction between the cannabinoid system and nicotine. Interestingly, it has been shown that co-administration of sub-threshold doses (that were not rewarding when administered solely) of tetrahydocannabinol and nicotine produced significant CPP (Valjent, Mitchell et al. 2002). The same study also showed that co-administration of the same compounds increased the expression of C-fos expression in the shell of the nucleus accumbens, central and basolateral nucleus of the amygdala, dorso-lateral bed nucleus of the stria terminalis, cingular and piriform cortex, and paraventricular nucleus of the hypothalamus. In contrast, using the same paradigm, pre-exposure to nicotine did not have an effect on subsequent locomotor and rewarding effects of repeated delta-9-tetrahydrocannabinol administration in Sprague-Dawley rats (Le Foll, Wiggins et al. 2006).

SECTION 3.2. INTRAVENOUS DRUG SELF ADMINISTRATION

Intravenous drug self-administration is considered the gold standard for assessing the direct reinforcing effects of drugs of abuse. It has the advantage of allowing the animal to voluntary control its intake and so it most closely approximates the human drug taking conditions. Humans as well as animals will readily administer drugs in a nondependent state, indicating that the reinforcing effects of drugs can drive drug-taking behaviour (Koob 2000).
The self-administration paradigm follows the principles of operant conditioning in that the drug taking behaviour is mediated by the its direct and immediate consequence (Bozarth 1990). Drugs serve as positive reinforcers through increasing the probability of behaviour (e.g. lever pressing or nose poking) upon which their presentation is contingent. In the self-administration paradigm, animals are first surgically prepared with chronic indwelling jugular catheters that will enable direct and rapid drug delivery to the animal. To facilitate drug self-administration, animals are initially trained to operantly respond for food.

In the body of work presented herein, we typically used an operant chamber equipped with two levers and environmental cues. Pressing on a lever designated as active results in the delivery of a set volume of drug infusion; pressing on the inactive lever, which acts as a control for non specific motor effects of the drug and whether the drug is controlling the behaviour, has no consequences (Gardner 2000). Self-administration sessions can vary in duration from one hour to continuous access, however limiting access to a discrete time period produces stable high drug intake and is a reliable method for assessing the effect of pharmacological manipulations in drug taking (Caine and Koob 1993). Often, drug delivery is paired with a discrete cue, such as a light or a tone stimulus, which acts as an indicator for drug availability and may develop conditioned reinforcing properties (Stewart, de Wit et al. 1984; Caggiula, Donny et al. 2002b). Drug associated cues are important in testing the motivational effects of discrete and environmental cues on drug taking behaviour (Stewart, de Wit et al. 1984).

The self-administration paradigm is useful in assessing the various processes involved in drug taking including acquisition, maintenance and relapse behaviours. Acquisition of drug self-administration is a direct measure of the ability of a drug to reinforce operant behaviour. The
operant behaviour becomes established and drug taking increases progressively across sessions through repeated pairings of the operant behaviour and subsequent drug delivery. Acquisition studies typically use fixed ratio (FR) 1 or continuous reinforcement schedules in which one lever press results in one infusion. This schedule is commonly used as it is easy to acquire and is an important screening tool for drug abuse liability (Richardson, Smith et al. 1994). However, the utility of this schedule is limited to qualitatively assessing whether the drug serves as a positive reinforcer and is not sensitive to changes in the drug's reinforcing efficacy.

A more direct and valid method for assessing the reinforcing efficacy of a drug is the progressive ratio schedule of reinforcement. In progressive ratio schedule, the animal is required to increase the effort in order to obtain successive drug infusions (Richardson and Roberts 1996; Stafford, LeSage et al. 1998). Progressive ratio schedule testing can be employed within or between sessions, with the former being more useful in providing a more rapid assessment of the reinforcing efficacy of a drug. Most PR schedules currently used are exponential in nature, and the animal's responding stabilizes quickly across a few sessions (Depoortere, Li et al. 1993). Break point is a measure typically used to assess the reinforcing efficacy of a drug. Break point is the largest ratio requirement an animal is willing to complete in order to obtain an infusion of a drug (Arnold and Roberts 1997).

Studies using the FR schedule have further demonstrated that self administration of nicotine is sensitive to changes in unit dose per infusion where the lower doses result in an increase in responding while higher doses result in a decrease in responding (Corrigall and Coen 1989). On the other hand, under the PR schedule of reinforcement, increasing the unit dose results in an increase in the last ratio schedule completed (i.e increase in break point) indicating that higher
doses are more reinforcing (Donny, Caggiula et al. 1999). The increased intake of lower doses of nicotine under FR schedule indicates that the animals are attempting to maintain a certain level of nicotine in the blood and brain. However, at higher infusion doses, the animals need a lesser number of infusions in order to maintain their target blood levels of nicotine and hence reduce their intake. Under the standard maintenance dose (30 ug/kg/infusion) of nicotine, rats self administer a cumulative dose of nicotine of 0.6 mg/kg/session that yield blood levels of approximately 65 ng/ml (Shoaib and Stolerman 1999). This is higher than typical levels obtained from smokers (15- 40 ng/ml) (Russell, Epstein et al. 1986), although higher levels have been reported in humans following heavy smoking (Benowitz 1983; Herning, Jones et al. 1983).

The self-administration model has shown to be a highly reliable paradigm with high face, construct and predictive validity. It has high concordance with drug taking behaviour in humans, since drugs that support self-administration in animals also possess a high abuse potential in humans (Griffiths and Balster 1979). Alterations in the dose of the drug of abuse produce reliable changes in the self-administration behaviour, indicating changes in the reinforcing effects of different doses (Koob 2000). The self-administration paradigm has important advantages, such as the active control of the animal over drug intake, controlling of discrete and environmental cues associated with drug taking and using a within subject design which allows more efficient utilization of animals compared to CPP.

SECTION 3.3. EXTINCTION AND REINSTATEMENT: AN ANIMAL MODEL OF RELAPSE

Relapse to drug taking is considered the most persistent problem in drug addiction and persists well beyond the withdrawal period (Hunt, Barnett et al. 1971; Childress, Mozley et al.)
1999; Grimm, Hope et al. 2001). In order to address the behavioural and neurobiological underpinnings of relapse behaviour, animal models derived from the original reinstatement model of relapse have been developed (de Wit and Stewart 1981; de Wit and Stewart 1983). In the reinstatement model, animals are initially trained to self administer a drug. Upon reaching stable responding, the animals are subjected to extinction procedure where the drug is no longer available. Measuring extinction of responding is important as it reflects the persistence of drug seeking behaviour when the drug is no longer available and provides a measure of the reinforcing effects of a drug (Bozarth 1990). After reaching extinction criteria (designated as a specific criterion for negligible responding e.g. < 20 presses on the lever previously associated with drug delivery), animals are then subjected to stimuli similar to those that lead to relapse in humans. These stimuli include priming injections of the drug itself, drug paired cues and stress (Epstein and Preston 2003; Epstein, Preston et al. 2006). Upon exposure to any of those three conditions, animals increase responding on the lever previously associated with drug delivery, also known as reinstatement (de Wit and Stewart 1983; Erb, Shahan et al. 1996; Shahan and Stewart 1996). Since the abstinence in the reinstatement paradigm is imposed (during extinction conditions) whereas in humans it is often a choice, it is considered to have considerable predictive validity yet, it may be limited in face and construct validity.

SECTION 3.4 REINFORCING EFFECTS OF NICOTINE: EVIDENCE FROM ANIMAL MODELS

Using the above-validated models, several studies have been conducted to improve our understanding of neurobiology and behavioural aspects of nicotine addiction. It is clearly evident that nicotine serves as a positive reinforcer in animals under a variety of conditions. Studies
utilizing the CPP paradigms have shown that nicotine can produce clear place preference (Fudala, Teoh et al. 1985; Fudala and Iwamoto 1986; Le Foll and Goldberg 2005b; Spina, Fenu et al. 2006). However, these findings have not been consistent among all studies (Clarke and Fibiger 1987; Shoaib, Stolerman et al. 1994; Pawlak and Schwarting 2005). Other studies have even shown that nicotine can induce CPA (Laviolette, Alexson et al. 2002; Laviolette and Van Der Kooy 2003). Most of the studies reporting nicotine induced CPP used the biased procedure, whereas the unbiased procedure has yielded negative results. These equivocal findings have directed researchers to suspect that nicotine does not possess rewarding properties and that the effects observed with the biased procedure are mainly due to the anxiolytic effects of nicotine (Torrella, Badanich et al. 2004). Compared to other drugs, e.g. potent psychostimulants, nicotine is considered a weak reinforcer and hence a narrow balance between its rewarding and aversive effects may play a significant role in the expression of CPP.

In fact several studies using the conditioned taste aversion paradigm have demonstrated that nicotine dose dependently suppresses the intake of the tastant previously paired with nicotine administration (Kumar, Pratt et al. 1983; Stolerman 1988; Parker and Gillies 1995; Laviolette, Alexson et al. 2002). Moreover, higher doses of nicotine (1.2- 2mg/kg) can elicit taste aversion (Parker and Gillies 1995). It appears that, with repeated exposure to nicotine, tolerance to the CTA effects of nicotine develops (Iwamoto and Williamson 1984). This tolerance has been observed in humans, and may play an important role in the continuation of smoking behaviour (Stolerman 1999).

Given the evidence stated above, the intravenous self-administration of nicotine is considered the preferred method for assessing and demonstrating the reinforcing effects of nicotine. Compared
to CPP, the intravenous self-administration paradigm possesses higher face and construct validity, and hence is closer mimic to the human drug taking condition. However, combined use of those two paradigms provides stronger evidence for the hypothesis to be tested.
Figure 1: The intravenous self-administration model. Rats are implanted with catheters into the jugular vein that exit between the scapulae. In the present study, acquisition of nicotine self-administration was performed under a fixed ratio schedule of reinforcement at a unit dose of 30 µg/kg/infusion of nicotine base. Session duration was 60 minutes. When an animal completed the schedule requirements, this would result in the infusion of nicotine followed by a time-out period of 60 seconds associated with dimming of the house light and illumination of the cue light above the active lever. During the timeout period, lever presses were recorded but did not count towards obtaining nicotine reinforcement. During the first week of acquisition, response requirements were FR1 (i.e. each active lever press during the time-in period resulted in an infusion of nicotine base). Response requirements were then gradually increased to reach a final value of FR5.
SECTION 4: NICOTINE

SECTION 4.1 PHARMACOLOGICAL AND PSYCHOLOGICAL EFFECTS OF NICOTINE

Administration of nicotine through smoking produces a wide variety of pharmacological and psychological effects, some of which contribute significantly to the addictive properties of nicotine. Nicotine produces an increase in blood pressure, heart rate, levels of epinephrine and cortisol (Stolerman and Jarvis 1995a). Nicotine intake also produces some desirable effects such as mild euphoria, increased concentration and alertness (Etter, Bergman et al. 2000; Pomerleau and Pomerleau 1992; Stolerman and Jarvis 1995a). These effects induce the reinforcing and drug-seeking behaviour associated with nicotine addiction i.e. positive reinforcing effects of nicotine. Several studies have shown that nicotine can maintain stable self-administration in animals and humans, induce conditioned place preference and lower brain reward threshold (thus inducing brain stimulation reward) (Le Foll and Goldberg 2009; Corrigall and Coen 1989; Fudala, Teoh et al. 1985; Goldberg and Henningfield 1988). Negative reinforcing effects are also critical in the motivational effect of nicotine in humans (Hughes, Gust et al. 1991) and animals (Epping-Jordan, Watkins et al. 1998). Acute withdrawal from nicotine has shown to produce negative symptoms such as anxiety, irritability and dysphoria in humans (Hughes, Gust et al. 1991). Similarly, rats and mice have shown somatic symptoms of withdrawal from nicotine whether spontaneous or precipitated (Epping-Jordan, Watkins et al. 1998; Malin, Lake et al. 1992).

Although the positive and negative reinforcing properties of nicotine are necessary for the initiation and maintenance of nicotine seeking behaviour, yet, there are other factors involved in the development of nicotine dependence. These factors include, smoking, conditioning properties and stress (Caggiula, Donny et al. 2002a; Buczek, Le et al. 1999).
Relapse to smoking is another key feature of nicotine dependence. It could be described as the return of nicotine seeking/nicotine taking behaviour after a certain period of abstinence (see (Fuchs, Lasseter et al. 2008). Although a high percentage of smokers express their desire to quit smoking and make attempts to achieve this goal, yet the relapse rates are still high (Fiore 2000). The long term abstinence rates (6 months) remain less than 10% (Hughes, Gulliver et al. 1992) despite the presence of a few pharmacotherapies for smoking cessation. Factors that predispose to the relapse to smoking include stress (Kassel, Stroud et al. 2003), re-exposure to environmental stimuli that were previously associated with smoking, and re-exposure to nicotine. In the current studies, we are primarily interested in studying how CB1, CB2 and endogenous cannabinoid ligands modulate the primary reinforcing, and reinstatement related effects of nicotine induced by re-exposure to nicotine and nicotine-associated cues.

SECTION 4.2 NICOTINE DEPENDENCE AND THE BRAIN REWARD PATHWAYS

Nicotine is believed to produce its psychoactive effects through activation of nicotinic acetylcholine receptors (nAChRs). There are twelve subunits that combine to form heteromeric or homomeric nicotinic receptors. These subunits are $\alpha_2-\alpha_{10}$ and $\beta_2-\beta_4$ (Gotti and Clementi 2004). A single nicotinic acetylcholine receptor is composed of 5 subunits that form an ion channel (Miyazawa, Fujiyoshi et al. 2003). Nicotinic acetylcholine receptors are widely spread in the brain and are not restricted to central cholinergic pathways (Paterson and Nordberg 2000). Chronic exposure to nicotine results in desensitization (Pidoplichko, DeBiasi et al. 1997) and up regulation (Flores, Rogers et al. 1992) of $\alpha 4\beta 2$ subtype of high affinity nicotinic acetylcholine receptors (Whiting, Liu et al. 1987). The $\alpha 4\beta 2$ nAchR type has been shown to play a critical role in
mediating the reinforcing and antinociceptive effects of nicotine. Studies have shown that β2 subunit knockout mice do not self-administer nicotine, and that the administration of nicotine does not result in an increase in dopamine levels in the ventral tegmental area compared to wild type mice (Picciotto, Zoli et al. 1998). Moreover, β2 subunit knockout mice have shown to have decreased signs of somatic withdrawal in mouse models of nicotine withdrawal (Salas, Cook et al. 2004). β2 subunit knockout mice have shown to have reduced antinociceptive effects as demonstrated by the hot plate and tail flick tests compared to the wild type mice (Marubio, del Mar Arroyo-Jimenez et al. 1999).

Nicotine acts on nAChRs located on nerve terminals of dopaminergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc). This results in the release of dopamine in the NAc and ventral striatum, specifically in the left ventral caudate/nucleus accumbens and left ventral putamen (Pidoplichko, DeBiasi et al. 1997). This has been demonstrated in PET imaging studies by the increase in the displacement of raclopride, a dopaminergic antagonist (Brody et al., 2004b).

Studies have shown that selective lesioning of the mesolimbic dopamine system (Corrigall, Franklin et al. 1992) or administration of selective dopamine antagonists of dopaminergic neurotransmission results in attenuation of nicotine self-administration in rats. Systemic administration of mecamylamine (MEC a non specific nAChR antagonist) decreased nicotine self administration in rats (DeNoble and Mele 2006) and nicotine induced elevations of dopamine in the NAc. However, local administration of MEC in the NAc does not block regional dopamine release in this area (Nisell, Nomikos et al. 1994), indicating that dopaminergic neurons in the VTA are vital in mediating dopamine release in the NAc and resulting nicotine self-administration.
Another alternative opposing hypothesis is that dopamine mediates the aversive effects of nicotine. This has been demonstrated using the CPP paradigm and direct injections of nicotine into the ventral tegmental area (Laviolle and van der Kooy 2003). In support of this hypothesis, the same study showed that administration of the dopamine receptor antagonist α-flupenthixol reversed the aversion produced by nicotine into preference (Laviolette and van der Kooy 2003). The authors interpreted their results to propose that blockade of mesolimbic dopamine signalling attenuates the aversive effects of nicotine and thus increase the vulnerability to nicotine’s rewarding and addictive properties. They further argue that these results may have bearing on the excessive smoking associated with antipsychotic treatment of schizophrenics. It should be noted that there is a major difference between these results and intravenous self-administration of nicotine, where blockade of dopaminergic activity attenuates, the reinforcing effects of nicotine (Corrigall and Coen 1991). To propitiate these positions may require using a range of doses of nicotine using the intravenous nicotine self-administration paradigm.

Other neurotransmitters have shown to play pivotal role in nicotine dependence. Studies have shown that nicotine induced dopamine release can be attenuated by atropine (muscarinic receptor antagonist), eticlopride (dopamine D1/2 receptor antagonist) and MK801 (N-Methyl- D-aspartate (NMDA) antagonist) (Sziraki, Sershen et al. 2002). It has been demonstrated that smoking increases plasma levels of endogenous opioids (Pomerleau, Fertig et al. 1983) and nicotine induces the release of b-endorphins in neuronal cell cultures (Boyadjieva and Sarkar 2010). Furthermore, µ-opioid knockout mice have shown to have a decrease in nicotine conditioned place preference (CPP) and nicotine induced antinociception compared to wild type mice (Berrendero, Kieffer et al. 2002) and naloxone blocks nicotine CPP in mice (Walters, Cleck et al. 2005).
The focus of the body of work presented herein is on the role of the cannabinoid CB1, CB2 receptors and the endogenous cannabinoid ligand anandamide in modulating the reinforcing effects of nicotine as well as, reinstatement of nicotine-seeking behaviour. Most of the evidence for the endocannabinoid system’s modulation of nicotine dependence has come from studies of CB1 receptor blockade of nicotine’s effects (see reviews by (Cohen, Kodas et al. 2005a; Le Foll and Goldberg 2005a; Scherma, Fadda et al. 2009).

SECTION 5 NEUROCIRCUITRY INVOLVED IN NICOTINE REWARD

SECTION 5.1. THE MESOLIMBIC DOPAMINERGIC SYSTEM

The dopaminergic system arises from the dopaminergic neurons located in the VTA and the substantia nigra pars compacta and projects to the NAc, amygdala and prefrontal cortex. These structures are in turn interconnected through the glutamatergic pathways. Moreover, these structures are connected to the basal forebrain structures, pedunclopontine nucleus and lateral dorsal tegmentum through cholinergic fibres (Kelly and Iversen 1976). The dopaminergic neurons are involved in innervation of other areas such the bed of the nucleus of the stria terminalis and the lateral septum (Di Chiara 1995). Therefore, the mesolimbic dopamine system appears to be located in the centre of a circuit involving the structures most implicated in reward processing and the mechanisms underlying the regulation of reinforcements.

The dopaminergic system has long been hypothesized to have a critical role in the expression of goal directed behaviour of drugs of abuse, including nicotine as well as other natural rewards (Carboni, Imperato et al. 1989; Corrigall, Franklin et al. 1992; Di Ciano and Everitt 2003). Moreover, the dopaminergic system is clearly implicated in the conditioned reinforcing properties
of drugs of abuse and their associated stimuli (Schultz, Tremblay et al. 1998; Stuber, Klanker et al. 2008). Dopamine has been also involved in the development of behavioural sensitization, which occurs following the repeated administration of drugs of abuse, as well as non drug stimuli (Kalivas and Stewart 1991).

A large body of evidence has demonstrated the role of the dopaminergic system in cue associations. In 1981, Miller and colleagues demonstrated an increase in neuronal firing in the VTA and substantia nigra using a discriminative stimulus and a conditioned stimulus as conditioning tasks associated with the availability of chocolate as a reward (Miller, Sanghera et al. 1981). Similarly, phasic neuronal responses were recorded in response to conditioned stimuli in dopaminergic neurons of monkeys (Schultz, Apicella et al. 1993). According to Stuber and colleagues, conditioned stimuli that predicted the availability of sucrose (in an appetitive Pavlovian task), did not only result in an increase in the firing of dopamine neurons, but also an increase in the synaptic strength of these neurons (Stuber, Klanker et al. 2008). In addition, the dopaminergic neurons are believed to mediate reward related error signals (Ljungberg, Apicella et al. 1992). In other words, there is an increase in firing of dopaminergic neurons following the presentation of an unexpected reinforcer. This increase in firing ceases to occur following the omission of an expected reinforcer (Fiorillo, Newsome et al. 2008).
Figure 2: Sagittal section through a representative rodent brain illustrating the pathways and receptor systems implicated in the acute reinforcing actions of nicotine. Nicotine activates nicotinic acetylcholine receptors in the ventral tegmental area, nucleus accumbens, and amygdala either directly or indirectly via actions on interneurons. Nicotine also may activate opioid peptide release in the nucleus accumbens or amygdala independent of the dopamine system. The blue arrows represent the interactions within the extended amygdala system hypothesized to have a key role in nicotine reinforcement. AC, anterior commissure; AMG, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; IF, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; N Acc., nucleus accumbens; OT, olfactory tract; PAG, periaqueductal gray; RPN, reticular pontine nucleus; SC, superior colliculus; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area. (Adapted with permission from The Neurobiology of Addiction by George Koob) ELSERVIER©
SECTION 5.2 INTRODUCTION TO THE CANNABINOID SYSTEM

SECTION 5.2.1 CANNABINOID 1 RECEPTORS

There are two types of cannabinoid receptors, CB1 and CB2. CB1 receptors are the most abundant G protein coupled receptors in the central nervous system. CB1 receptors are believed to be the main mediators of the psychoactive properties of Δ9THC (the main psychoactive component of cannabis) (Buckley, McCoy et al. 2000). Cannabinoids act at CB1 receptors to induce changes in the synaptic plasticity of central neuronal circuits that are involved in several processes including reward (Freund, Katona et al. 2003).

Autoradiographic studies using radiolabeled [3H]CP 55,940 showed the most dense binding to cannabinoid receptors in the hippocampus, some olfactory regions, caudate, putamen, nucleus accumbens (ventral striatum), the substantia nigra pars reticulata (SNr), globus pallidus, and the horizontal limb of the diagonal band (Herkenham, Lynn et al. 1990; Herkenham, Lynn et al. 1991). The presence of CB1 receptors in the hippocampus in high density is hypothesized to result in the frequently described disruptive effects of cannabinoids on memory and cognition (Freund, Katona et al. 2003). The CB1 receptor expression was more pronounced in regions affecting a number of key functions, including mood, motor coordination, autonomic function, memory, sensation and cognition. Electron microscopy studies demonstrated CB1 receptors predominantly on presynaptic terminals (Katona, Sperlagh et al. 1999; Marsicano and Lutz 1999; Tsou, Mackie et al. 1999), but they were found also on postsynaptic structures and glia (Rodriguez de Fonseca, Navarro et al. 2001).
The presence of CB1 receptors in the basal ganglia and the effects of cannabinoid receptor activation on these structures denote that endogenous cannabinoid ligands may play prominent role in the fine-tuning of motor control. Indeed, several studies have shown disturbances in CB1 receptor expression and binding in neurological disorders of the extrapyramidal system (Glass, Faull et al. 1993; Richfield and Herkenham 1994). Thus, there is a decrease in CB1 receptor binding observed in neurodegenerative diseases such as Parkinson's and Huntington's disease (Sanudo-Pena, Romero et al. 2000).

The CB1 receptors, among other functions, play an important role in the central and peripheral regulation of food intake, fat accumulation, and metabolism of lipids and glucose. CB1 receptor system hyperactivity leads to the alteration of these functions in both CNS and peripheral tissues (adipocytes, skeletal muscle cells, liver, gastrointestinal tract) (Gelfand and Cannon 2006). Activation of CB1 receptors located in the hypothalamus interacts with neuropeptides involved in the regulation of energy homeostasis, food intake and lipogenesis in visceral tissues (Cota, Marsicano et al. 2003). The activity of the central CB1 receptors increases also when levels of leptin released from adipose tissues are elevated (Pagotto and Pasquali 2005). Additionally, CB1 receptors are highly expressed in areas like the periaqueductal gray that are involved in pain modulation (Tsou, Mackie et al. 1999) and the dorsal horn of the spinal cord (Farquhar-Smith, Egertova et al. 2000). Studies involving CB1 knockout mice confirmed the important role of CB1 receptors in mediating the behavioural effects of cannabinoids. When challenged with ∆9THC, CB1 knockout mice showed no analgesia, no increase in locomotor activity and no precipitated cannabinoid withdrawal in animals chronically treated with ∆9THC (Ledent, Valverde et al. 1999).
SECTION 5.2.2 CANNABINOID 2 RECEPTORS

CB2 receptor gene expression transcripts were primarily isolated in the spleen, thymus, tonsils and mast cells (Berdyshev 2000; Munro, Thomas et al. 1993; Sugiura and Waku 2000; Wilson and Nicoll 2002) and were previously referred to as the peripheral CB receptors (Klein, Newton et al. 2003).

In contrast to the predominant presynaptic localization of CB1 receptors in the brain, immunoreactivity suggests a more likely postsynaptic localization of CB2 receptors (Gong, Onaivi et al. 2006; Onaivi, Ishiguro et al. 2006). First, the expression of CB2 receptors centrally was demonstrated in rat microglial cells and other cells in the brain associated with inflammation (Golech, McCarron et al. 2004; Ibrahim, Deng et al. 2003; Nunez, Benito et al. 2004; Benito, Kim et al. 2005).

CB2 receptors were also identified in peripheral structures as the peripheral nerve terminals in mouse (Griffin, Fernando et al. 1997; Lu, Straiker et al. 2000). Activation of CB2 receptors produced immune consequences including modulating cytokine release from immune cells and migration of immune cells throughout the central nervous system (Cabral and Staab 2005). Later, CB2 receptor mRNAs were detected in some regions of the rat brain (cerebellum, cortex, and brainstem) using reverse transcription polymerase chain reaction (RT-PCR) (Van Sickle, Duncan et al. 2005). Moreover, Van Sickle et al. (2005) were able to detect CB2 receptor protein using Western blotting and immunohistochemistry and were able to demonstrate that CB2 receptors have antiemetic activity using intracranial ligand infusion (Van Sickle, Duncan et al. 2005). It should be noted that the levels of expression of central CB2 receptors are much lower than those of CB1 receptors (Gong, Onaivi et al. 2006; Onaivi, Ishiguro et al. 2006).
Furthermore, studies have shown that CB2 receptor activation by 2-arachidonoylglycerol (endogenous cannabinoid ligand), JWH015 or JWH133 (CB2 receptor agonists) produced an inhibitory effect on locomotion (Van Sickle, Duncan et al. 2005; Guindon and Hohmann 2008), and morphine-glucuronide-induced emesis (Van Sickle, Duncan et al. 2005) and attenuated neuropathic pain (Guindon and Hohmann 2008; Jhaveri, Elmes et al. 2008). On the other hand, CB2 receptor activation in turn activates neural progenitor proliferation (Goncalves, Suetterlin et al. 2008) and produces neuroprotective effects (Viscomi, Oddi et al. 2009; Sagredo, Gonzalez et al. 2009). Furthermore, studies have proposed that CB2 receptor activation inhibits neuronal firing in dorsal-root ganglia and spinal cord (Elmes, Jhaveri et al. 2004; Sagar, Kelly et al. 2005).

It has been proposed that the CB2 receptors may be involved in mental disorders, including drug addiction (Ishiguro, Iwasaki et al. 2007; Onaivi, Ishiguro et al. 2008; Ishiguro, Horiuchi et al. 2010). Onaivi and colleagues showed that activation of CB2 receptors increases alcohol intake in C57Bl/6 mice subjected to chronic mild stress, while selective CB2 receptor blockade decreased alcohol intake in the same strain of mice using the same testing conditions (Onaivi, Ishiguro et al. 2008). More recently, Xi, Peng et al. (2011) demonstrated that CB2 receptor activation produced a dose dependent decrease in cocaine self administration, cocaine induced hyperlocomotion and cocaine induced increase in extra cellular nucleus accumbens dopamine in wild type and CB1 receptor knockout mice but not in CB2 receptor knockout mice (Xi, Peng et al. 2011). These findings challenge some views that CB2 receptors are absent from the CNS and that CB receptor ligands lack CNS effects and further suggest that brain CB2 receptors may be a potential target for the pharmacotherapy of drug abuse and addiction. However, to date there is
no clear evidence supporting a modulatory role of CB2 receptors on the reinforcing and motivational effects of nicotine.

CB1 and CB2 receptors are coupled to Gi and Go G-proteins. This interaction leads to the inhibition of adenyl cyclase (Howlett and Fleming 1984; Howlett, Qualy et al. 1986; Koob, Rocio et al. 1998). This leads to a subsequent reduction in cAMP and an increase in mitogen activated protein kinase (Ameri 1999). Cannabinoids also enhance A-type potassium channels leading to an increase in the outward potassium current (Deadwyler, Hampson et al. 1993), inhibit voltage-activated N-type calcium channels (Caulfield and Brown 1992), and inhibit presynaptic P/Q calcium channels (Sullivan and Kendler 1999; Yamamoto and Takada 2000).

In summary, activation of CB2 receptors can in turn lead to inhibition of presynaptic release via multiple molecular pathways including inhibition of adenyl cyclase and thus reduce the amount of cAMP and decrease the protein kinase A-mediated phosphorylation of A-type potassium channels, or direct inhibition of P/Q and N type calcium channels through inhibition of G proteins.

SECTION 5.2.3 ENDOGENOUS CANNABINOID LIGANDS

The discovery of cannabinoid receptors subsequently raised the hypothesis of the potential existence of endogenous ligands for these receptors, and a search of lipid extracts from porcine brain led to the isolation and discovery of five endocannabinoid ligands. These ligands include, AEA [N-arachidonoylethanolamine] (anandamide) (Devane, Hanus et al. 1992), 2-AG (2-arachidonoylglycerol) (Mechoulam, Ben-Shabat et al. 1995; Sugiura, Kondo et al. 1995), 2-arachidonoylglcelyl ether (noladin) (Hanus, Abu-Lafi et al. 2001; Fezza, Bisogno et al. 2001).
O-arachidonylethanolamine (virodhamine) (Porter, Sauer et al. 2002) and NADA (N-arachidonoyldopamine) (Bisogno, Melck et al. 2000; Huang, Bisogno et al. 2002). Among the five endocannabinoid ligands, anandamide and 2-AG are most well studied and the details of their biosynthesis and degradation will be the focus of this section.

Since these endocannabinoids are proposed to function as neuromodulators (Di Marzo, Melck et al. 1998), they require the existence of specific biosynthetic and metabolic mechanisms subject to regulation during physiological and pathological conditions. It has been clearly demonstrated that anandamide and 2-AG produce overlapping functions (Sugiura, Kondo et al. 1999), and that they both bind to the CB receptors (Devane, Hanus et al. 1992; Sugiura, Kondo et al. 1995). 2-AG possesses a high affinity for the CB receptors in synaptosomal membranes, and is much more abundant in the brain than anandamide (Sugiura, Kondo et al. 1995). However, 2-AG has its distinct structure, different biosynthesis and degradation pathways (Figure 3). Moreover, 2-AG appears to be formed under conditions that are different from those required for the synthesis of anandamide and is modulated by different pharmacological manipulations (Freund, Katona et al. 2003; Piomelli 2003; Di Marzo, Bifulco et al. 2004; Fride, Bregman et al. 2005). Interestingly, it has been recently demonstrated that anandamide enhances the metabolism and in turn attenuates the effects of 2-AG levels in the striatum (Maccarrone, Rossi et al. 2008). These findings suggest that anandamide and 2-AG might have different roles according to the different physiological or pathophysiological conditions in which they are synthesized (Piomelli 2003).

Anandamide synthesis is thought to be regulated through the conversion of a minor phosphoglyceride, N-arachidonylphosphatidylethanolamine (N-arachPE), through 2 possible
mechanisms. The first pathway is the phospholipase D (NAPE-PLD) (Okamoto, Tsuboi et al. 2009) and the second is mediated through two enzymes, the alpha, beta hydrolase (ABH4) and the glycerophosphodiesterase (GDE1) (Simon and Cravatt 2008). To date, the exact mechanisms that modulate the activities of these enzymes and their involvement in the synthesis of AEA are not fully elucidated. Cessation of endocannabinoid signalling is hypothesized to be through a two-step mechanism consisting of (1) transport inside the cell and (2) degradation by specific enzymes. It is further hypothesized that anandamide and 2-AG use the same intracellular transport as the first step of degradation (Freund, Katona et al. 2003; Piomelli 2003; Di Marzo, Bifulco et al. 2004; Fride, Bregman et al. 2005). In contrast, it has been clearly shown that their metabolic degradation is distinct (Freund, Katona et al. 2003; Piomelli 2003; Di Marzo, Bifulco et al. 2004; Fride, Bregman et al. 2005).

Anandamide and 2-AG are able to diffuse passively through lipid membranes aided by their lipophilic nature. However, it appears that diffusion is facilitated by a selective carrier system. After anandamide’s uptake inside the cell it is degraded into arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH), which has been cloned and molecularly characterized (Desarnaud, Cadas et al. 1995; Hillard, Wilkison et al. 1995; Cravatt, Giang et al. 1996; Bracey, Hanson et al. 2002). FAAH and CB1 receptors are widely distributed in the CNS and show partial overlap. FAAH is mainly available at the postsynaptic neurons and CB1 receptors at the presynaptic neurons (Tsou, Nogueron et al. 1998; Egertova, Cravatt et al. 2003). The availability of FAAH-deficient mice (Cravatt, Demarest et al. 2001), and selective FAAH inhibitors (Kathuria, Gaetani et al. 2003), have allowed a better understanding of the critical role played by this enzyme in the degradation of anandamide (Cravatt, Demarest et al. 2001;
FAAH is the key enzyme involved in the degradation of anandamide however there are two other enzymes involved in the degradation of anandamide (Cravatt, Giang et al. 1996), a lysosome-localized fatty acyl amide hydrolase (Tsuboi, Sun et al. 2005) and a recently identified FAAH-2 localized in lipid droplets (Kaczocha, Glaser et al. 2009).

On the other hand, 2-AG is synthesized in response to cellular activation from arachidonic acid containing membrane phospholipids. The most important pathway for 2AG synthesis is the phosphatidylinositol (PI)-phospholipase C (PLC)/DAG lipase [diglyceride lipase (DAGL)] pathway. This pathway consists of hydrolysis of inositol phospholipids containing arachidonic acid by PLC. The second pathway for producing 2-AG is through the sequential hydrolysis of PI via lyso PI, which is catalyzed by PLA1 and lyso PI-specific PLC (Ueda, Kobayashi et al. 1993).

Although a large number of enzymes are involved in the hydrolysis of monoacyl glycerols, evidence has shown that MAG lipase [monoglyceride lipase (MAGL)] is considered to play the most important role in the 2-AG degradation in brain (Blankman, Simon et al. 2007). The remaining 2-AG is hydrolysed by ABHD6 and ABHD12 (Blankman, Simon et al. 2007), enzymes about which very limited information is known.

It is strongly hypothesized that for endocannabinoids to become substrates for intracellular enzymes that facilitate their degradation, they need to be taken up by the cells. It has been suggested that there is a common mechanism responsible for the facilitation of this uptake for all endocannabinoids (see Hillard and Jarrahian 2000 for a review) according to the concentration gradient across the cell membrane. This suggestion, is based on several observations: (i) AEA, 2-
AG, noladin and NADA are rapidly taken up by both neuronal and non-neuronal cells in a saturable and temperature-dependent and energy-independent manner (Fezza, Bisogno et al. 2002; Huang, Bisogno et al. 2002; Di Marzo, Fontana et al. 1994; Hillard, Edgemond et al. 1997; Beltramo and Piomelli 2000; Bisogno, MacCarrone et al. 2001), (ii) 2-AG, noladin, NADA and virodhamine compete with AEA cellular uptake, and AEA competes with 2-AG, noladin and NADA uptake (Fezza, Bisogno et al. 2002; Bisogno, MacCarrone et al. 2001; Huang, Bisogno et al. 2002; Beltramo and Piomelli 2000) and (iii) AEA and 2-AG uptakes are subject to selective inhibition by some AEA analogues and to stimulation by nitric oxide (Bisogno, MacCarrone et al. 2001; Beltramo, Stella et al. 1997; Maccarrone, van der Stelt et al. 1998). We will discuss the several lines of evidence that are in support of the existence of the transport system for anandamide in the discussion (see page 154).

The endocannabinoids are synthesized and released from neurons on demand, bind to cannabinoid receptors, activate neuronal transduction mechanisms, and possibly possess a reuptake system (Yamamoto and Takada 2000). Endocannabinoids are synthesized when required in response to depolarization by receptor-stimulated cleavage of membrane lipid precursors and released from cells immediately after their production, playing a role of neuromodulators rather than neurotransmitters (Piomelli, Giuffrida et al. 2000). Once released, endocannabinoids act on cannabinoid receptors or are taken back into cells via an energy-independent transport system (Beltramo, Stella et al. 1997). Once inside the postsynaptic neurons, their degradation enzymes can hydrolyze both anandamide and 2-AG. Such a non-synaptic release mechanism and rapid breakdown of both anandamide and 2-AG suggest that
these compounds may play an important regulatory effect on primary neurotransmitters such as dopamine (Piomelli, Giuffrida et al. 2000; Elphick and Egertova 2001).

Endocannabinoids function as non-conventional neuromodulators whose functions include retrograde signalling through mediating several types of synaptic plasticity (Kano, Ohno-Shosaku et al. 2009). Once released, the endocannabinoids activate cannabinoid receptors and subsequently inhibit neurotransmitter release modulating both short and long-term forms of synaptic plasticity.

Dopamine neurons utilize endocannabinoids as retrograde neurotransmitters (Melis, Pistis et al. 2004). This property allows endocannabinoids to have the ability to attenuate the activity of external afferents (presynaptic neurons) and allow dopamine neurons (postsynaptic neurons) to regulate their own function in order to respond to specific stimuli in an optimum manner.

The effect of endocannabinoids in modulating synaptic plasticity has been widely studied in excitatory afferents arising from rostral/cortical regions. This effect has been demonstrated in VTA dopamine neurons which release endocannabinoids to decrease further release of glutamate from presynaptic afferents (Melis, Pistis et al. 2004b; Marinelli, Rudick et al. 2006 b; Kortleven, Fasano et al. 2011).

Studies have shown that endocannabinoids act as key signalling molecules that mediate depolarization induced suppression of excitation (Melis, Pillolla et al. 2006), a form of short-term plasticity that serves as a protective mechanism to limit pathological excitation of VTA dopamine neurons.

Hence, it is hypothesized that endocannabinoids act locally at the VTA as a tool for dopamine neurons to switch their firing pattern and activity in response to stimuli (Melis 2004a).
Moreover, it has been shown that a brief burst of excitatory synaptic activity on to dopaminergic neurons leads to the stimulation of mGluR1 and increases intracellular Ca²⁺ levels, subsequently leading to anandamide and 2-AG release. Once released, these endocannabinoids selectively and temporarily dampen excitatory inputs and dopaminergic neuron spike and burst activity (Pillolla, Melis et al. 2007).

Despite the large body of research in this area, the translation of endocannabinoid modulation of dopaminergic neuronal firing into behaviour is yet to be established. The endocannabinoid system has recently gained significant attention in being involved in different aspects of compulsive seeking. Although pharmacological manipulation of the endocannabinoid system has been shown to modulate drug seeking behaviour, yet the direct involvement of the dopamine system in the observed behaviours is less clear. For example, blocking the CB1 receptor selectively through the compound rimonabant attenuates nicotine, cocaine and alcohol induced rises in dopamine release in the ventral striatum (Cheer, Wassum et al. 2007). Moreover, using micro dialysis techniques (Cohen, Perrault et al. 2002) have shown that rimonabant reduces nicotine induced dopamine release in the shell of the nucleus accumbens.

Despite the evidence that drugs of abuse increase dopamine release, electrophysiological data failed to demonstrate the effects of CB1 receptor blockade on drug induced increases in dopaminergic firing (Melis, Gessa et al. 2000; Melis, Pillolla et al. 2008), with alcohol as the only exception (Perra, Pillolla et al., 2005). Hence, it could be inferred that endocannabinoids are able to modify dopamine release at the level of the synaptic cleft with no apparent modification of firing rate of dopaminergic neurons. It is possible that endcannabinoids are mainly involved in modulating the long term rather than short term forms of synaptic plasticity, that is observed.
after chronic drug administration i.e. modulating long term depression (LTD) of GABA neurons (Pan, Hillard et al. 2008 a,b), LTD of dopaminergic neurons (Haj-Dahmane and Shen 2010) or their long term potentiation (LTP)( Kortleven, Fasano et al. 2011). At the present, it is not yet clear how endocannabinoids are able to produce two functionally opposing effects and more importantly at which stage of the addiction process they are mostly involved.

Progress in the understanding of the neuropharmacology of endocannabinoids has been facilitated by the identification of drugs that can block the synthesis, reuptake, and inactivation of both anandamide and 2-AG. Anandamide reuptake is blocked by the anandamide transport inhibitor N-(4-hydroxyphenyl)-arachidonamide (AM 404) and, (5Z, 8Z, 11Z, 14Z)-N-(4-hydroxy-2-methylphenyl)-5, 8, 11, 14-eicosatetraenamide (VDM11) and, as such, the effects of exogenously mediated anandamide are potentiated (Beltramo, Stella et al. 1997). AM 404 has been shown to elevate levels of circulating anandamide and to decrease locomotor activity (Gonzalez, Romero et al. 1999; Piomelli, Giuffrida et al. 2000). There also are inhibitors of fatty acid amide hydrolase such as cyclohexyl carbamic acid 3’-carbamoyl-3-yl ester (URB597) which can increase the levels of anandamide by blocking its breakdown.

Pharmacological blockade of FAAH activity has shown to prolong many behavioural and neurobiological effects of anandamide. URB 597 (FAAH inhibitor) administration reversed the abuse-related behavioural and neurochemical effects of nicotine in rats (Scherma, Medalie et al. 2008a; Forget, Coen et al. 2009a). While demonstrating no rewarding effects per se, URB597 prevented nicotine-induced conditioned place preference as well as inhibited nicotine-taking and nicotine–seeking behaviour induced by nicotine priming (Scherma, Medalie et al. 2008a) and nicotine associated cues (Forget, Coen et al. 2009a). The surprising similarity between
rimonabant and URB597 may be explained by the ability of both compounds to reduce nicotine-induced elevations of dopamine levels in the nucleus accumbens (Cohen, Perrault et al. 2002; Scherma, Medalie et al. 2008a).

Although the initial point of interest in FAAH inhibition research was the modulation of anandamide concentration and effect, it is worth mentioning that FAAH inhibition has shown to increase levels and to prolong effects of non-cannabinoid acylethanolamides, namely oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), which are endogenous ligands for the peroxisome proliferator-activated nuclear receptors (PPAR-α) (Fegley, Gaetani et al. 2005; Bond, P. Leff et al. 1995; Astarita, Di Giacomo et al. 2006a). Interestingly, PPAR-α agonists dose-dependently decrease nicotine self-administration and nicotine-induced reinstatement in rats as well as decrease nicotine-induced elevations of dopamine levels in the nucleus accumbens shell (Mascia, Pistis et al. 2011).

Apparently, there is a growing need to delineate effects of anandamide versus OEA/PEA on nicotine-taking and nicotine-seeking behaviour, and a promising approach was to inhibit the transport system that re-uptakes anandamide into cells for further FAAH-mediated hydrolysis (Freund, Katona et al. 2003; Piomelli 2003; Di Marzo, Bifulco et al. 2004; Glaser, Kaczocha et al. 2005; Moore, Nomikos et al. 2005). For this purpose, in the body of the work presented, we used the selective anandamide transport inhibitor (5Z, 8Z, 11Z, 14Z)-N-(4-hydroxy-2-methylphenyl)-5, 8, 11, 14-eicosatetraenamide (VDM11). In contrast to the inverse agonists rimonabant and AM251, anandamide reuptake inhibitors have shown to be of potential therapeutic benefit in the treatment of pain, motor impairments and anxiety disorders (Bortolato, Campolongo et al. 2006; Fernandez-Espejo, Caraballo et al. 2004; La Rana, Russo et al. 2006).
Hence, these compounds could be a promising pharmacological therapeutic tool for treatment of drug dependence.

VDM11 is an analogue to the better known compound AM404. AM404 is the most studied synthetic inhibitor of AEA transport that has been shown to selectively increase brain levels of AEA in vivo, without significantly affecting brain levels of PEA or OEA (Giuffrida and Piomelli 2000; Fegley, Kathuria et al. 2004; Bortolato, Campolongo et al. 2006). Moreover, the recent studies with the conditioned place preference (CPP) model demonstrated that AM404 prevented development of nicotine-induced CPP and impeded reinstatement of extinguished CPP (Scherma, Justinova et al. 2011). It should be noted that the reason we preferred to use VDM11 is that it is more selective and does not bind to TRPV1 receptors as reported with AM404 (De Petrocellis, Bisogno et al. 2000)

It should be noted that besides binding to cannabinoid receptors, endocannabinoids also interact with other GPCR (Mackie 2008) like the vanilloid receptor-type1 (TRPV-1) , K+ channels, 5-HT3 receptors and alpha7nicotinic receptors (Szallasi and Di Marzo 2000; Oz 2006). Whether these interactions are a result of the physiologic effects of cannabinoids, or just a consequence of their lipophilic character, is yet to be clarified (Mackie 2008)
Figure 3: The endocannabinoid system in the brain. The metabolic pathways of the two major endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG) are shown, with their most likely localization in presynaptic and postsynaptic neurons. Anandamide biosynthesis occurs from a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE), which is synthesized from phosphatidylethanolamine (PE) and another phospholipid by an N-acyltransferase (NAT). NAPE is then hydrolyzed to anandamide by a specific phospholipase D (NAPE-PLD) (Di Marzo, Fontana et al. 1994; Okamoto, Morishita et al. 2004). These enzymes are localized in intracellular membranes, although it is not known whether they are presynaptic or postsynaptic. The biosynthesis of 2-AG occurs through the formation from phospholipids of a diacylglycerol (DAG) precursor, which is catalyzed by a phospholipase C (PLC), followed by the hydrolysis of DAG by DAGLs (Bisogno, Howell et al. 2003). Similar to PLC, DAGLs are in the plasma membrane (postsynaptic in the adult brain and presynaptic in the developing brain) (Bisogno, Howell et al. 2003). Degradation of anandamide by fatty acid amide hydrolase (FAAH) occurs postsynaptically at intracellular membranes (Cravatt, Giang et al. 1996; Piomelli 2003; Di Marzo, Bifulco et al. 2004), whereas degradation of 2-AG by monoacylglycerol lipases (MAGLs) (Dinh, Carpenter et al. 2002) occurs presynaptically in the cytosol and at intracellular membranes. Endocannabinoids diffuse through the plasma membrane depending on their intracellular–extracellular concentration gradient by an endocannabinoid membrane transporter or binding protein (EMT) that is still to be characterized (Fowler, Tiger et al. 2004). The endocannabinoid system is a regulatory apparatus that is present in the brain and most of the tissues and organs that have been studied. It is activated on demand to re-establish transient perturbations of the homeostasis of other mediators (such as neurotransmitters, hormones and cytokines) Adapted with permission from (Di Marzo 2006)
SECTION 5.3 CANNABINOID SYSTEM AND STRESS

There are two main physiological mechanisms involved in the organism’s response to stress, an autonomic response and a neuroendocrine response. The autonomic response involves activation of motor and hormonal sympathetic outputs that are mediated via neuronal circuits originating in the hypothalamic preautonomic control centres. On the other hand, the neuroendocrine response to stress is mediated via activation of the hypothalamic pituitary adrenal axis (HPA). Activation of HPA, results in activation of multiple target systems as a result of an increase in the levels of circulating corticosteroids (Pecoraro, Ginsberg et al. 2006). As a response to stress, the HPA activates neuronal circuits that control the hypothalamic paraventricular nucleus (PVN) which in turn increases the levels of corticotrophin releasing hormone (CRH) and vasopressin release from the axon terminals in the basal hypothalamus into the pituitary portal circulation. CRH and vasopressin stimulate adrenocorticotropic hormone (ACTH) producing cells in the anterior pituitary, release ACTH into the systemic circulation, and subsequently stimulate the synthesis and release of corticosteroids into the blood stream. Systemic corticosteroids then elicit both rapid and protracted actions in target tissues throughout the organism, including in the brain (Pecoraro, Ginsberg et al. 2006; Tasker and Herman 2012).

Endogenous cannabinoid ligands have shown to play a significant role in the modulation of HPA’s response to stress. Evidence has shown that attenuation of anandamide signal in the BLA is involved in the activation of the HPA axis in response to stress. Furthermore, it has been demonstrated that exposure to stressful events specifically reduces the levels of anandamide in the amygdala (Patel, Cravatt et al. 2005b; Rademacher, Meier et al. 2008; Hill, Miller et al. 2009a). The mechanism by which anandamide levels are reduced is hypothesized to be through rapid
induction of FAAH mediated anandamide hydrolysis (Hill, Miller et al. 2009a). The degree of reduction of levels of anandamide is in negative correlation with the degree of HPA stimulation and subsequent increases in the level of cortisone (Hill, Miller et al. 2009a).

Furthermore, it has been demonstrated that increasing the levels of anandamide locally through the administration of a FAAH inhibitor in the BLA results in attenuation of stress (Hill, Miller et al. 2009a). These data are in agreement with the hypothesis that anandamide signalling in the BLA acts as a gatekeeper and is modified via exposure to stress in order to facilitate the neuroendocrine response to stress. Moreover, it has been shown that local administration of a CB1 receptor agonist directly into the BLA similarly attenuates stress-induced activation of the HPA axis (Ganon-Elazar and Akirav 2009; Hill, Miller et al. 2009a). These findings support the hypothesis that CB1 receptor activity in the BLA counteracts the HPA axis activity.

Under normal conditions, anandamide produces a tonic control of BLA signalling that controls incoming excitatory neurotransmission. This has been demonstrated in several studies, which have shown that disruption of this AEA tone through the blockade of CB1 receptor signalling, or through a reduction in AEA content, leads to an increase in the levels of glucocorticoids in the circulation through activation of neuronal circuits in the BLA and subsequent activation of HPA axis. Furthermore, it has been demonstrated that there is greater ACTH and corticosteroid response following exposure to acute stress in CB1 receptor knockout mice. This suggests that absence of CB1 receptors increases the magnitude and duration of the HPA axis response to acute stress as a result of the reduction in the fast feedback inhibition observed in the presence of CB1 receptors (Hill, Hillard et al. 2011a; Barna, Zelena et al. 2004; Uriguen, Perez-Rial et al. 2004; Aso, Ozaita et al. 2008; Steiner and Wotjak 2008). Together, these
data provide compelling evidence that endocannabinoids are responsible for mediating a fast negative feedback inhibition of the HPA axis by glucocorticoids inside the PVN. Glucocorticoids are hypothesized to induce endocannabinoid mobilization to suppress excitatory input to CRH neurons. Additionally, evidence has shown that endocannabinoids are involved in glucocorticoid-feedback inhibition of the HPA axis outside the PVN for example in the amygdala. Exposure to acute stress reduces levels of AEA in the basolateral nucleus of the amygdala (Patel, Cravatt et al. 2005b; Rademacher, Meier et al. 2008; Hill, Miller et al. 2009a). On the other hand, in the absence of stress, administration of glucocorticoids causes a rapid increase in AEA content within the amygdala (Hill, McLaughlin et al. 2010a). One interpretation of these data is that stress, possibly through a CRH or norepinephrine pathway, rapidly reduces AEA within the BLA to facilitate activation of the HPA axis. Once the levels of glucocorticoids begin to rise, they act to normalize the reduction of anandamide levels, induced by stress exposure. However, this process remains to be determined experimentally.

A recent study using BLA slices from stressed animals has shown that when corticosterone is added in vitro, it leads to suppression of afferent glutamatergic input through an endocannabinoid mechanism (Karst, Berger et al. 2011). Interestingly, a genetic study has also found that individuals with FAAH gene that carries the C385A polymorphism resulting in rapid degradation of FAAH (Sipe, Chiang et al. 2002; Sipe, Scott et al. 2010) exhibit an attenuation of amygdala activation in response to threatening stimuli (Hariri, Gorka et al. 2009). Preclinical studies have demonstrated similar findings to human data. For example, Hill and colleagues have shown that local inhibition of FAAH within the amygdala can attenuate the stress response (Hill, Miller et al. 2009a). Recently is has been demonstrated in humans that administration of a CB1 receptor

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antagonist may increase cortisol levels when delivered at high doses (Goodwin, Baumann et al. In press), suggesting that endocannabinoid signalling may constrain basal activation of the HPA axis in humans, similar to what has been found in rodents. Taken together, these data provide some initial evidence that endocannabinoid signalling may operate to regulate the HPA axis in humans as it does in rodents (for further review, see the article by (Hillard, Weinlander et al. In press).

SECTION 5.4 CANNABINOID SYSTEM AND MOOD

For centuries, cannabis sativa has been used for its mood elevating and euphoric effects (Williamson and Evans 2000). Frequent users of cannabis have shown to exhibit less depressed mood and more positive affect than non users of cannabis (Denson and Earleywine 2006). It has been reported in some case studies that cannabis use produces antidepressant effects in some clinically depressed individuals (Gruber, Pope et al. 1996). Given that the CB1 receptors are the main mediators of the psychoactive effects of cannabis (Huestis, Gorelick et al. 2001), it is hypothesized that activation of CB1 receptors could thereby produce antidepressant effects. The cannabinoid system including the cannabinoid receptors and enzymes involved in the synthesis and degradation of endocannabinoid ligands are abundant throughout the brain areas and circuits involved in mediating depression. These areas include, the prefrontal cortex, hippocampus, amygdala, hypothalamus and forebrain monoaminergic circuits (Herkenham, Lynn et al. 1991). Furthermore, deletion of CB1 receptors in CB1 knockout mice has shown to produce a phenotype that is very similar to the symptomatic profile of depression (Hill and Gorzalka 2005). Moreover, these transgenic mice exhibit significantly higher levels of anxiety and depressive-like behaviours, motivated behaviour impairments, reduced reward salience, disruption of neurovegetative functions.
functioning and cognitive deficits in tasks requiring high functioning (Hill and Gorzalka 2005). Furthermore, CB1 receptor knockout mice exhibit increased vulnerability to the anhedonic effects of chronic stress (Martin, Ledent et al. 2002) and an accentuated neuroendocrine response to stress (Aso, Ozaita et al. 2008; Steiner, Marsicano et al. 2008). Together, these data indicate that the wide distribution of the endocannabinoid system throughout the brain circuitry involved in emotional processing, could explain the hypothesis that deficits in endocannabinoid signalling could subsequently lead to a depression like profile. This has been consistently demonstrated in rodent and human studies. Studies have shown that chronic mild stress results in widespread reductions in AEA levels all over the brain; along with reductions in CB1 receptor binding site density and/or signal transduction in the hippocampus, hypothalamus and striatum (Hill, Patel et al. 2005; Hill, Carrier et al. 2008; Rossi, De Chiara et al. 2008). These reductions in endocannabinoid signalling, in turn, have been found to modulate reward salience and cognitive processing (Bortolato, Mangieri et al. 2007; Hill, Patel et al. 2005; Rademacher and Hillard 2007). This indicates that down regulation of limbic endocannabinoid signalling as a result of stress produces functional manifestations that are similar to behavioural characteristics of human depression. In support of the above findings, human studies have also demonstrated a crucial role of endocannabinoid signalling in regulating mood and emotions. This has been demonstrated by the significantly high incidences of depression and anxiety in patients that were taking the CB1 receptor inverse agonist rimonabant for the treatment of obesity (Nissen, Nicholls et al. 2008). This effect was of a serious enough concern that it resulted in the discontinuation of rimonabant both within North America and Europe (Hill, Miller et al. 2009a). This finding also suggests that mood and anxiety are regulated by tonic endocannabinoid signalling in a subset of the human population (Hill, Miller et al. 2009a).
In addition to the above evidence, it has been shown that there was a reduction in the levels of circulating endocannabinoids in two independent populations diagnosed with major depression (Hill, Miller et al. 2008; Hill, Miller et al. 2009a). Moreover, recent studies have demonstrated that individuals with certain CB1 receptor haplotypes show a higher vulnerability to depression as a result of adverse life events (Juhasz, Chase et al. 2009) and increase the risk of resistance to antidepressants via modulating subcortical responsiveness to social reward stimuli (Domschke, Dannlowski et al. 2008). Collectively, these studies support the hypothesis that disruption of endocannabinoid signalling in humans could lead to the development of disease and that a subset of depressed population exhibits impairments in this system.

Monoaminergic neurotransmission has also been shown to be modulated via endogenous cannabinoids. Activation of CB1 receptors, either directly through agonists or indirectly through inhibitors of AEA hydrolysis, enhances the firing activity of the dorsal raphe neurons which contain the largest cohort of 5-HT neurons (Gobbi, Bambico et al. 2005; Palazzo, de Novellis et al. 2006; Bambico, Katz et al. 2007). Although CB1 receptors are expressed along the dorsal raphe (Haring, Marsicano et al. 2007), it has been demonstrated through trans-sectional and microinjection studies that stimulation of CB1 receptors within the prefrontal cortex enhances the neuronal activity of dorsal raphe neurons through a multi-synaptic circuit connecting these brain structures (Bambico, Katz et al. 2007). This notion is supported from studies demonstrating that activation of prefrontal cortex CB1 receptors produces antidepressant-like behavioural responses that require intact serotonergic signalling (Bambico, Katz et al. 2007). Furthermore, CB1 receptors are essential for serotonergic mediated negative feedback, and genetic deletion of the CB1 receptor blocks the rise in synaptic serotonin following administration of an SSRI (Aso, Renoir et al. 2009).
Additionally, cannabinoid receptor activation has also been found to increase firing activity of neurons in the locus coeruleus, which has the highest content of noradrenergic neurons, and consequently NE efflux (Gobbi, Bambico et al. 2005; Muntoni, Pillolla et al. 2006) in the forebrain. Studies using synaptosomal preparations have found that cannabinoids inhibit the reuptake of serotonin, dopamine and norepinephrine (Banerjee, Snyder et al. 1975; Steffens and Feuerstein 2004). This indicates that cannabinoids share some essential pharmacological properties of conventional antidepressants.

SECTION 5.5 CANNABINOID SYSTEM AND COGNITION

There is growing interest in the role of endocannabinoids in modulating cognitive processes which has been stimulated by evidence that central CB1 receptors and endocannabinoids (anandamide and 2-AG) occur in high concentration and are highly expressed in the hippocampus (Herkenham, Lynn et al. 1991; Di Marzo, Breivogel et al. 2000), which plays a critical role in learning and memory. Effects of cannabinoid manipulations on various stages of learning memory including acquisition, consolidation and retrieval have been studied extensively using animal models (Riedel and Davies 2005; Varvel, Wise et al. 2009). In summary, the exogenous and endogenous cannabinoid agonists impair working memory and long-term memory acquisition. In contrast, cannabinoid antagonist and inverse agonists and genetic deletion of cannabinoid receptors improve learning and memory. Endocannabinoid signalling has a wide range of behavioural and physiological effects. Hence, it is important to use the appropriate models in order to demonstrate that the effect observed after alteration of endocannabinoid signalling is specifically attributed to memory per se and not due to non specific modulation of motivation, emotions or locomotion.
Most of the evidence indicating that cannabinoid receptor activation can cause learning impairment comes from studies using water maze, which focuses on spatial memory. In mice, systemic administration of Δ9-THC (8 mg/kg, IP) before the training session impairs acquisition in the water maze test without affecting locomotion; an effect that is prevented by the CB1 inverse agonist rimonabant (DaSilva and Takahashi 2002). Similar findings have also been reported in rats after being treated repeatedly with Δ9-THC (8mg/kg, IP) or acutely with Δ8-THC (Diana, Malloni et al. 2003) or with HU-210, a synthetic CB agonist (Ferrari, Ottani et al. 1999), but not with nabilone (Diana, Malloni et al. 2003). However, in these experiments, the effects of CB1-receptor blockade were not tested. Similar to HU 210, another synthetic cannabinoid agonist, WIN 55, 212-2 (1and 3mg/kg), has been found to impair the acquisition of animals in the water maze, however, this effect was prevented by CB1 antagonists, suggesting there might be more than one mechanism by which WIN55212-2 may impair learning (Robinson, Goonawardena et al. 2010). The water maze paradigm has been used to study the effects of Δ9-THC on memory retrieval. It has been reported by two laboratories that, once established, memory retrieval is not affected by cannabinoid receptor activation (Mishima, Egashira et al. 2001; Varvel, Hamm et al. 2001; Varvel, Wise et al. 2007).

It has been recently proposed that the endocannabinoid system is involved in extinction learning, which is extinguishing of a learned response when the conditions that induced the learning cease to exist. For example, after rats are initially exposed to shock in contextual fear conditioning, there will be a gradual fade in the conditioned freezing response if the animal is exposed to the context repeatedly without receiving the electric shock. This extinction of the learned response might be described as forgetting, or as the acquisition of new learning experience
appropriate to the current situation. Marsicano, Wotjak et al. 2002 were the first to report impaired extinction learning in CB1-knock out mice and in wild type mice given rimonabant (3mg/kg) using fear conditioning with a discrete cue. Interestingly, these findings have been linked to a decrease in the long term depression of neurons in the amygdala which is known to play a critical role in extinction learning (Quirk and Mueller 2008). Moreover, there was an increase in the levels of anandamide in the basolateral amygdala of wild type mice after presentation of the shock-associated tone during extinction, which suggests that endocannabinoid neurotransmission is involved in extinction learning (Marsicano, Wotjak et al. 2002).

Several laboratories using rat and mice models have shown that, the effects observed with genetic and pharmacological blockade of CB1 receptors can impair extinction learning, but not acquisition of long-term and short-term fear-related memory, (Suzuki, Josselyn et al. 2004; Chhatwal, Davis et al. 2005; Kamprath, Marsicano et al. 2006; Niyuhire, Varvel et al. 2007; Pamplona, Bitencourt et al. 2008). Supporting the notion that CB1 receptors are involved in modulating extinction learning and fear conditioning, activation of CB1 receptors has been shown to facilitate fear conditioning and to produce effects opposite to those of CB1 antagonists. Enhancing the cannabinoid tone through administration of the anandamide uptake inhibitor AM404 (IP:10mg/kg;1.0μg/μL, I.C.V.) during extinction training accentuated the extinction of startle elicited by a shock-associated context (Chhatwal, Davis et al. 2005; Bitencourt, Pamplona et al. 2008; Pamplona, Bitencourt et al. 2008). This effect has been blocked by the CB1 inverse agonist rimonabant and hence is proposed to be CB1 receptor dependent (Bitencourt, Pamplona et al. 2008). On the other hand, under the same conditions, low doses (0.25mg/kg, IP) but not a high dose (5mg/kg) of the CB agonist WIN 55, 212-2 produced an impairment in contextual fear
conditioning where rimonabant enhanced it (Chhatwal, Davis et al. 2005; Pamplona and Takahashi 2006). Moreover, Ganon-Elazar and Akirav have shown that intraccumbal injection of a low dose of WIN 55, 212-2 that has no effect by itself can reverse the impairment produced by a stressor on extinction of passive avoidance (Ganon-Elazar and Akirav 2009). Using the water maze, Varvel and colleagues was able to confirm the effects of cannabinoids on extinction learning. This group demonstrated that treatment with rimonabant (3mg/kg) or genetic CB1 deletion impaired extinction learning, however, administration of THC did not affect extinction (Varvel, Anum et al. 2005b; Varvel, Wise et al. 2007).

It has been suggested that the effects observed with of CB1 antagonism in extinction procedures may be due to its effects on perseverance. Animals treated with rimonabant or CB1 knock out mice show impairment in learning when the platform is relocated to a new place in the watermaze test (Varvel and Lichtman 2002; Pamplona and Takahashi 2006). However this view is not supported by Hill, Froese et al. (2006b) in which certain doses of CB1 agonists facilitated and antagonists impaired flexibility between different strategies. The effects of FAAH inhibition on extinction fear have been studied using FAAH knockout mice and mice treated with the FAAH inhibitor OL135. These mice show enhanced extinction learning in the watermaze test (Varvel, Wise et al. 2007). Thus, FAAH inhibitors are unique in their facilitative effects on both acquisition and extinction processes. However, until the present it is not clear whether the effects observed with FAAH inhibition are mediated through anandamide or PEA and OEA. Indeed, it has been shown that administration of 30 mg/kg of OEA can facilitate extinction of passive avoidance in rats (Murillo-Rodriguez, Giordano et al. 2001).
It has been shown that memories are destabilized by retrieval and require destabilization in order to persist (Lewis 1979; Nader 2003). Memory reconsolidation is thought to be pivotal in pairing drug exposure to conditioned stimuli (Stewart, de Wit et al. 1984; O'Brien, Childress et al. 1992; Everitt, Parkinson et al. 1999). This pairing is key in the process of relapse to drug seeking and, in turn, drugs that disrupt drug associated memory could be beneficial in preventing relapse to drug seeking (Lee, Di Ciano et al. 2005; Lee, Milton et al. 2006). In conclusion, there is mounting evidence that activation of the endocannabinoid system plays a facilitative role in extinction learning related to aversive conditioning. These roles, along with its role in modulating conditioned stimuli, suggest that drugs that enhance endogenous cannabinoid signalling might be useful for treating drug addiction.

SECTION 5.6 ACUTE CANNABINOID NICOTINE INTERACTIONS

A wealth of studies has shown that there is a significant interaction between Δ9THC and nicotine on locomotion, heart rate, body temperature, anxiety and nociception (Pryor, Larsen et al. 1978; Valjent, Mitchell et al. 2002). It has been shown that acute administration of nicotine enhanced Δ9THC induced hypothermia, bradycardia, hypolocomotion and impaired rotarod performance (Pryor, Larsen et al. 1978). This interaction has been also demonstrated biochemically. Co-administration of both nicotine and Δ9THC potentiated the enhancement of c-FOS immunoreactivity in specific brain regions such as the shell of the nucleus accumbens, basolateral and central amygdala, bed of the nucleus stria terminalis, cingular and pyriform cortex and paraventricular nucleus of the hypothalamus (Valjent, Mitchell et al. 2002). Most of these
areas are highly innervated by the dopaminergic neurons, suggesting that the interaction between Δ9THC and nicotine could possibly be via activation of the mesolimbic dopaminergic circuitry.

The possible involvement of the cannabinoid system in the reinforcing effects of nicotine has been assessed using the conditioned place preference (CPP) and self-administration paradigm. Using the CPP paradigm, it has been demonstrated that co-administration of sub-threshold doses of Δ9THC and nicotine induced rewarding effects despite being not reinforcing when administered per se (Valjent, Mitchell et al. 2002). This finding indicates that low doses of cannabinoids co-administered with nicotine could exhibit a higher capacity to induce behavioural responses related to addictive processes than when administered alone.

Clinical and animal studies have shown that chronic administration of nicotine produced physical dependence revealed by the presence of withdrawal symptoms when nicotine intake is discontinued or mecamylamine (NACHR antagonist) is administered (Malin 2001; Damaj, Kao et al. 2003). The most characteristic somatic signs of nicotine withdrawal in rodents are tremors, wet dog shakes, teeth chatters, ptosis, abdominal constriction and scratching (Isola, Vogelsberg et al. 1999). Mice co-treated with nicotine and Δ9THC exhibited an enhancement in expression of the withdrawal syndrome induced by administration of a cannabinoid antagonist (Valjent, Mitchell et al. 2002). However administration of CB1 antagonist rimonabant failed to induce withdrawal syndrome in nicotine dependant animals (Balerio, Aso et al. 2004) suggesting that the cannabinoid system is not involved in the expression of nicotine physical dependence. These findings were further supported by studies showing no modification of the number of nicotine receptors following chronic exposure to nicotine (Balerio, Aso et al. 2004; Gonzales, Bjornson et al. 2002).
However, administration of cannabinoid agonists seems to attenuate the magnitude of severity of nicotine withdrawal (Balerio, Aso et al. 2004).

**SECTION 5.7 CANNABINOID SYSTEM AND NICOTINE DEPENDENCE**

Most of the evidence for the endocannabinoid system’s modulation of nicotine dependence has come from studies of CB1 receptor blockade of nicotine’s effects. See reviews by Cohen, Kodas et al. 2005a; Scherma, Fadda et al. 2009; Le Foll and Goldberg 2005a. The CB1 receptor inverse agonists rimonabant and AM251, have been shown to attenuate nicotine self-administration behaviour (Shoaib 2008), nicotine-induced conditioned place preference (Le Foll and Goldberg 2004; Forget, Hamon et al. 2005). In addition, these compounds have also attenuated drug (nicotine) and cue-induced reinstatement of nicotine-seeking behaviour (Cohen, Kodas et al. 2005a; Forget, Coen et al. 2009a) and nicotine-induced dopamine release in the shell of the nucleus accumbens and in the bed nucleus of the stria terminalis of rats (Cohen, Perrault et al. 2002). Furthermore, nicotine has significant rewarding effects in rodents (Le Foll and Goldberg 2004) that appear CB1 mediated because CB1 knockout mice do not display nicotine induced place preference (Castane, Berrendero et al. 2005). In contrast, the absence of CB1 receptors does not appear to modify self-administration of nicotine in knockout mice (Cossu, Ledent et al. 2001), which suggests that compensatory mechanisms become involved in response to the absence of cannabinoid receptors under the conditions of this study.
SECTION 5.8 THE CANNABINOID SYSTEM AND FOOD INTAKE

There are several reports indicating that the endogenous cannabinoid ligands AEA and 2-AG promote hyperphagia (Kirkham et al., 2002), confirming the critical role of the endocannabinoid system in the regulation of food intake. Furthermore, since the mid 1980s, THC has been approved as an orexigenic medication for the treatment of chemotherapy induced anorexia and weight loss in patients suffering from, cancer Alzheimer and HIV by the U.S. Food and Drug Administration (Pagotto et al., 2006). The role of CB1 receptors in modulating the reinforcing effects of food is supported by the presence of CB1 receptors in areas that are known to modulate the reinforcing effect of natural reward. These areas include the olfactory bulb, cortical and limbic regions (neocortex, pyriform cortex and hippocampus), several parts of the basal ganglia, thalamic and hypothalamic nuclei cerebellar cortex and brainstem nuclei (Cota, 2007).

Furthermore, studies have clearly shown that CB1 receptor blockade using the selective CB1 inverse agonist rimonabant improves metabolic parameters such as lipid metabolism sensitivity to insulin (Scheen & Paquot, 2009). These findings prompted the use of rimonabant as an antiobesity agent in Europe (Nathan, O'Neill et al. 2011). However, rimonabant was later withdrawn from the market as a result of increased incidence of psychiatric adverse events such as anxiety, depression and increased suicide (Moreira, Grieb et al. 2009). These psychiatric side effects could be explained by extensive prescription of rimonabant in inadequately screened groups (i.e. in subjects with mood disorder risk) as well as by the nature of the compound itself – inverse agonist as opposed to neutral antagonist (Le Foll, Gorelick et al. 2009). As a consequence, rimonabant was withdrawn from the European market at the end of 2008 (Scheen, Finer et al. 2006).
Studies looking at levels of endogenous cannabinoid ligands in response to food deprivation and satiety have found that levels of 2AG and anandamide show a gradual rise in response to prolonged intervals between meals, thus augmenting the feelings of hunger and encouraging feeding (Gardner and Vorel 1998; Kirkham, Williams et al. 2002). Moreover, there is a significant decline in the hypothalamic levels of 2AG following feeding while the levels of 2-AG remain unchanged in areas not believed to be related to feeding behaviour like the cerebellum implying that these changes in endocannabinoid levels are regional (Kirkham, Williams et al. 2002).

Studies have also shown that besides stimulating food intake, hypothalamic endocannabinoids inhibit the release of other neurotransmitters and neuropeptides involved in regulating appetite such as opioids (Gallate and McGregor 1999; Corchero, Manzanares et al. 2004; Spanagel and Weiss 1999), serotonin, (Rowland, Mukherjee et al. 2001) and γ-aminobutyric acid (GABA) (Jo, Wiedl et al. 2005). This inhibition has been shown to be CB1 receptor mediated (Di Marzo and Maccarrone 2008). Moreover, studies have demonstrated that there is a clear interaction between CB1 receptors and dopaminergic neurotransmission (Melis, Pistis et al. 2004). This interaction is supported by the anatomical co-localisation of dopamine receptors (D1 and D2) and CB1 receptors in mouse hippocampus (CB1 and D2), striatum and olfactory tubercle (CB1, D1and D2) (Hermann, Marsicano et al. 2002). In addition, there is a correlation between levels of endocannabinoids and dopamine and intake of food that is believed to activate the limbic forebrain (Gardner and Vorel 1998; Bisogno, Berrendero et al. 1999).

It is also proposed that several central and peripheral hormones involved in energy homeostasis like cholecystokinin (CCK), leptin and gherlin play a regulatory role in endocannabinoid signalling (Di Marzo 2011).
In addition to the central role of cannabinoid receptors on energy homeostasis, endocannabinoids play a critical role in the peripheral regulation of energy and feeding regulation. This has been shown in studies in which CB1 inverse agonist rimonabant was able to persistently produce significant weight loss in a manner that is independent from lowering food intake (Colombo, Agabio et al. 1998). More evidence is now supporting the role of endogenous cannabinoids in modulating peripheral processes such as gastric emptying, lipogenesis, and glucose uptake inside the cells, through CB1 receptors located in the gastrointestinal tract, adipose tissue and skeletal muscles. It is hypothesized that CB1 receptors serve as a communication circuit incorporating signals coming from peripheral organs and converging them centrally allowing the brain to constantly monitor the organism’s metabolic status and hence serve as a feedback circuit that ultimately controls energy homeostasis and food intake (Flier 2004).

SECTION 5.9 THE CANNABINOID SYSTEM AND DIFFERENT DRUGS OF ABUSE

There is mounting evidence indicating that cannabinoid receptors and genetic and pharmacological manipulation of endogenous cannabinoids modulates the reinforcing properties of most drugs of abuse. CB1 receptor blockade similarly attenuates cocaine-, heroin- and methamphetamine-induced reinstatement of drug seeking behaviour (De Vries, Shaham et al. 2001; Fattore, Spano et al. 2003; Anggadiredja, Nakamichi et al. 2004). This blockade was also noticed on reinstatement produced by cues associated with various drugs of abuse (De Vries, Shaham et al. 2001; Forget, Coen et al. 2009a). This suggests that the effects of CB1 receptor blockade are due to actions on common neural pathways mediating reinstatement behaviour and not on direct pharmacological blockade of the individual drug effects. All of these effects of CB1
receptor blockade are remarkably consistent across rat strains, schedules of reinforcement, and routes of CB1 antagonist administration.

Although the precise neural mechanisms underlying relapse to drug seeking after a period of abstinence are not fully elucidated, the hypothesis that CB1 receptor stimulation plays a key role in relapse to drug seeking for different drugs of abuse is currently acquiring considerable momentum. CB1 receptor activation has shown to stimulate cocaine (De Vries, Shaham et al. 2001), heroin (Fattore, Spano et al. 2003) and amphetamine seeking (Anggadiredja, Nakamichi et al. 2004).

In contrast, activation of the CB receptor through enhancing the endogenous cannabinoid tone produced the opposite effects. Administration of the FAAH inhibitor URB597 has shown to attenuate cocaine-seeking behaviour induced by cocaine priming and cocaine associated cues (Adamczyk, Golda et al. 2008). Moreover, co-administration of the FAAH inhibitor URB 597 (0.3 mg/kg) and anandamide reuptake inhibitor AM404 (3mg/kg) decreased the break points for heroin self-administration under progressive ratio schedule of reinforcement indicating a decrease in the motivation to self-administer heroin (Solinas, Panlilio et al. 2005). On the other hand, URB597 did not modify alcohol self administration under fixed or progressive ratio schedules of reinforcement nor did it modify reinstatement of alcohol seeking behaviour in response to stress or presentation of alcohol associated cues (Cippitelli, Cannella et al. 2008). Moreover, administration of URB597 and CB agonist WIN 55,212-2 attenuated alcohol induced increase in dopamine levels and neuronal firing in the nucleus accumbens (Perra, Pillolla et al. 2005).

In conclusion, activation of CB receptors through systemic administration of CB agonists can enhance the motivational properties of different drugs of abuse and could lead to reinstatement of drug seeking behaviour. In contrast, enhancing the endogenous cannabinoid tone through
inhibition of enzymatic degradation of the endogenous cannabinoid anandamide produces the opposite effects in certain drugs of abuse. However, the exact down stream mechanism remains to be elucidated.

SECTION 6: CRAVING AND RELAPSE

Cravings and cue induced relapse are common and prominent features of all drugs of abuse including nicotine. Drug craving is a term used to describe a state of intense desire to obtain and consume a certain drug. This desire is thought to be directed by the urge to obtain pleasurable and rewarding effects of the drug as well as to avoid experiencing the negative consequences of withdrawal. Craving is commonly triggered by the exposure to environmental stimuli that were previously paired with the intake of the drug (Carter and Tiffany 1999; Childress AR 1993; O'Brien, Childress et al. 1992; O'Brien and McLellan 1996) and results in an increase in the motivation for drug seeking ending ultimately in relapse. Moreover, repeated and effective pairing of certain of stimuli along with drug taking has been shown to add motivational salience to these stimuli (see, Fuchs, See et al. 2003). Over time these conditioned stimuli gain the quality of being secondary reinforcers themselves and ultimately lead to an increase in drug taking behaviour (Caggiula, Donny et al. 2002b) and subsequent induction of drug seeking behaviour in cases of abstinence; thus acquiring control over drug seeking behaviour (Everitt and Wolf 2002).

Although the primary reinforcing effects of nicotine, such as euphoria, are less pronounced than with other drugs, the abuse potential of nicotine and rates of relapse remain relatively high compared to other drugs (Goldberg, Spealman et al. 1981). This discrepancy could possibly be explained by the presence of other non-pharmacological factors that acquire incentive salience.
through being associated with nicotine intake. This association is believed to further enforce nicotine-seeking behaviour and later predispose to the high relapse rates observed in human smokers. This has been supported by several animal studies which indicated that environmental stimuli play a strong role in mediating nicotine intake and reinstatement of nicotine seeking behaviour (Donny, Caggiula et al. 1999; Rose and Corrigall 1997). Caggiula and coworkers demonstrated that self-administration of nicotine that was not paired with cues was at a significantly lower level than nicotine self administration that was paired with cues (Caggiula, Donny et al. 2001). Furthermore, reintroduction of nicotine paired with its associated cues following a period of extinction, reinstated nicotine-seeking behaviour at a higher level than that observed following the reintroduction of nicotine alone. Taken together, evidence strongly supports an important role of nicotine-associated cues in the maintenance of nicotine seeking as well as in relapse.
SECTION 7 EXPERIMENTAL RATIONALE

Section 7.1 Rationale for investigating the effects of CB receptor activation on nicotine self-administration under fixed and progressive ratio schedules of reinforcement and on reinstatement of nicotine seeking behaviour.

Emerging evidence has shown that the endocannabinoid system has been implicated in several neuropsychiatric diseases including drug addiction. This involvement has been reported in several drugs of abuse including nicotine. It has been reported that cannabinoid agonists activate the mesolimbic dopamine reward system (French, Dillon et al. 1997) and demonstrate rewarding effects in preclinical animal models of drug addiction (Martellotta, Cossu et al. 1998; Fattore, Cossu et al. 2001). It has been previously shown that genetic deletion of cannabinoid receptors as well as specific pharmacological blockade of CB1 receptors reduces self administration of nicotine and nicotine induced elevations in dopamine levels in the nucleus accumbens shell (Cohen, Perrault et al. 2002). Similar findings have been observed with other drugs of abuse such as heroin (Navarro, Carrera et al. 2001) and alcohol.

Together these findings are supporting the notion of a specific interaction between the cannabinoid and the nicotinic acetylcholine systems in the modulating the reward processes. Therefore, it is of particular significance to extend our investigation of the neurobiological mechanisms underlying this interaction in the context of nicotine taking and reinstatement of nicotine seeking after periods of extinction. The cannabinoid system has been implicated in relapse to cocaine (De Vries, Shaham et al. 2001) and heroin seeking (Fattore, Spano et al. 2003). No study so far has addressed the role of cannabinoid system activation in nicotine taking or reinstatement of nicotine seeking. Our study was undertaken to study the specific effect of CB
receptor activation on nicotine self-administration under fixed and progressive ratio schedules of reinforcement. We also extended our study to investigate the effect of CB receptor activation on reinstatement of nicotine seeking if administered alone and with contingent presentation of nicotine-associated cues. We hypothesized that activation of CB receptors would increase nicotine self-administration under a progressive ratio schedule of reinforcement. We also hypothesized that activation of CB receptors per se would lead to reinstatement of nicotine seeking as well as enhance cue induced reinstatement of nicotine seeking behaviour through a CB1 receptor mechanism.

Section 7.2 Rationale for investigating the effect of CB2 receptor activation and blockade on nicotine self-administration and reinstatement of nicotine seeking behaviour

Several reports have indicated that the behavioral and psychoactive effects of cannabinoid ligands are mediated by activation of brain cannabinoid receptors (Parolaro and Rubino 2008; Mackie 2008). To date, two major cannabinoid receptors have been identified and cloned (CB1 and CB2).

It has been widely hypothesized that the behavioural and psychotropic effects of cannabinoids are mediated by CB1 receptors (Parolaro and Rubino 2008; Mackie 2008) and that CB2 receptors do not possess psychoactive properties (Malan, Ibrahim et al. 2003). However, the proposed lack of central CB2 receptors has been recently challenged by reports of low densities of CB2 receptors on neuronal cells (Baek, Zheng et al. 2008; Gong, Onaivi et al. 2006; Onaivi, Ishiguro et al. 2006; Van Sickle, Duncan et al. 2005) in several brain regions that are related to memory and reward. These areas include the anterior olfactory nucleus, cerebral cortex,
cerebellum, hippocampus, striatum and brainstem. Furthermore, it has been shown that activation of CB$_2$ receptors by 2-AG, and the selective CB2 agonists JWH015 or JWH133 produce centrally mediated inhibition of locomotion (Onaivi, Ishiguro et al. 2006; Van Sickle, Duncan et al. 2005), centrally block morphine-6-glucuronide–induced emesis (Van Sickle, Duncan et al. 2005) and suppress neuropathic pain (Guindon and Hohmann 2008; Jhaveri, Elmes et al. 2008).

More recent studies have suggested that CB$_2$ receptor activation inhibits neuronal firing in the dorsal-root ganglia and spinal cord (Elmes, Jhaveri et al. 2004; Sagar, Kelly et al. 2005) and GABAergic transmission in rat cerebral cortex (Morgan, Stanford et al. 2009). These data indicate that functional CB$_2$ receptors may be expressed on CNS neuronal cells, prompting us to re-examine the role of CB$_2$ receptors in drug reward and addiction in the context of nicotine addiction.

We hypothesized that activation of CB2 receptors would enhance nicotine self administration and reinstatement of nicotine seeking. Furthermore, we hypothesized that blocking CB 2 receptors would attenuate nicotine self administration as well as reinstatement of nicotine seeking behaviour induced by nicotine priming as well as nicotine associated cues.

**Section 7.3 Rationale for investigating the effect of anandamide reuptake inhibitor VDM11 on nicotine self-administration and reinstatement of nicotine seeking behaviour induced by nicotine priming and nicotine associated cues.**

A more functionally selective way to alter endocannabinoid activity is to inhibit fatty acid amide hydrolase (FAAH), the main enzyme responsible for degradation of the endocannabinoid
anandamide (AEA), when and where it is synthesized and released. It has been recently reported that the FAAH inhibitor cyclohexyl carbamic acid 3’-carbamoyl-biphenyl-3-yl ester (URB597) can counteract abuse-related effects of nicotine in several animal models (Melis, Pillolla et al. 2008; Scherma, Panlilio et al. 2008b; Forget, Coen et al. 2009a). In rats, FAAH inhibition suppresses the development of nicotine-induced conditioned place preference (CPP) and intravenous (I.V.) nicotine self-administration, two widely used animal models of nicotine’s habit-forming rewarding effects (Scherma, Panlilio et al. 2008b). Inhibition of FAAH also suppresses reinstatement of nicotine seeking, an animal model of relapse (Scherma, Panlilio et al. 2008b; Forget, Coen et al. 2009a). In addition to these behavioural effects, FAAH inhibition reduces nicotine-induced excitation of dopamine neurons in the ventral tegmental area (VTA), the brain area where nicotine appears to trigger its rewarding effects (Melis, Pillolla et al. 2008). Moreover, FAAH inhibition reduces nicotine-induced elevation of dopamine levels in the shell of the nucleus accumbens, the terminal area of the brain’s mesolimbic reward system (Scherma, Panlilio et al. 2008b).

Although research with FAAH inhibitors has generally focused on enhancement of cannabinoid signalling mediated through prolongation of AEA’s effects, FAAH inhibition also increases brain levels, magnifies and prolongs effects of the non-cannabinoid fatty acid ethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). OEA and PEA are endogenous ligands for the peroxisome proliferator-activated receptors alpha (PPAR-a) (Fegley, Gaetani et al. 2005; Astarita, Di Giacomo et al. 2006a).

Mascia et al. (2011) showed that the selective PPAR-a agonists WY14643 and methyloleoylethanolamide (methOEA; a long-lasting form of OEA) dose-dependently counteract
the rewarding effects of nicotine in rats and monkeys (Mascia, Pistis et al. 2011). These findings converge to suggest that URB597 modulates the rewarding effects of nicotine by elevating levels of the endogenous PPAR-α ligands OEA and PEA; further studies are needed to delineate the role of AEA.

A way to selectively increase endogenous levels of AEA without altering levels of OEA or PEA is to inhibit uptake of AEA into cells where it is degraded by FAAH, by administering VDM11, a selective synthetic inhibitor of endocannabinoid transport that has been shown to increase endogenous brain levels of AEA, without significantly affecting brain levels of PEA or OEA (Fegley, Kathuria et al. 2004; Bortolato, Campolongo et al. 2006). Anandamide reuptake inhibitors have the property of potentiating many effects elicited by AEA in vitro and in vivo (Beltramo, Stella et al. 1997; Calignano, La Rana et al. 1997). However, they do not closely mimic the spectrum of pharmacological responses produced by direct cannabinoid agonists, like WIN 55,212-2 and tetrahydrocannabinol (THC), because they do not elicit catalepsy or hypothermia (Beltramo, Stella et al. 1997; Beltramo and Piomelli 2000) and do not produce THC-like discriminative effects or alter dopamine levels in the shell of nucleus accumbens in rats (Solinas, Tanda et al. 2007). These differences have been attributed to the ability of anandamide reuptake inhibitors to increase AEA levels in the brain by inhibition of AEA transport into cells without directly activating cannabinoid receptors (Beltramo, Stella et al. 1997; Beltramo and Piomelli 2000; Bortolato, Campolongo et al. 2006). We hypothesized that similar to URB597; administration of VDM11 will not affect nicotine nicotine self-administration under fixed or progressive ratio schedule of reinforcement. We also hypothesized
that VDM11 will attenuate reinstatement of nicotine seeking induced by nicotine priming and nicotine associated cues.

We were particularly interested in studying the role of the cannabinoid system in cue and nicotine induced reinstatement. The main reason is that we had a priori hypothesis based upon results from different reinstatement studies that cue and drug induced reinstatement are mediated via the mesolimbic dopaminergic system and that both types of reinstatement have distinct yet overlapping neuronal circuits (Shaham, Shalev et al. 2003). On the other hand, stress induced reinstatement is mediated through neuronal pathways (CRF and noradrenergic neurons) that are completely separate from those involved in drug and cue induced reinstatement. This hypothesis is also supported by the fact that the CB1 inverse agonist rimonabant was able to attenuate cue and cocaine induced reinstatement but not stress induced reinstatement (Shaham, Shalev et al. 2003).
Chapter 2: Cannabinoid receptor stimulation increases motivation for nicotine and nicotine seeking


Based on Addiction Biology, 2012

All the animal training, animal feeding, intravenous catheterization surgeries, self administration training and drug treatment for the self administration studies were preformed by Gamaleddin I except the experiment testing the effect of WIN 55,212 on nicotine self administration using different unit doses of nicotine which was performed by Zhu AZ. Gamaleddin I performed all the statistical analysis and wrote the manuscript. Coen KM was responsible for training Gamaleddin I to perform the intravenous catheterization surgeries and drug administration. Drs. Vemuri and Makriyannis synthesized and provided us with study drugs. Dr. Le Foll B and Wertheim C performed the drug discrimination experiments. Drs. Le Foll B and Goldberg SR. performed the study design for the all the experiments and made the necessary revisions to the manuscript.

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ABSTRACT

The cannabinoid system appears to play a critical facilitative role in mediating the reinforcing effects of nicotine and relapse to nicotine-seeking behaviour in abstinent subjects based on the actions of cannabinoid (CB) receptor antagonists. However, the effects of CB receptor stimulation on nicotine self-administration and reinstatement have not been studied systematically.

Here, we studied the effects of WIN 55,212-2, a CB1/2 agonist, on intravenous nicotine self-administration under fixed-ratio (FR) and progressive-ratio (PR) schedules of reinforcement in rats. The effects of WIN 55,212-2 on responding for food under similar schedules were also studied. In addition, the effects of WIN 55,212-2 on nicotine- and cue-induced reinstatement of nicotine seeking were also studied, as well as the effects of WIN 55,212-2 on nicotine discrimination.

WIN 55,212-2 decreased nicotine self-administration under the FR schedule. However, co-administration of WIN 55,212-2 with nicotine decreased responding for food, which may suggest that this effect was rather a non specific effect on locomotion. In contrast, WIN 55,212-2 increased both nicotine self-administration and responding for food under the PR schedule, produced a dose-dependent reinstatement of nicotine seeking, and enhanced the reinstatement effects of nicotine-associated cues. Some of these effects were reversed by the CB1 antagonist rimonabant, but not by the CB2 antagonist AM630. In the drug discrimination tests between saline and 0.4 mg/kg nicotine, WIN 55,212-2 produced no nicotine-like discriminative effects but significantly potentiated discriminative stimulus effects of nicotine at the low dose through a CB1-receptor-dependent mechanism. These findings indicate that cannabinoid CB1-receptor
stimulation increases the reinforcing effects of nicotine and precipitates relapse to nicotine-seeking behaviour in abstinent subjects. Thus, modulating CB1-receptor signalling might have therapeutic value for treating nicotine dependence.
INTRODUCTION

A large body of evidence has indicated that the endocannabinoid system plays a prominent role in the development and persistence of different types of drug dependence (De Vries, Shaham et al. 2001; Anggadiredja, Nakamichi et al. 2004; Spano, Fattore et al. 2004). Most of the evidence for the endocannabinoid system's modulation of nicotine dependence has come from studies of CB1 receptor blockade of nicotine's effects (see reviews by (Cohen, Kodas et al. 2005a; Le Foll and Goldberg 2005a; Scherma, Panlilio et al. 2008b), but the effects of CB1-receptor stimulation by selective agonists has seldom been studied.

The CB1 receptor antagonists rimonabant and AM251, have been shown to attenuate nicotine self-administration behaviour (Cohen, Perrault et al. 2002; Shoaib 2008), nicotine-induced conditioned place preference (Le Foll and Goldberg 2004; Forget, Barthelemy et al. 2006). Moreover CB1 receptor blockade, significantly attenuates drug- (nicotine) and cue-induced reinstatement of nicotine-seeking behaviour (Cohen, Perrault et al. 2005b; Forget, Coen et al. 2009a), and nicotine-induced dopamine release in the shell of the nucleus accumbens and in the bed nucleus of the stria terminalis of rats (Cohen, Perrault et al. 2002). Furthermore, nicotine has significant reinforcing effects in rodents (Le Foll and Goldberg 2004) that appear CB1 mediated because CB1 knockout mice do not display nicotine-induced place preference (Castane, Berrendero et al. 2005). CB1 receptor blockade has also shown to attenuate WIN 55,212-2-induced reinstatement of cannabinoid-seeking behaviour (Spano, Fattore et al. 2004). In contrast, the absence of CB1 receptors does not appear to modify self-administration of
nicotine in knockout mice (Cossu, Ledent et al. 2001), which suggests that compensatory mechanisms become involved in response to the absence of cannabinoid receptors under the conditions of this study. Interestingly, CB1 receptor blockade similarly attenuates cocaine-, heroin- and methamphetamine-induced reinstatement of drug-seeking behaviour (De Vries, Shaham et al. 2001; De Vries, Homberg et al. 2003; Fattore, Spano et al. 2003; Anggadiredja, Nakamichi et al. 2004). This blockade was also noticed on reinstatement produced by cues associated with various drugs of abuse (De Vries, Shaham et al. 2001; Forget, Coen et al. 2009a), which suggests that the effects of CB1 receptor blockade are due to actions on common neural pathways mediating reinstatement behaviour and not on direct pharmacological blockade of the individual drug effects. All of these effects of CB1 receptor blockade are remarkably consistent across rat strains, schedules of reinforcement, and routes of CB1 antagonist administration.

To the present, the precise neural mechanisms underlying relapse to drug seeking after a period of abstinence are not fully elucidated. However, the hypothesis that CB1 receptor stimulation plays a key role in relapse to drug seeking for different drugs of abuse is currently acquiring considerable momentum (De Vries, Shaham et al. 2001; Fattore, Spano et al. 2003; Anggadiredja, Nakamichi et al. 2004; Le Foll and Goldberg 2005a; Scherma, Panlilio et al. 2008b).

Although cannabinoid receptor agonists do not produce nicotine-like discriminative stimulus effects and do not alter the discriminative stimulus effects of nicotine (Zaniewska, McCreary et al. 2006), the psychoactive ingredient in marijuana, delta-9-tetrahydrocannabinol (THC), reduces dysphoric effects associated with nicotine withdrawal as well as anxiogenic-like effects of
nicotine in rats (Balerio, Aso et al. 2006; Balerio, Aso et al. 2004). On the other hand, acute administration of nicotine can potentiate hypothermic, antinociceptive and hypolocomotor effects of THC (Valjent, Mitchell et al. 2002). Furthermore, the sub-threshold doses of THC and nicotine that did not produce conditioned place preference when administered separately were able to induce conditioned place preference and enhance C-fos expression when co-administered. In the same study, co-administration of THC and nicotine twice a day for 5 days potentiated the expression of THC withdrawal signs precipitated by a cannabinoid CB1 antagonist (Valjent, Mitchell et al. 2002).

The aim of the present study was to examine the effects of CB1 receptor activation in rats self-administering nicotine and in rats trained to discriminate nicotine from saline. We used the synthetic cannabinoid CB1 receptor agonist WIN 55,212-2; (R),2,3-dihydro-5-methyl-3-(4 morpholinylmethyl), pyrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1 naphthalenylmethanone mesylate) (Pertwee, Stevenson et al. 1993). WIN 55,212-2 is a potent agonist for both CB receptors (CB1 and CB2) and is the most frequently studied synthetic compound from this class of drugs. To assess which of the CB receptors is involved in the observed effects; we used rimonabant and AM630 to selectively block CB1 and CB2 receptors, respectively.

We first tested the effects of pretreatment with graded doses of WIN 55,212-2 on the reinforcing effects of nicotine using an intravenous self-administration procedure with both a fixed-ratio (FR) schedule and a progressive-ratio (PR) schedule of reinforcement. We then studied the effects of WIN 55,212-2 pretreatment on reinstatement of nicotine-seeking behaviour during abstinence by both drug (nicotine) priming and presentation of nicotine-associated visual
cues. To serve as controls for the nicotine self-administration experiments, we also studied the
effect of WIN 55,212-2 pretreatment on operant food responding using both an FR and a PR
schedule of reinforcement. Finally, we tested the effects of WIN 55,212-2 pre-treatment in rats
trained to discriminate injections of nicotine from injections of saline vehicle using a two-lever
choice discrimination procedure with a food reinforced FR schedule of reinforcement as a
baseline.
Materials and Methods

Animals

Experimentally naïve male Long Evans rats (Charles River, Lachine, PQ, Canada) initially weighing 250–275 g were used for food reinforcement and nicotine intravenous self-administration studies. All rats were individually housed in a temperature-controlled environment on a 12-hour reverse light/dark cycle (lights off from 0700 to 1900 hours). Prior to any experimental manipulation, animals were given a minimum of 7 days to habituate to the colony room during which they were weighed, handled and received unlimited access to both food and water.

After habituation, all rats were diet restricted to five pellets (20 g) daily, but still received unlimited access to water. Diet restriction in the experiments conducted here in has shown to be essential in maintaining a stable and robust operant behaviour without restricting the animals ability to grow and gain weight. All the experimental procedures described in this study were carried out in compliance with the guidelines of the Canadian Council on Animal Care (compatible with NIH guidelines), and were reviewed and approved by the Institutional Animal Care Committee.

Male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) initially weighing 290–350 g were used for the drug discrimination studies. Rats were housed individually, and were allowed 7 days of free feeding and water upon arrival to the animal facility. Before the start of the study, rats were diet restricted (3 NIH07 biscuits/day) for 10 days (weight was 270–340 g at
the start of the study) and the diet restriction was maintained throughout the study. Enrichments (fresh fruits and vegetables) were provided on Saturdays. Water was available ad libitum.

Twelve of the rats used in the drug discrimination studies were initially naïve at the beginning of the experiments; the remainder received previous drug administration. Given that similar results were obtained, the results were combined. Animals used for the drug discrimination study, were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All experimentation was conducted in accordance with the guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, NIH, and the Guide for Care and Use of Laboratory Animals (National Research Council 2003).

**Apparatus**

Food reinforcement studies and nicotine intravenous self-administration studies were carried out in commercially available experimental chambers (Medical Associates, St. Albans, VT, USA) cased in sound attenuating boxes, and equipped with two levers, a house light and two cue lights (one located above each lever). For half the animals, the left lever was the active lever and for the other half the right lever was the active lever. The start of a session was signalled by the illumination of the house light and presentation of the levers. Pressing on the active lever resulted in the delivery of nicotine (30 μg/kg/infusion) when schedule requirements were met, and accompanied by the dimming of the house light and illumination of the cue light above the active lever. This continued for 60 seconds (timeout period) during which further pressing of the
active lever were recorded, but had no consequences. Pressing on the inactive lever was also recorded, but had no programmed consequences throughout the session.

For the drug discrimination studies, 12 standard operant-conditioning chambers (Coulbourn Instruments, Lehigh Valley, PA, USA) were used. Each chamber contained a white house light and two levers separated by a recessed tray into which a pellet dispenser could deliver 45-mg food pellets (F0021, Bioserv, Frenchtown, NJ, USA). Each press of a lever with a force of 0.4 N through 1 mm was recorded as a response and was accompanied by an audible click. The operant-conditioning chambers were controlled by microcomputers using the MED Associates MED-PC software package (MED Associates Inc., East Fairfield, VT, USA).

**Experimental procedures**

**Food-maintained behaviour**

Techniques for initial acquisition of food-maintained behaviour and surgery were similar to those already reported (Corrigall and Coen 1989; Forget, Coen et al. 2009a; Forget, Pushparaj et al. 2010a; Khaled, Farid Araki et al. 2010; Forget, Pushparaj et al. 2010a). Animals learned to press the lever for food reinforcement on a continuous reinforcement schedule in which each press on the active lever resulted in the delivery of a 45-mg food pellet. During the acquisition sessions, the house light was on and there was no illumination of the cue lights above the levers. Daily 1-hour acquisition sessions were conducted for 5 days. Once food-maintained behaviour was acquired, intravenous catheters were surgically implanted.
Intravenous catheterization

Surgical procedures for implantation of chronic intravenous catheters were similar to those already reported (Corrigall and Coen 1989; Khaled, Farid Araki et al. 2010). Briefly, catheters were implanted into the jugular vein and exited between the scapulae. Surgery was performed under anaesthesia induced by xylazine (10 mg/kg, intraperitoneal, IP) and ketamine hydrochloride (90 mg/kg, IP). Incision sites were infiltrated with the local anaesthetic marcaine (0.125%). Buprenorphine was given for post-operative analgesia (0.03 mg/kg, subcutaneous, SC), and a single dose of penicillin (30,000 units, intramuscular, IM) was administered at the completion of the surgical procedures. The animals had a 1-week recovery period before the initiation of the drug self-administration sessions. Patency of the catheters was verified 1 day before the start of the self-administration sessions using 0.1 ml of methohexital 10 mg/ml and was performed every 2 weeks of self-administration. Catheters were considered patent and venous if the animal experienced mild drowsiness and or very brief sleep.

Self-administration procedures

Acquisition of nicotine self-administration was performed under an FR schedule of reinforcement at a unit dose of 30 µg/kg/infusion of nicotine base. Session duration was 60 minutes. When an animal completed the schedule requirements this would result in the infusion of nicotine followed by a time-out period of 60 seconds associated with dimming of the house light and illumination of the cue light above the active lever. During timeout period, lever presses were recorded but did not count towards nicotine reinforcement. During the first week of acquisition, response requirements were FR1 (i.e. each active lever press during the time-in
period resulted in an infusion of nicotine base). Response requirements were then gradually increased to reach a final value of FR5, by which time the self-administration behaviour was stable and the animals had a 15- to 20-day history of nicotine self-administration. The self-administration sessions were mainly conducted 5 days a week.

**Testing under the FR5 schedule of reinforcement**

Rats were considered to have acquired stable nicotine self-administration when they pressed the active lever more than twice the number of times they pressed the inactive lever, received a minimum of 10 infusions per 1-hour session, and had less than 20% variation in the number of infusions earned per session during two consecutive sessions. Once stability was achieved, the animals were given intraperitoneal injections of vehicle for 3 days to habituate them to the injection procedure. The rats \((n = 13)\) were then tested using vehicle or WIN 55,212-2 at 0.1, 0.3, and 1.0 mg/kg) in a counterbalanced within-subject design.

After the completion of tests under the FR schedule, the unit dose of nicotine was changed from 30 to 10µg/kg/infusion. Once responding stabilized for 3 consecutive days, testing started using pre-session treatment with a 1 mg/kg dose of WIN 55,212-2. After testing for the effects of WIN 55,212-2 on nicotine (10µg/kg/infusion) self-administration, the dose of nicotine was changed to 60 µg/kg/infusion. Once the responding stabilized for 3 days, testing using the 1 mg/kg dose of WIN 55,212-2 was performed.
Testing under the PR schedule of reinforcement

Another group of animals (n = 8) learned to self-administer nicotine at 30 µg/kg per infusion under the FR schedule and then were directly switched to a PR schedule wherein the response requirement increased with each successive infusion. The response requirement progression was based on the formula \[5 \times \exp(0.25 \times \text{infusion number}) - 5\], with the first two values replaced by 5 and 10 (modified from (Roberts and Bennett 1993). Thus, the response requirements for successive infusions were 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. The breaking point (BP) was defined as the highest ratio completed prior to the first 30-minute period without a response on the active lever. The progressive ratio sessions lasted a maximum of 4 hours. The animals were allowed 10 days of nicotine self-administration under the PR schedule before testing began with the pharmacological compounds. All animals reached their break points during the 4-hour sessions within the 10-day training period, which was then followed by the testing of WIN 55,212-2 (0.1–1 mg/kg) using a counterbalanced within-subject design.

Food reinforcement under FR and PR schedules of reinforcement

A separate group of animals (n = 12) was used to test for the effects of WIN 55,212-2 on food self-administration under FR and PR schedules of reinforcement. Procedures used for food self-administration were similar to those used for nicotine self-administration (e.g. session duration, time-out periods, acquisition training and stabilization requirements before testing), except that the group did not undergo intravenous catheterization and the reinforcer after completing the schedule requirement was a 45-mg food pellet.
Extinction

Following the acquisition of nicotine self-administration, an extinction phase was conducted by withholding nicotine and its associated cues (house light stayed on and cue lights stayed off throughout the session). Responses on the active and inactive lever were recorded, but had no programmed consequences. An animal was considered to have met extinction criteria when their total active lever presses was less than 20 per session for two consecutive sessions. All animals reached extinction criteria within an average of 12 extinction sessions.

Reinstatement of nicotine seeking

For these experiments, two groups of rats were used. All tests were carried out in a counterbalanced within-subject design. After each test, extinction procedures were repeated until extinction criteria were satisfied for at least two consecutive sessions.

A separate group of animals (n = 7) was used to determine the effects of WIN 55,212-2 on reinstatement of nicotine seeking. Rats were pre-treated with vehicle, or 0.1, 0.3 or 1 mg/kg WIN 55,212-2. WIN 55,212-2 induced reinstatement tests were conducted under conditions similar to the extinction sessions (no cues and no nicotine delivery).

A subset of the rats that were used to determine the effects of WIN 55,212-2 on nicotine self-administration under the FR5 schedule (n = 7) were used for further testing of nicotine reinstatement. The effects of WIN 55,212-2 on cue-induced reinstatement of nicotine-seeking behaviour were assessed using the same doses as mentioned above. The cue-induced reinstatement tests were conducted under conditions identical to that of self-administration,
except that the responses on the active lever (on an FR5 schedule) resulted in contingent presentation of the cues (light above the active lever on and house-light off for 60 seconds) without nicotine availability (no infusions). Responses on the inactive lever were also recorded, but had no programmed consequences. The testing sessions lasted 1 hour.

**Drug-discrimination procedure**

Rats acquired food-maintained behaviour as described previously (Le Foll, Sokoloff et al. 2005; Le Foll, Wertheim et al. 2008c; Justinova, Ferré et al. 2009). Under a discrete-trial schedule of food-pellet delivery, they learned to respond on one lever after an injection of a training dose of 0.4 mg/kg nicotine and on the other lever after an injection of 1 ml/kg of saline vehicle (n = 36). Injections of nicotine or saline were given subcutaneously 10 minutes before the start of the session. At the start of the session, a white house light was turned on and in its presence the rats were required to make 10 consecutive responses (FR10 schedule of food delivery) on the lever appropriate to the pre-session treatment. The completion of 10 consecutive responses on the correct lever produced the delivery of a 45-mg food pellet and initiated a 45-second time-out period during which lever-press responses had no programmed consequences and the chamber was dark. Responses on the incorrect lever had no programmed consequences other than to reset the FR requirement on the correct lever. After each time-out period, the house light was again turned on and the next trial began. Each session ended after the completion of 20 FR trials or after 30 minutes elapsed, whichever occurred first. Discrimination-training sessions were conducted 5 days per week under a double alternation schedule (i.e. DDSSDDSS, etc., D = drug; S = saline). Training continued until there were eight consecutive sessions during
which rats completed at least 90% of their responses during the session on the correct lever and 
no more than four responses occurred on the incorrect lever during the first trial. Test sessions 
with other doses and other drugs were then initiated.

During the test sessions, a range of doses of WIN 55,212-2 were substituted for the training 
dose of nicotine. WIN 55, 212-2 was also administered together with 0.01 mg/kg nicotine to 
assess potentiation of discriminative stimulus effects induced by a sub-threshold dose of 
nicotine. The influence of co-administration with rimonabant was also evaluated. In addition, to 
assess for possible disruption of rates of responding, response rates were analyzed after pre-
treatment with various doses of WIN 55, 212-2 co-administered with various doses of nicotine. 
Test sessions were identical to training sessions, with the exception that both levers were active 
and 10 consecutive responses on either one of the two levers resulted in the delivery of a food 
pellet. Switching responses from one lever to the other lever reset the FR requirement to 10. In a 
test phase, a single alternation schedule was introduced and test sessions were usually conducted 
on Tuesdays and Fridays. Thus, a 2-week sequence starting on Monday was: DTSDTSTDST 
(T = test). In this way, test sessions occurred with equal probability after saline and drug 
sessions. Test sessions were conducted only if the criterion of 90% accuracy and not more than 
four incorrect responses during the first trial was maintained in the two preceding training 
sessions.

For the drug-discrimination studies, two independent measures of behaviour were 
collected: a measure of discrimination performance expressed as the percentage of nicotine-
associated responses and a measure of motor performance expressed as response rate. The
percentage of nicotine-associated responses during each session (training or test) reflected the percentage of the number of responses emitted on the nicotine-associated lever relative to the total number of responses emitted on both levers during a session. The percentage of nicotine-associated responses was individually calculated for each rat and then expressed as groups mean (±SEM). Nicotine-associated lever selection data were excluded from analysis if a rat emitted fewer than 10 responses during the test session. Full generalization to the nicotine cue was defined as a percentage of responding on the nicotine-associated lever of 80% or higher. No generalization to the nicotine cue was defined as a percentage of responding on the nicotine-associated lever of 20% or lower. Partial generalization to the nicotine cue was defined as a percentage of responding on the nicotine-associated lever ranging from >20% to <80%.

Response rates (responses/second) during each session were calculated by dividing the total number of responses emitted on both levers during a session by the total session length. Response rates were individually calculated for each rat and then expressed as group means (±SEM)

Drugs

(-)Nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline, the pH adjusted to 7.0 (±0.2) and the solution filtered through a 0.22-mm syringe filter (Fisher Scientific, Pittsburgh, PA, USA) for sterilization purposes. All nicotine doses are reported as free base concentrations. Nicotine was administered IV in a volume of 100 µl/kg/infusion.
WIN 55, 212-2 was suspended in 0.3% Tween and administered intraperitoneally 15 minutes prior to the test session. Solutions were sonicated for 10 minutes in case of imperfect dissolution and WIN 55, 212-2 injected in a volume of 1 ml/kg.

Rimonabant (provided by NIH-NIDA) was suspended in 0.3% Tween and was sonicated for 10 minutes. The drug was administered intraperitoneally 30 minutes prior to testing in a volume of 1 ml/kg (except the 3 mg/kg dose which was administered in a volume of 2 ml/kg).

AM630 (Tocris Bioscience, Ellisville, MO, USA) was dissolved in 10% dimethyl sulfoxide and then suspended in 10% Tween in distilled water. The drug was administered intraperitoneally 30 minutes prior to testing in a volume of 2 ml/kg.

Data analysis

For food and nicotine reinforcement studies the number of active and inactive lever presses, and the number of food pellets or nicotine infusions were recorded and analyzed. To analyze the effects of WIN 55, 212-2 on the number of nicotine infusions and on the number of food pellets earned under the FR and the PR schedule of reinforcement, a one-way analysis of variance (ANOVA) was performed followed when appropriate by post hoc Dunnett's test for comparisons with the baseline condition (the baseline score is the mean of the scores the day before each test session with an injection with the appropriate vehicle). The ability of WIN 55, 212-2 administration to induce nicotine reinstatement was analyzed using a one-way ANOVA with repeated measures. The analysis on the effect of WIN 55, 212-2 on nicotine self-administration using 10, 30 and 60 µg/kg per infusion was done using a Student's t-test. The ability of nicotine-
associated stimuli presentation to induce reinstatement was evaluated using a one-way ANOVA with repeated measures followed by Dunnett's test for comparisons with baseline condition or Newman–Keuls test for multiple comparisons (analysis of the effect of drugs on reinstatement of nicotine seeking). For the nicotine-discrimination study, one- and two-way ANOVAs, as appropriate, were used to analyze the experimental data. A post-hoc analysis using LSD tests was performed following detection of a significant main effect. Statistical analyses were performed on raw (rates of responding) or transformed (percentages of nicotine-associated lever selections) data. SigmaStat (SPSS Inc., Chicago, IL, USA) and Statistica computer programs were used for statistical analysis of the experimental data. Data were considered statistically significant at \( P < 0.05 \)

Results

Effects of WIN 55,212-2 on nicotine self-administration under the FR5 schedule of reinforcement

Analysis of variance showed a main effect of treatment on the number of nicotine infusions \((F_{3,36} = 2.967, P < 0.05)\), and Dunnett's post-hoc pairwise comparisons with the baseline level indicated that pre-administration of 1 mg/kg WIN 55,212-2 significantly reduced the number of nicotine infusions received during the session (Fig. 4a). Student t-test analysis of the effects of WIN 55,212-2 on nicotine self-administration maintained by different unit doses of nicotine (Fig. 4b) indicated that 1 mg/kg WIN 55,212-2 significantly reduced the number of infusions when responding was maintained by a unit dose of 30 µg/kg per infusion of nicotine \((N = 13)\) \((df = 12 \ t = 2.262, P < 0.05)\), whereas no significant effects were noticed with lower or higher unit doses of nicotine \((df = 6 , t = 2.137, P = 0.07,)\) and \((P = 0.74, df = 7, t = 0.346)\) for 10 and 60 µg/kg per infusion, respectively).
Figure 4. Effects of WIN 55, 212-2 on nicotine self-administration under a FR5 schedule of reinforcement. (a) Effects of pre-treatment with WIN 55,212-2 (0.1–1 mg/kg, IP) on nicotine (0.03 mg/kg/infusion) self-administration under FR5 schedule. Data are expressed as means (±SEM) of the number of infusions obtained during the one-hr session. * $P < 0.05$ versus vehicle pre-treatment (Dunnett’s test after significant ANOVA for repeated measures $n = 14$). (b) Effect of WIN 55,212-2 (1 mg/kg, IP, H 15 min) on nicotine self-administration (0.01–0.06 mg/Kg/infusion, $n = 8–14$) under the FR5 schedule.
Effects of WIN 55,212-2 on nicotine self-administration under the PR schedule of reinforcement

Analysis of variance showed a main effect of treatment on the number of nicotine infusions with the dose of 1 mg/kg WIN 55, 212-2 (N = 8) (F_{3, 21} = 6.333, P < 0.05) in significantly increasing the break point, as compared to vehicle (Fig. 5a). Figure 5b provides the typical responding obtained following vehicle and Fig. 5c provides the typical responding after administration of 1 mg/kg WIN 55, 212-2 pre-treatment during the 4-hour sessions under the PR schedule of reinforcement. The graph represents the cumulative responding on active (upper curve) or inactive levers (lower curve). The pattern of response on the active and inactive levers is also represented on the axis provided below the graphs.
Figure 5. Effects of WIN 55,212-2 on nicotine self-administration under a PR schedule of reinforcement. Effects of pre-treatment with WIN 55,212-2 (0.1–1 mg/kg, IP) on nicotine (0.03 mg/kg/infusion) self-administration under PR schedule. (a) Data are expressed as means (±SEM) of the number of infusions obtained during the 4-hr sessions ** $P < 0.01$ versus vehicle pre-treatment (Dunnett’s test after significant ANOVA for repeated measures $n = 9$). (b and c) Typical cumulative responses on active and inactive levers during a nicotine (0.03 mg/kg/infusion) self-administration session under a PR schedule with vehicle pre-treatment (b) or 1 mg/kg WIN 55,212-2 pre-treatment (c) in an individual rat. Each short upward mark on the cumulative lever-press records indicates one nicotine infusion. Break-point values are indicated and the pattern of response across time on active and inactive levers are provided below.
Effect of WIN 55,212-2 on responding for food under FR5 and PR schedules

WIN 55,212-2 significantly increased the number of food pellets obtained under the FR5 schedule ($N = 12$) ($F_{3, 33} = 8.349$, $P < 0.05$). Dunnett's post-hoc analysis indicated that only 0.1 and 0.3 mg/kg WIN 55,212-2 increased responding (Fig. 6a). It should be noted that the maximal number of pellets that could be obtained under the FR5 schedule was limited to 60 due to the time-out duration of 60 s and the one hour session limit. Analysis of the number of lever presses performed during the time-out period indicated that WIN 55,212-2 significantly increased the number of lever presses performed during the period (662.3 ± 140 versus 498.6 ± 145 for WIN 55,212-2 and vehicle conditions, respectively; $P = 0.04$).

WIN 55,212-2 produced a dose-dependent increase in the breaking point under the PR schedule of food reinforcement compared to vehicle (Fig 6b). This effect was significant with the 1 mg/kg dose of WIN 55, 212-2 as shown by Dunnett's test after significant ANOVA ($N = 11$) ($F_{3, 30} = 1.890$, $P < 0.05$).
Figure 6. Effects of WIN 55, 212-2 on food reinforcement. Effects of pre-treatment with WIN 55,212-2 (0.1–1 mg/kg, IP, H 15 min) on food self-administration under FR5 (a) and PR (b) schedules of reinforcement. Data are expressed as means (±SEM) of the number of food pellets earned during the sessions and corresponding break-point values for the PR sessions. Session durations were 1 and 4 hr for FR5 and PR, respectively. *P < 0.05, *** P < 0.01 versus vehicle pre-treatment (Dunnett’s test after significant ANOVA for repeated measures)
Effects of WIN 55,212-2 on reinstatement of nicotine seeking

The ability of WIN 55,212-2 to induce reinstatement of nicotine-seeking behaviour was assessed on a specific group of rats (Fig. 7a). An ANOVA test indicated a main effect of treatment ($N = 7$) ($F_{4, 24} = 12.55, P < 0.05$). Dunnett's post-hoc analysis indicated that the 0.3 and 1 mg/kg doses WIN 55,212-2 significantly increased the number of lever presses on the active lever ($P < 0.05$ for both doses). An ANOVA test on lever-presses on the inactive lever indicated it to be not significant ($P > 0.05$).

Effects of CB1 and CB2 blockade on WIN 55,212-2 induced reinstatement of nicotine-seeking behaviour

Analysis of variance performed on active lever presses indicated a main effect of WIN 55,212-2 (1 mg/kg) compared to vehicle treatment ($P < 0.01$) as seen in Fig. 7b. The ANOVA performed on active lever presses indicated a main effect of treatment ($N = 7$) ($F_{3, 18} = 13.05$, $P < 0.05$). Dunnett's multiple comparison post-hoc analysis showed that the pre-treatment with 1 mg/kg rimonabant significantly decreased WIN 55,212-2-induced reinstatement ($P < 0.05$). However, pre-treatment with AM630 did not attenuate the reinstatement obtained with 1 mg/kg WIN 55,212-2 ($P > 0.05$). The number of inactive lever presses was unaffected by the pre-treatment's rimonabant and AM630 ($F_{3, 18} = 2.015$, $P > 0.05$).
Figure 7. Effects of WIN 55, 212-2 on nicotine seeking. (a) Rats previously trained to self-administer nicotine and for which responding for nicotine was extinguished were pre-treated with various doses of WIN 55,212-2 (0.1–1 mg/kg, IP, H 15 min). Responses on the active lever (top) and inactive lever (bottom) were recorded. WIN 55, 212-2 (0.3 and 1 mg/kg) produced a significant reinstatement of nicotine seeking, assessed by the number of responses on the active lever (* \(P < 0.05\) and ** \(P < 0.001\)) (Dunnett’s test after significant ANOVA for repeated measures). There was no significant change in responding on the inactive lever \((P > 0.05)\). (b) Reversal of reinstatement of nicotine seeking induced by WIN 55,212-2 by pre-treatment with the CB1 antagonist rimonabant (1 mg/kg, IP) (* \(P < 0.05\)) (New Man Keuls multiple comparison test after significant ANOVA), but not by pre-treatment with the CB2 antagonist AM630 (5 mg/kg, IP) \((P > 0.05)\).
Effects of WIN 55,212-2 on cue-induced reinstatement

Analysis of variance performed on active lever presses indicated a main effect of cues alone on reinstatement of nicotine seeking compared to baseline conditions ($P < 0.001$; Fig. 8a).

ANOVA performed on the active lever presses indicated a main effect of WIN 55,212-2. Newman–Keuls post-hoc analysis showed that pre-treatment with 0.3 and 1 mg/kg WIN 55,212-2 15 min before the start of the session enhanced the effect of the cues ($P < 0.05$) compared to reinstatement under vehicle pre-treatment ($N = 7$) ($F_{4, 24} = 16.94$, $P < 0.05$). Neither cues nor WIN 55,212-2 altered responding on the inactive lever ($F_{4, 24} = 1.442$, $P > 0.05$).

Effect of rimonabant on WIN 55,212-2-induced potentiation of reinstatement of nicotine seeking

Student's $t$-test performed on active lever presses indicated significant reinstatement of nicotine seeking after pre-treatment with WIN 55,212-2 and introduction of cues ($P < 0.05$). The ANOVA performed on active lever presses indicated a main effect of rimonabant ($F_{3, 18} = 6.161$, $P < 0.05$) (Fig.8b). Post-hoc analysis showed that pre-treatment with 1 and 3 mg/kg rimonabant significantly reduced reinstatement induced by WIN 55,212-2 associated with cues. Introduction of cues and pre-treatment with either WIN 55,212-2 or rimonabant did not affect responding on the inactive lever ($F_{4, 24} = 3.144$, $P > 0.05$).
Figure 8. Effects of WIN 55,212-2 on reinstatement of nicotine seeking induced by presentation of nicotine associated cues. (a) Effects of pre-treatment with WIN 55,212-2 (0.1–1 mg/kg, IP) on cue-induced reinstatement of nicotine seeking. A significant reinstatement of nicotine seeking was found by cues alone (**P < 0.01) (Dunnett's test after significant ANOVA for repeated measures). Pre-treatment by WIN 55,212-2 significantly enhanced cue induced reinstatement (**P < 0.01) (New Man Keuls multiple comparison after significant ANOVA). (b) Rimonabant (0.3–3 mg/kg, IP, H 45 min) significantly reduced reinstatement of nicotine seeking induced by pre-treatment with WIN 55,212-2 (0.3 mg/kg) and presentation of visual cues. Data are expressed as means (±SEM) of the number of active lever presses during extinction (BSL), vehicle pre-treatment (visual cues, no WIN 55,212-2) sessions with cue presentation and WIN 55,212-2 pre-treatment, and sessions with rimonabant and WIN 55,212-2 pre-treatment and cue presentation.
Generalization tests with WIN 55,212-2

Figure 9 shows the percentage of responses made on the drug lever (Fig. 9a) and overall rates of responding (Fig. 9b) obtained during sessions when WIN 55,212-2 was tested for its ability to substitute for the training dose of 0.4 mg/kg of nicotine. Overall, the 20% criterion for partial generalization was not reached when WIN 55,212-2 was substituted for nicotine. One-way ANOVA indicated no significant difference of discrimination performance \(F_{5,89} = 1.71, P = 0.14\), although a few rats did have some responding on the nicotine associated lever after administration of 1 mg/kg WIN 55,212-2 (Fig. 9a). One-way ANOVA indicated a significant effect of WIN 55,212-2 on rates of responding \(F_{6,122} = 10.4, P < 0.0001\). Post-hoc tests indicated that 1 and 3 mg/kg WIN 55,212-2 significantly decreased rates of responding \(P = 0.009\) and \(0.002\), respectively).

WIN 55,212-2 administration potentiates nicotine discriminative effects of the 0.01 mg/kg subthreshold dose of nicotine

Various doses of WIN 55,212-2 (0, 0.1, 0.3 and 1 mg/kg) were administered in combination with the 0.01 mg/kg subthreshold dose of nicotine with or without 3 mg/kg rimonabant. Since 3 mg/kg WIN 55,212-2 with 0.01 mg/kg nicotine completely abolished responding (not shown), this group was not included in the two-way ANOVA test. Analysis of rates of responding using a two-way ANOVA indicated a significant effect of rimonabant pre-treatment \(F_{1,111} = 9.1, P = 0.003\), a significant effect of WIN 55,212-2 administration \(F_{3,111} = 8.4, P < 0.0001\), but no interaction between rimonabant pre-treatment and WIN 55,212-2 administration \(F_{3,111} = 1.6, P = 0.2\). Post-hoc analysis indicated that rates of responding of rats receiving 1 mg/kg WIN 55,212-2 with 0.01 mg/kg nicotine were significantly decreased compared to rates of responding
of rats receiving nicotine (0.01 mg/kg) alone (P = 0.05). Rates of responding were significantly higher in rats receiving 3 mg/kg rimonabant with 0.01 mg/kg nicotine, compared to those receiving nicotine alone (P = 0.03). Analysis of discrimination performance using a two-way ANOVA indicated a significant effect of rimonabant pre-treatment (F_{1, 83} = 6.4, P = 0.01), a significant effect of WIN 55,212-2 administration (F_{3, 83} = 4.6, P < 0.01), but no interaction between rimonabant pre-treatment and WIN 55,212-2 administration (F_{3, 83} = 1.7, P = 0.2).

Post-hoc analysis of discrimination performance indicated that the combination of 0.01 mg/kg nicotine with 0.3 and 1 mg/kg WIN 55,212-2 (P < 0.01 and P = 0.013, respectively) produced nicotine-like discriminative stimulus effects compared to rats receiving 0.01 mg/kg nicotine alone (Fig. 9c). In contrast, no significant change of discrimination performance was noticed in rats receiving 3 mg/kg rimonabant pre-treatment (all P > 0.3) compared to rats receiving 0.01 mg/kg nicotine alone. In contrast, 3 mg/kg rimonabant significantly blocked the potentiation of discriminative stimulus effects of 0.01 mg/kg nicotine induced by 0.3 mg/kg WIN 55,212-2 (P < 0.01) and by 1 mg/kg WIN 55,212-2 (P = 0.03) (Fig. 9c).
Figure 9 WIN 55,212-2 significantly decreased rates of responding and potentiates nicotine discrimination of sub-threshold dose of nicotine. Effects of different doses of WIN 55,212-2 given alone (A, B) or in combination with 0.01 mg/kg nicotine (C, D) in rats previously trained to discriminate 0.4 mg/kg nicotine from saline. WIN 55,212-2 did not substitute for nicotine in rats trained to discriminate 0.4 mg/kg nicotine (A), but it produced a potentiation of the ability to discriminate low doses of nicotine when co-administered with nicotine and this effect was reversed by the CB1 antagonists at the dose of 3 mg/kg (C). The percentage of responses on the lever associated with nicotine administration is shown as a function of dose during test sessions (upper panels), and response rates are expressed as responses per second averaged over the session (bottom panels). Data are means ± S.E.M. (* P<0.05)
We compared the response rates of rats while various doses of WIN 55,212-2 were administered together with various doses of nicotine (Table 2). The analysis of rates of responding using a two-way ANOVA indicated a significant effect of WIN 55,212-2 pre-treatment ($F_{3, 325} = 62.3, \ P < 0.0001$), no significant effect of nicotine administration ($F_{3, 325} = 0.3, \ P = 0.8$), and a significant interaction between WIN 55,212-2 and nicotine ($F_{9, 325} = 2.5, \ P < 0.01$). Post-hoc analysis indicated a significant decrease in rates of responding in rats receiving 0.3 and 1 mg/kg WIN 55,212-2, and this decrease was more pronounced in rats receiving nicotine at the highest doses (see Table 2 for individual results and P values).
Table 1: Responses rates of rats responding under a FR 10 schedule of food delivery (drug discrimination testing). Results are presented as Mean ± SEM (and individual number of rats for each testing is indicated).

<table>
<thead>
<tr>
<th>WIN 55212-2</th>
<th>Nicotine (mg/kg)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0 mg/kg</td>
<td>1.4 ± 0.1 (n=5)(^a)</td>
</tr>
<tr>
<td>0.1 mg/kg</td>
<td>1.4 ± 0.1 (n=21)</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>1.2 ± 0.5 (n=25)(^e)</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>0.5 ± 0.1 (n=26) (^**)</td>
</tr>
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\(^*\) P<0.05, \(^**\) P<0.01, \(^****\) P<0.0001 using Post-hoc analysis.
Discussion

Here we report that stimulation of CB₁/₂ receptors by WIN 55,212-2 increased both nicotine self-administration and responding for food under a PR schedule, produced dose-dependent reinstatement of nicotine-seeking behaviour, and enhanced the reinstatement effects of nicotine-associated cues. The reinstatement of nicotine seeking was reversed by the CB₁ antagonist rimonabant, but not by the CB₂ antagonist AM630. However, WIN 55,212-2 decreased nicotine self-administration under the FR schedule and co-administration of WIN 55,212-2 with nicotine decreased responding for food, suggesting that the effect was non-selective. In rats discriminating 0.4 mg/kg nicotine from saline, WIN 55,212-2 produced no nicotine-like discriminative effects, but it significantly potentiated the discriminative stimulus effect of the low threshold dose of nicotine and this effect was blocked by rimonabant.

Contrary to our hypothesis, 1 mg/kg WIN 55,212-2 significantly decreased the number of nicotine infusions that were self-administered under the FR5 schedule of reinforcement (Fig. 4a). Changing the unit dose of nicotine available under the FR schedule strongly affects rates of responding and the number of infusions earned by the animals (Donny, Caggiula et al. 1998; Le Foll, Wertheim et al. 2007) In agreement with previous studies, peak responding was maintained by a unit dose of 30 µg/kg/infusion of nicotine (Corrigall and Coen 1989). Studies have shown that changes in responding under FR schedule can be rather difficult to interpret. An increase or a decrease in responding could be as result of an increase or decrease in the reinforcing efficacy of the drug (Arnold and Roberts 1997). To determine if the decrease in responding under fixed ratio schedule induced by WIN 55,212-2 was due to a decrease or an increase in the reinforcing effects of nicotine, we assessed the effects of WIN 55,212-2 on responding maintained by a
lower and a higher unit dose of nicotine (Fig. 4b). Neither potentiation, nor blockade was noted under those conditions. This could be attributed to the floor effect as the responding of the animals on the 10 and 60 µg/kg per infusion was resting at a significantly lower level than the 30 µg/kg per infusion dose.

Given that there is a more linear relationship between the unit dose of nicotine and the motivation to self-administer nicotine when a PR schedule is used (Donny, Caggiula et al. 1999; Le Foll, Wertheim et al. 2007), we assessed the effects of WIN 55,212-2 on responding for nicotine under a PR schedule (Fig. 5). WIN 55,212-2 significantly increased the motivation for nicotine under this schedule, as reflected by the average breaking point values of 56 versus 95 obtained under vehicle and 1 mg/kg WIN 55,212-2, respectively (Fig. 5a). The apparent opposite results obtained under the FR and PR schedule may appear difficult to reconcile. The decrease in responding for nicotine under the FR schedule did not appear to be due to motor-disruptive effects of WIN 55,212-2 administered alone since responding for food was if anything increased by WIN 55,212-2 using a FR5 or a PR schedule (Fig. 6). The increase in responding for food is consistent with previous reports (Higgs, Barber et al. 2005; Solinas and Goldberg 2005a).

In contrast, analysis of the rate of responses obtained using the drug discrimination paradigm, indicated that the co-administration of WIN 55,212-2 and nicotine resulted in a strong dose-dependent decrease in the rates of responses (Table 2). The decrease in responding observed with nicotine self-administration under the FR5 schedule could be explained by the fact that WIN 55,212-2 co-administered with nicotine decreased responding rate in the drug discrimination paradigm (figure 4b,d). The absence of disruption noted with WIN 55,212-2
under the PR schedule could be related to the lower intake of nicotine under this schedule (7.1 ± 0.8 infusions received in 4 hours under the PR schedule compared to 23 ± 2.2 infusions in 1 hour under the FR schedule). Therefore, it appears that WIN 55,212-2 significantly increased the reinforcing effects of nicotine under the PR schedule, and that this effect of WIN 55,212-2 was not apparent under the FR5 schedule due to non-specific motor impairment. This is of importance as the vast majority of studies performed suggest that blocking CB₁ receptors results in a blockade of the reinforcing effects of drugs of abuse, including nicotine (Le Foll and Goldberg 2005a) and heroin (Solinas and Goldberg 2005a). In agreement with those studies, our present results suggest that stimulation of CB₁ receptors increases the motivation to self-administer nicotine under a PR schedule and we have previously shown that stimulation of CB₁ receptors increases the motivation to self-administer heroin under a similar PR schedule (Solinas and Goldberg 2005a). However, it should be noted that it would be have been interesting to test the effect of WIN 55, 212-2 on different infusion doses of nicotine under PR schedule. other doses of nicotine infusion using only one dose of nicotine (30 ug/kg/infusion) it should be noted that WIN 55,212-2 administration significantly decreases cocaine self-administration under an FR schedule (Fattore, Martellotta et al. 1999), an effect that has been proposed as reflecting a blockade of the reinforcing effects. Further experiments comparing the effects of WIN 55,212-2 on responding maintained by cocaine under different schedules of reinforcement are needed to delineate the possible involvement of non-specific motor effects.

This is the first report to demonstrate that stimulation of CB₁ receptors induces reinstatement of nicotine seeking (Fig. 7). These results are consistent with the findings of (De Vries, Shaham et al. 2001) that CB₁ agonist administration reinstates cocaine seeking (De Vries,
A large body of evidence has shown that blockade of CB1 receptors blocks reinstatement of drug-seeking behaviour and that this effect is seen with a large variety of drugs of abuse (De Vries and Schoffelmeer 2005). It appears clear that WIN 55,212-2 is producing its effects through CB1 receptors, as the effects of WIN 55,212-2 were abolished by a CB1 antagonist, but not by a CB2 antagonist (see Fig. 7b). Therefore, our results support the critical role of CB1 receptors in reinstatement of drug seeking across various drugs of abuse.

Administration of WIN 55,212-2 also potentiated the effects of the presentation of nicotine-associated cues on reinstatement of nicotine seeking (Fig. 8). However, as WIN 55,212-2 also directly induced nicotine seeking, we cannot exclude the possibility that some of those effects were also mediating the potentiation of the effects of nicotine-associated cues.

A critical role of CB1 receptors on reactivity to drug associated cues has been previously suggested (Le Foll and Goldberg 2005a). Notably, this influence has been previously reported by us and others on reactivity to nicotine-associated stimuli using the nicotine-induced place preference paradigm (Forget, Hamon et al. 2005; Le Foll and Goldberg 2005b) and the reinstatement paradigm (Forget, Coen et al. 2009a). It is interesting to note that delta-9-tetrahydrocannabinol (THC), a CB1/2 partial agonist, enhances cue-induced reinstatement of amphetamine seeking (Anggadiredja, Nakamichi et al. 2004). It is clear that anxiogenic stimuli such as foot-shock (Buczek, Le et al. 1999) can produce reinstatement of nicotine seeking. One limitation of the current study is the possible anxiogenic effect of WIN 55,212-2 that could lead to an increase the responding on the active lever. Studies with mice have shown that WIN 55,212-2 produces anxiolytic effects (Haller, Varga et al. 2004). In agreement, cannabinoids have been shown to decrease amphetamine induced anxiety-like behaviours (Hayase, Yamamoto
et al. 2005). In contrast, in rats the same compound produced the opposite effects (Haller, Varga et al. 2004).

In the present experiments, WIN 55,212-2 did not produce nicotine-like discriminative effects, but it significantly potentiated the discriminative stimulus effects of a low subthreshold nicotine dose. This potentiation was blocked by 3 mg/kg of the CB1 antagonist rimonabant (Fig. 9c). It has been reported that the discriminative stimulus effects of nicotine are likely mediated by neuronal nicotinic acetylcholine receptors (Pratt, Stolerman et al. 1983; Stolerman, Garcha et al. 1984; Kumar, Reavill et al. 1987). We and others have previously reported that blockade of CB1 does not alter the discriminative stimulus effects of nicotine (Cohen, Perrault et al. 2002; Le Foll and Goldberg 2004). Considering that discriminative stimulus effects are mediated by nicotinic acetylcholine receptor stimulation, the potentiation observed in our experiments could be mediated directly by effects on acetylcholine, as it has been reported that CB1-receptor ligands can modulate acetylcholine release in the brain (Pisanu, Acquas et al. 2006). Another possibility could be that stimulation of CB1 receptors produces a compensatory decrease in anandamide levels, which have been shown to have an inhibitory role on 4β2 nicotinic acetylcholine receptors (Spivak, Lupica et al. 2007). However, our results are not in agreement with a previous report indicating the absence of potentiation of nicotine discrimination with CB agonists (Zaniewska, McCready et al. 2006). Further studies are needed to delineate which factors could explain the different results obtained between the two studies.
Conclusion

Our results show that cannabinoid receptor stimulation by WIN 55,212-2 potentiates abuse-related properties of nicotine. Cannabinoid receptor stimulation increased the motivation to self-administer nicotine, enhanced nicotine seeking and enhanced the discriminative stimulus effects of low doses of nicotine. Several of those responses were reversed by a CB1 antagonist, but not by a CB2 antagonist, confirming the critical role of CB1 receptors in mediating drug-dependent processes. In contrast, we and others have previously shown that CB1 receptor blockade attenuated reinforcement/reward induced by nicotine (Forget, Hamon et al. 2005; Cohen, Perrault et al. 2002; Forget, Coen et al. 2009a), as well as reinstatement of nicotine seeking (Forget, Coen et al. 2009a).

Taken together, those findings indicate that the endocannabinoid system has a bi-directional role on nicotine reinforcement and nicotine seeking. However, CB1 antagonists have been withdrawn from the market because of an enhanced risk of depression/anxiety and suicide concerns (Le Foll, Gorelick et al. 2009). Further studies are needed to determine alternative strategies to modulate the CB transmission that would be better tolerated. Neutral CB1 antagonists or approaches targeting the enzyme fatty acid amide hydrolase (Kathuria, Gaetani et al. 2003; Gobbi, Bambico et al. 2005; Scherma, Panlilio et al. 2008b), or the endocannabinoid membrane transporter system (Beltramo, Stella et al. 1997), may have therapeutic utility notably for nicotine addiction (Scherma, Panlilio et al. 2008b; Forget, Hamon et al. 2009b). These approaches have the potential advantage of having less psychiatric side effects. In summary, our results demonstrate for the first time the critical influence of CB1 receptor activation on an array of abuse-related effects of nicotine.
Acknowledgments

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Chapter 3: Effects of a selective cannabinoid CB2 agonist and antagonist on intravenous nicotine self administration and reinstatement of nicotine seeking behaviour

Gamaleddin I, Zvonok A, Makriyannis A, Goldberg SR & Le Foll B

Based on PLoS ONE, 2012

All the animal training, animal feeding, intravenous catheterization surgeries, self administration training and drug treatment for the self administration studies were preformed by Gamaleddin I. Gamaleddin I performed all the statistical analysis and wrote the manuscript. Drs. Zvonok A and Makriyannis synthesized the AM1241 compound. Drs. Le Foll B and Goldberg SR. were involved in the study design for the all the experiments and made the necessary revisions to the manuscript.

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ABSTRACT

Over the last decade there have been significant advances in the discovery and understanding of the cannabinoid system along with the development of pharmacologic tools that modulate its function. Characterization of the crosstalk between nicotine addiction and the cannabinoid system may have significant implications on our understanding of the neurobiological mechanisms underlying nicotine dependence.

Two types of cannabinoid receptors (CB1 and CB2) have been identified. CB1 receptors are expressed in the brain and modulate drug taking and drug seeking for various drugs of abuse, including nicotine. CB2 receptors have been recently identified in the brain and have been proposed to play a functional role in mental disorders and drug addiction.

Our objective was to explore the role of CB2 receptors on intravenous nicotine self administration under two schedules of reinforcement (fixed and progressive ratio) and on nicotine seeking induced by nicotine priming or by nicotine associated cues. For this, we evaluated the effects of various doses of the selective CB2 antagonist AM630 (1.25 to 5 mg/kg) and CB2 agonist AM1241 (1 to 10 mg/kg) on these behavioural responses in rats.

Different groups of male Long Evans rats were trained to lever press for nicotine at a unit dose of 30µg/kg/infusion. Subsequently, animals were randomized using a Latin-square design and injected with either AM1241 or AM630 using a counterbalanced within subject design. Administration of the CB2 ligands did not affect either nicotine-taking nicotine-seeking behaviour. Our results do not support the involvement of CB2 receptors in nicotine-taking or nicotine-seeking behaviour.
Cigarette smoking is responsible for 5 million deaths worldwide every year. The mechanisms underlying tobacco smoking are of wide interest and clearly there is still a need for more effective medications to help in smoking cessation and prevent relapse (Le Foll and George 2007). The cannabinoid system appears to play a critical role in mediating the reinforcing effects of nicotine as well as relapse to nicotine-seeking behaviour. The cannabinoid system consists of CB1 and CB2 receptors and the endogenous cannabinoid receptor ligands, anandamide, and 2-arachidonoylglycerol (2-AG) (Howlett, Barth et al. 2002; Di Marzo, Bifulco et al. 2004), in addition to the enzymes responsible for their degradation which are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase 2-AG, respectively (De Petrocellis, Cascio et al. 2004; Di Marzo, Bifulco et al. 2004).

CB1 receptors are highly expressed in the CNS, and are the most abundant G protein coupled receptors in the brain (Cinar and Szucs 2009). Cannabinoids act at CB1 receptors located presynaptically to elicit changes in the synaptic efficacy of central neuronal circuits that are involved in several processes including reward (Freund, Katona et al. 2003). The CB2 receptors are predominantly expressed outside the central nervous system on immune tissues (Klein, Newton et al. 2003).

Recently, the expression of CB2 receptors has been reported in the brain. First, the expression of CB2 receptors was demonstrated in rat microglial cells and other cells in the brain associated with inflammation (Ibrahim, Deng et al. 2003; Golech, McCarron et al. 2004; Nunez, Benito et al. 2004; Benito, Kim et al. 2005). Then, CB2 receptor mRNAs were detected in rat brain (cerebellum, cortex, and brainstem) using reverse transcription polymerase chain reaction
(RT-PCR) (Van Sickle, Duncan et al. 2005). Moreover, CB2 receptor protein was detected using western blotting and immunohistochemistry. Evidence shows that CB2 receptors are functional and have antiemetic activity using intracranial ligand infusions of anandamide, THC and 2-AG. This antiemetic effect was reversed through selective CB2 receptor blockade (Van Sickle, Duncan et al. 2005).

More recently, it has been suggested that CB2 receptors may be involved in mental disorders and drug addiction (Ishiguro, Iwasaki et al. 2007; Ishiguro, Horiuchi et al. 2010). It has been reported that selective blockade of CB2 receptors prevented the development of alcohol preference, while selective activation of CB2 receptors enhanced alcohol preference, in mice subjected to chronic mild stress (Ishiguro, Iwasaki et al. 2007). In addition, it has been recently reported that selective activation of CB2 receptors reduced the reinforcing effects of cocaine and reduced levels of dopamine in the nucleus accumbens in wild type and CB1 receptor knockout mice, but not in CB2 receptor knockout mice (Xi, Peng et al. 2011). These findings support the notion that CB2 receptors are involved in modulating the reinforcing effects of drugs of abuse.

Most of the studies conducted so far, have explored the effects of activation or inactivation of CB1 receptors on drug-taking and drug-seeking behaviour for various drugs of abuse, including nicotine (Cohen, Perrault et al. 2002; Le Foll and Goldberg 2004; Cohen, Kodas et al. 2005a; Forget, Hamon et al. 2005; Shoaib 2008; Gamaleddin, Wertheim et al. 2012). However, to our knowledge, no studies have examined the role of CB2 receptors on nicotine-taking and reinstatement of nicotine-seeking behaviour. Here, we explored the impact of selective blockade and/or activation of CB2 receptors on nicotine self-administration behaviour under fixed-ratio
and progressive-ratio schedules of reinforcement and on reinstatement of nicotine-seeking behaviour induced by reintroduction of nicotine-associated cues and by nicotine priming.

**MATERIALS & METHODS:**

**Animals:** Male Long Evans rats (Charles River, Lachine, PQ, Canada) experimentally naive at the start of the study and initially weighing 250 to 275 g were used. All rats were individually housed in a temperature-controlled environment on a 12-h reverse light/dark cycle (lights off from 07:00 hours to 19:00 hours). Prior to any experimental manipulation, animals were given a minimum of 7 days to habituate to the colony room, during which they were weighed, handled and received unlimited access to both food and water. After habituation, all rats were diet restricted to 5 Pellets or 20gms daily and had free access to water. Food restriction continued until all the experiments were completed. All the experimental procedures described in this report were carried out in compliance with the guidelines of the Canadian Council on Animal Care (compatible with NIH guidelines), and were reviewed and approved by the Centre for Addiction and Mental Health (CAMH) Animal Care Committee (Protocol no. 543).

**Apparatus:** Nicotine intravenous self-administration studies were carried out in commercially available experimental chambers (Med Associates, St. Albans, Vt., USA) enclosed in sound attenuating boxes and equipped with two levers, a house light and 2 cue lights, one located above each lever. For half the animals, the left lever was the active lever and for the other half the right lever was the active lever. Session start was signalled by the illumination of the house-light and presentation of the levers. Pressing on the active lever resulted in the delivery of nicotine (30 μg/kg/infusion) when schedule requirements were met, accompanied by dimming of the house
light and illumination of the cue light above the active lever. This continued for 60 seconds (time out period), during which further pressing on the active lever was recorded but had no programmed consequences. Pressing on the inactive lever was recorded, but had no programmed consequences throughout the session.

**Experimental Procedures:**

**Food-maintained behaviour**

Techniques for initial acquisition of food-maintained behaviour were similar to those already reported (Khaled, Farid Araki et al. 2010; Forget, Coen et al. 2009a; Corrigall and Coen 1989; Gamaleddin, Wertheim et al. 2012). Animals learned to lever press for food reinforcement on a continuous reinforcement (CRF) schedule, in which each press on the active lever resulted in the delivery of a 45 mg food pellet. During the acquisition sessions, the house light was on and pressing the active lever resulted in the delivery of food with no illumination of the cue light above the levers. Daily 1-h acquisition sessions were conducted for 5 days. Once animals acquired food-maintained behaviour, intravenous catheters were surgically implanted.

**Intravenous catheterization**

Surgical procedures for implantation of chronic intravenous catheters were similar to those reported previously (Corrigall and Coen 1989). Briefly, catheters were implanted into the jugular vein, exiting between the scapulae. Surgery was performed under anaesthesia induced by xylazine (10 mg/kg, intraperitoneal (IP) and ketamine hydrochloride (90 mg/kg, IP). Incision sites were infiltrated with the subcutaneous (SC) local anaesthetic marcaine (0.125%). Buprenorphine was given for post-operative analgesia (0.03 mg/kg, SC), and a single dose of
penicillin (30,000 units, IM) was administered at the completion of surgical procedures. Animals were allowed to recover for a 1-week period before starting drug self-administration sessions.

Self-administration procedures

Acquisition of nicotine self-administration behaviour was performed under a fixed-ratio (FR) schedule of reinforcement at a unit dose of 30 μg/kg/ infusion of nicotine base. Session duration was 60 min. The start of each 60 min session was signalled by illumination of the house light. In the presence of the illuminated house light, completion of the schedule requirement on the active lever (i.e. 1 to 5 lever presses under FR1 to FR5) resulted in the delivery of a nicotine infusion. Each infusion was followed by a time out (TO) period of 60 seconds, during which the house light was dimmed, the cue light above the active lever illuminated, and lever press responses had no programmed consequences.

During the first five days of acquisition, response requirements were FR1 (i.e., each active lever press during the time-in period resulted in the delivery of a nicotine infusion), then FR2 for three days, then increased to reach a final value of FR5. Training was continued until the self-administration behaviour was stable and the animals had a 15-20 day history of nicotine self-administration. Self-administration sessions were conducted mostly 5 days a week.

Testing under the FR5 schedule of reinforcement

Animals were considered to have acquired stable nicotine self-administration when they: (1) pressed the active lever more than twice the number of times they pressed the inactive lever, (2) received a minimum of 10 infusions per 1-h session and (3) had less than 20% variation in the number of infusions earned per session over 2 consecutive sessions. Once stability was reached, the animals were given IP injections of vehicle 30 minutes before the start of the session, to
habituate them to the injection procedure for an additional three days. Rats were randomized using a Latin-square design and were then tested with vehicle (0 mg/kg) and different doses of AM630 or AM1241 in a counter-balanced, within-subject design. Drugs were administered intraperitoneally 30 min before the session. Two separate groups of animals were used, one for testing the effects of the CB2 agonist AM1241 (N=10) and the other for testing the CB2 antagonist AM630 (N=12) on nicotine self-administration behaviour under the fixed-ratio schedule, with drugs or vehicle administered 30 min before the session. Animals in each group were allowed at least two days of stable responding before they were retested with a different dose of either AM630 or AM1241.

Testing AM1241 under the PR schedule of reinforcement

A separate group of animals (N= 8) was trained to self-administer 30 μg/kg/infusion nicotine under the FR1 schedule for 5 days, then the FR2 schedule for 3 days and the FR5 schedule for another 2 days and then were directly switched to a progressive-ratio (PR) schedule where the response requirement during the session increased with each successive injection. The response requirement progression was based on the formula $[5 \times \exp(0.25 \times \text{inj number}) - 5]$, with the first two values replaced by 5 and 10 (modified from (Roberts and Bennett 1993). Thus, the response requirements for successive injections were 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. PR sessions lasted a maximum of 4 h. However, if the animal ceased to press the active lever for 30 minutes, the session automatically ended and the last ratio completed by the animal was defined as the break point. The animals were allowed 10 days of nicotine self-administration under the PR schedule and testing was performed only after stabilization of the responding on
the active lever for at least 2 consecutive sessions before testing with AM1241 compound began. All animals reached their break points during the 4-h sessions within this 10-day training period and testing of vehicle (0 mg/kg) and AM1241 (1, 3 and 10 mg/kg, IP, 30 min before the session) was then performed.

**Testing AM630 under the PR schedule of reinforcement**

The same group of animals that were tested with AM630 under the FR schedule of reinforcement (N=12) were switched to the PR schedule. After stabilization of behaviour under the PR schedule for 2 successive sessions, animals were tested using vehicle (0 mg/kg) and the highest dose of AM630 (5 mg/kg) in a counterbalanced, within-subject design, in a similar fashion to that described with AM1241. Only 7 animals completed testing under the PR schedule; 5 animals were excluded due to catheter blockade.

**Extinction**

After acquisition of nicotine self-administration behaviour, as described above, an extinction phase was conducted by withholding nicotine and its associated cues (house light remained on and cue lights remained off throughout the session). Responses on the active and inactive lever were recorded, but had no programmed consequences. An extinction criterion was established for each animal individually and was defined as total active lever responses during the session being less than 20 presses. This extinction criterion had to be maintained for 2 consecutive days before testing. All animals reached the extinction criterion within an average of 12 extinction sessions.
Effects of AM1241 on cue induced reinstatement of nicotine-seeking behaviour

All tests were carried out in a counter-balanced within-subject design. After each test, extinction was re-established until extinction criteria were obtained for at least two consecutive days. Animals (N=11) were pre-treated 30 min before the session with vehicle (0 mg/kg) and 1, 3 and 10 mg/kg AM1241 in a counterbalanced order to measure the effects of AM1241 on cue-induced reinstatement of nicotine-seeking behaviour. Cue induced reinstatement tests were conducted under conditions identical to that of self-administration, except that responses on the active lever (under a FR5 schedule) resulted in contingent presentation of the cues (light above the active lever on and house-light off for 60 s) without nicotine availability (no infusions). Responses on the inactive lever were recorded but had no programmed consequences. The testing sessions lasted for 60 minutes.

Effects of AM1241 on nicotine induced reinstatement of nicotine seeking

A new group of animals (N=13) underwent a similar acquisition and extinction training procedure, as described above with cue-induced reinstatement. This group was tested for effects of vehicle (0 mg/kg) and AM1241 (1, 3 and 10mg/kg IP 30 min before the session) on nicotine-induced reinstatement. Nicotine priming was performed as in Forget, Pushparaj et al. (2010a) by administering 0.15 mg/kg nicotine SC, 10 min before the start of the test session.

Effects of AM630 on cue-induced and nicotine-induced reinstatement of nicotine-seeking behaviour

Two separate groups of animals were tested for effects of AM630 on reinstatement of nicotine-seeking behaviour induced by cues (N=9) and by nicotine priming (N=9). Animals were
pre-treated with vehicle (0 mg/kg) and AM630 (1.25, 2.5 and 5 mg/kg) IP 30 minutes before the start of the session. Cue-induced reinstatement tests were conducted under conditions identical to that of self-administration, except that responses on the active lever (on an FR5 schedule) resulted in contingent presentation of the cues (light above the active lever on and house-light off for 60 s) without nicotine availability (no infusions). Responses on the inactive lever were recorded but had no programmed consequences. Testing the effects of AM630 on nicotine-induced reinstatement was performed as in Forget, Pushparaj et al. (2010a) and Forget, Wertheim et al. (2010b) by administering 0.15 mg/kg nicotine SC, 10 min before the start of test session, in the same manner and using the same methodology as described above with AM1241. All extinction and reinstatement sessions lasted for 60 minutes.

Data Analysis

The number of active and inactive lever presses and the number of nicotine infusions were recorded and analyzed. To analyze the effects of AM1241 and AM630 on the number of nicotine infusions earned under the FR and the PR schedules of reinforcement, one way ANOVA analysis was performed. For reinstatement studies, one-way repeated measures analysis of variance (ANOVA) was used to assess the effects of AM1241 and AM630 on reinstatement induced by nicotine priming and by nicotine-associated cues. Student-t test was used to assess the effect of 5 mg/kg of AM630 pre-treatment compared to vehicle pre-treatment on nicotine self-administration behaviour under the PR schedule of reinforcement.

Drugs

(-) Nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, Mo., USA) was dissolved in saline, the pH was adjusted to 7.0 (±0.2), and the solution was filtered through a 0.22 mm syringe filter
(Fisher Scientific, Pittsburgh, Pa., USA) for sterilization purposes. All nicotine doses are reported as free base concentrations. Nicotine was administered IV in a volume of 100 μl/kg/injection for self-administration studies or was administered SC at the dose of 0.15mg/kg for reinstatement studies. AM1241 (2-iodo-5-nitrophenyl)-(1-(1-methylpiperdin-2-ylmethyl)-1h-indol-3-yl) methanone was dissolved in 20% DMSO in saline and injected IP 30 min before the start of the session and was synthesized by the group of Dr. Alexandros Makriyannis, the Centre for Drug discovery at Northeastern University, Boston, MA, USA. AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl)(4-methoxyphenyl)methanone (Tocris Bioscience, Missouri USA) was dissolved in 10% DMSO, 10% tween in distilled water and injected IP in a volume of 1ml/kg 30 min before the start of the session.

Results:

Acquisition of nicotine self-administration behaviour under fixed ratio schedule of reinforcement

During the first week of acquisition, responding on the active lever decreased to low levels, then gradually increased when the ratio requirement was increased up to FR5; in contrast, responding on the inactive lever remained low (Fig. 10a). Over the next 2 weeks, responding on the active lever under the FR5 schedule that was reinforced by nicotine infusion increased to the high levels previously maintained by food, while responding on the inactive lever remained low. The number of nicotine infusions throughout the different schedules of reinforcement (FR1 – FR5) showed a consistent level of nicotine self administration (above 10 infusions/session) (Fig. 10b).
Extinction

The data presented in Fig 10c, reflect the extinction pattern for the group of animals (N = 12) used in the experiment testing the effect of AM1241 on cue-induced reinstatement (only 11 animals completed testing on cue-induced reinstatement and 1 animal was excluded due to failure of extinction). Most animals reached extinction criteria within 8-9 days and testing with AM1241 on reinstatement was started (extinction training was pursued for the remaining rats until they reached the extinction criteria)
Figure 10: Pattern of responding during acquisition and extinction phases

a. Acquisition of nicotine self-administration (30 µg /kg/infusion). The total number of active (●) and inactive (■) lever presses (means ± SEM) received in each session (during time in and time out periods) under the different schedules of reinforcement (FR- 1, FR-2, FR-5,)

b. Number of nicotine infusions (means ± SEM) earned during acquisition phase in the same group of animals represented as fig.1A.

c. The number of active (●) and inactive (■) lever presses (means ± SEM) received in each extinction session in the same group of animals represented in figures 13a & 13b.
Effects of AM1241 on nicotine self-administration behaviour under the FR5 schedule

ANOVA analysis showed no significant effect of AM1241 pre-treatment on the number of nicotine infusions ($F_{3, 27}=1.13$, $P=0.35$), indicating that administration of AM1241 (1, 3 and 10 mg/kg) did not affect the number of nicotine infusions received during the session ($N = 10$) (Fig. 11a).

Effects of AM1241 on nicotine self-administration behaviour under the PR schedule

ANOVA analysis showed no significant effect of AM1241 pre-treatment on the number of nicotine infusions ($F_{3, 21}= 0.20$, $P=0.89$). Administration of various doses of AM1241 (1, 3 and 10 mg/kg) failed to produce any change in break point values, as compared to vehicle (0 mg/kg; $N = 8$) (Fig. 11b).
Figure 11. Effects of AM1241 on nicotine self-administration under FR5 and PR schedules of reinforcement.

a. Effects of pre-treatment with AM1241 (1, 3 and 10 mg/kg, IP H 30) on nicotine (30 μg/kg/infusion) self-administration under the FR5 schedule. Data are expressed as means (±SEM) of the number of nicotine infusions obtained during the 60-min session. All doses of AM1241 did not affect responding vs. vehicle (0 mg/kg) pre-treatment (N=10); P=0.35

b. Effects of pre-treatment with AM1241 (1, 3 and 10mg/kg) on nicotine (30μg/kg/infusion) self-administration under PR schedule. A, Data are expressed as means (±SEM) of the number of nicotine infusions obtained during the 4-hr sessions. AM1241 did not affect break point P>0.05 compared to vehicle (0 mg/kg) pre-treatment. (N=8) P=0.89
Effect of AM1241 on reinstatement of nicotine-seeking behaviour induced by nicotine-associated cues

ANOVA analysis performed on active lever presses indicated a main effect of cues per se on reinstatement of nicotine seeking compared to extinction (Ext) conditions ($P<0.001$). Newman-Keuls Multiple Comparison Test performed on the active lever presses indicated no effect of AM1241 administration ($F_{4, 40}=19.75; P>0.05$), compared to cue-induced reinstatement after vehicle (0 mg/kg) administration. Neither presentation of nicotine-associated cues nor AM1241 administration had a significant effect on responding on the inactive lever ($F_{4, 40}=1.34, P=0.27$) ($N = 11$) (Fig. 12a).

Effect of AM1241 on reinstatement of nicotine-seeking behaviour induced by nicotine priming.

ANOVA analysis performed on active lever presses indicated a main effect of 0.15 mg/kg nicotine priming on nicotine-seeking behaviour, as compared to extinction (Ext) conditions ($P<0.001$). ANOVA analysis performed on the active lever presses indicated no effect of AM1241 ($F_{3, 36}=6.64; P>0.05$), as compared to nicotine-induced reinstatement after vehicle (0 mg/kg) pre-treatment. Neither priming injections of nicotine, nor AM1241 (1, 3 and 10 mg/kg) administration, had a significant effect on responding on the inactive lever ($F_{3, 36}=1.80; P = 0.14$) ($N = 13$) (Fig. 12b)
Figure 12 Effects of AM1241 on reinstatement of nicotine-seeking behaviour induced by presentation of nicotine-associated cues and by nicotine priming.

a. A significant reinstatement of nicotine-seeking behaviour was produced by presentation of nicotine associated cues alone compared to extinction condition (Ext) (* P<0.001). ANOVA showed that pre-treatment with AM1241 (1, 3 and 10 mg/kg, IP, H 30 min) did not modify cue induced reinstatement of nicotine-seeking behaviour compared to vehicle (0 mg/kg) (P>0.05) N=11. Data are expressed as means (±SEM) of the number of active and inactive lever presses during extinction (Ext); vehicle (0 mg/kg) pre-treatment (visual cues) and after pre-treatment with AM121 (1, 3 and 10mg).

b. A significant reinstatement of nicotine-seeking was produced by pretreatment with nicotine (0.15 mg/kg) compared to extinction condition (Ext) (* P<0.001). ANOVA showed that AM1241 (1, 3, 10 mg/kg, IP, H 30 min) did not modify reinstatement of nicotine-seeking behaviour induced by a priming injection of 0.15 mg/kg nicotine administered 1 min before the session compared to vehicle (0 mg/kg) pre-treatment (P>0.05). Data are expressed as means of the number of active and inactive lever presses during extinction (Ext); vehicle (0 mg/kg) pre-treatment and after pre-treatment with AM121 (1, 3 and 10mg) N=13
Effects of AM630 on nicotine self-administration behaviour under the FR5 schedule

ANOVA showed no effect of AM630 pre-treatment on the number of nicotine infusions received during the session ($F_{3, 33} = 0.51, P=0.67$), and pair wise comparisons with vehicle (0 mg/kg) pre-treatment indicated that administration of AM630 (1.25, 2.5 and 5mg/kg) did not affect the number of nicotine infusions received during the session ($N = 12$) (Fig. 13a).

Effects of AM630 on nicotine self-administration behaviour under the PR schedule

Student-t test showed no effect of AM630 pre-treatment on the number of nicotine infusions received during the session ($P=0.73$). Administration of 5 mg/kg AM630 failed to produce any change in the break point values, as compared to vehicle (0 mg/kg) (Fig. 13b)($N = 7$).
Figure 13 Effect of AM630 on nicotine self administration under FR5 and PR schedules of reinforcement.

a. Effects of pre-treatment with AM630 (1.25, 2.5 and 5 mg/kg, IP, H 30) on nicotine (30 μg/kg/infusion) self administration under the FR5 schedule. Data are expressed as means (±SEM) of the number of nicotine infusions obtained during the 60-min session. AM630 did not affect responding vs. vehicle (0 mg/kg) pre-treatment (N=12); P=0.67

b. Effects of pre-treatment with AM630 (5 mg/kg, IP) on nicotine (30μg/kg/infusion) self administration under PR schedule. A, Data are expressed as means (±SEM) of the number of nicotine infusions obtained during the 4-hr sessions. AM630 did not affect break point P>0.05 vs. vehicle (0 mg/kg) pre-treatment. (N=7) P= 0.73
Effects of AM630 on reinstatement of nicotine-seeking behaviour induced by nicotine-associated cues

ANOVA analysis performed on active lever presses indicated a main effect of cues per se on reinstatement of nicotine-seeking behaviour compared to extinction (Ext) conditions ($P<0.001$). ANOVA performed on the active lever presses indicated no effect on cue-induced reinstatement of different doses of AM630 (1.25, 2.5 and 5mg/kg) ($F_{4, 32}=14.94; P>0.05$), compared to vehicle (0 mg/kg). Neither presentation of nicotine-associated cues, nor administration of AM630, had a significant effect on responding on the inactive lever ($F_{4, 32}=0.50 P=0.73$) ($N=9$) (Fig. 14a).

Effects of AM630 on reinstatement of nicotine-seeking behaviour induced by nicotine priming

ANOVA analysis performed on active lever presses indicated a main effect of 0.15 mg/kg nicotine priming on nicotine-seeking behaviour, compared to extinction (Ext) conditions ($P<0.001$). ANOVA analysis performed on the active lever presses showed no effects of administration of AM630 (1.25, 2.5 and 5 mg/kg) ($F_{4, 32}=8.33; P>0.05$), as compared to vehicle (0 mg/kg) pre-treatment. Neither priming injections of nicotine nor AM630 administration, had a significant effect on responding on the inactive lever ($F_{4, 32}=0.73; P=0.57$) ($N=9$) (Fig. 14b).
Figure 14 Effects of AM630 on reinstatement of nicotine-seeking behaviour induced by presentation of nicotine associated cues and by nicotine priming.

a. Effects of pre-treatment with AM630 (1.25, 2.5 and 5 mg/kg, IP H 30 min) on cue-induced reinstatement of nicotine-seeking behaviour. A significant reinstatement of nicotine-seeking behaviour was produced by presentation of nicotine-associated cues alone (* P<0.001). ANOVA showed that pre-treatment with AM630 (1.25, 2.5 and 5 mg/kg, IP, H 30 min) did not modify cue induced reinstatement of nicotine-seeking behaviour compared to vehicle (0 mg/kg) pre-treatment (P>0.05). Data are expressed as means (±SEM) of the number of active and inactive lever presses during extinction (Ext); vehicle (0 mg/kg) pre-treatment and after pre-treatment with AM630 (1.25, 2.5 and 5mg).

b. A significant reinstatement of nicotine-seeking was also produced by pre-treatment with nicotine (0.15 mg/kg) (* P<0.001). ANOVA showed that AM630 (1.25, 2.5, 5 mg/kg, IP, H 30 min) did not modify reinstatement of nicotine-seeking behaviour induced by a priming injection of 0.15 mg/kg nicotine administered 1 min before the session (P>0.05). Data are expressed as means (±SEM) of the number of active and inactive lever presses during extinction (Ext); vehicle (0 mg/kg) pre-treatment and after pre-treatment with AM630 (1.25, 2.5 and 5mg).
Discussion

This study is the first to evaluate the impact of selective CB2 receptor ligands on an animal model of nicotine-taking and nicotine-seeking behaviour. Neither activation of CB2 receptors by the selective CB2 agonist AM1241, nor blockade using the selective CB2 antagonist AM630 produced significant effects on nicotine-taking behaviour under fixed-ratio or progressive-ratio schedules of reinforcement. Moreover, both compounds failed to modulate nicotine-seeking behaviour induced by reintroduction of nicotine-associated cues or by priming injections of nicotine just before the start of the session.

To our knowledge, data on the behavioural properties of AM1241 are relatively scarce and are mostly limited to studying its effects on motor function and pain. We selected a dose range that covers the different doses used in several previous studies (Malan, Ibrahim et al. 2002; Ibrahim, Porreca et al. 2005; Rahn, Makriyannis et al. 2007), doses that had potent antinociceptive effects, but, no locomotor, cataleptic or motor side effects (Yamamoto, Mikami et al. 2008; Khasabova, Gielissen et al. 2011 ). Similarly, AM630 has seldom been tested in drug dependence paradigms. Similar to AM1241, we used a relatively wide range of AM630 doses similar to doses previously tested (Garcia-Gutierrez, Perez-Ortiz et al. 2010; Sticht, Long et al. 2011). Choice of AM630 was due to its high potency and affinity for rat CB2 receptors (Mukherjee, Adams et al. 2004).

Our results with AM1241 on nicotine self administration under the fixed-ratio schedule of reinforcement are in agreement with previous results with the CB2 agonist JWH015, which failed to modulate alcohol intake in C57Bl/6 mice under a fixed-ratio schedule of reinforcement.
Furthermore, selective blockade of CB2 receptors by AM630 did not affect alcohol intake in the same strain of mice under the same schedule of reinforcement (Ishiguro, Iwasaki et al. 2007). However, both JWH015 and AM630 were able to increase and decrease alcohol intake, respectively, in mice subjected to chronic mild stress, which is a paradigm outside the scope of this study (Ishiguro, Iwasaki et al. 2007). These findings were later replicated by the same group which also reported that blockade of CB2 receptors decreased food consumption in C57Bl/6 mice but failed to produce significant changes in food intake for Balb/c and DBA/2 mice (Onaivi, Ishiguro et al. 2008).

In contrast to our findings, Xi and colleagues have recently shown that systemic intranasal and local intra-accumbens administration of the selective CB2 agonist JWH133 produced a dose dependent decrease in intravenous cocaine self-administration behaviour. Furthermore, the same compound attenuated cocaine-induced hyperlocomotion, and cocaine-induced increases in extracellular levels of dopamine in the nucleus accumbens in wild-type and CB1 receptor knockout mice, but not in CB2 knockout mice (Xi, Peng et al. 2011). The selective CB2 antagonist AM630 reversed the effects observed with CB2 receptor activation (Xi, Peng et al. 2011). The discrepancy between our findings and the findings of Xi et al may be due to several methodological differences such as: (i) Differences in the neurobiological substrates of the drug of abuse studied (nicotine vs. cocaine), (ii) Differences in the role of CB2 receptors based on the animal strain (rats vs. mice), (iii) Differences in the pharmacological effects of the CB2 agonist used (JWH133 vs. AM1241) or, (iv) Differences in the schedule of reinforcement used (FR5 vs. FR1). Further work addressing those factors would be needed to clarify the role of CB2 receptors in drug reinforcement.
The behavioural findings in this study are in line with our recent findings that stimulation of CB1/2 receptors using the mixed CB1/2 receptor agonist WIN 55,212-2 increases nicotine self-administration behaviour under a progressive-ratio schedule of reinforcement. Moreover, in the same study, we demonstrated that administration of WIN 55,212-2 per se reinstates nicotine-seeking behaviour, an effect that was reversed by the selective CB1 inverse agonist/antagonist rimonabant but not by the selective CB2 antagonist AM630, indicating that this enhancement of nicotine-seeking behaviour was mediated by CB1 receptors. WIN 55,212 also significantly enhanced reinstatement of nicotine-seeking behaviour induced by reintroduction nicotine associated cues, an effect that was also reversed by rimonabant (Gamaleddin, Wertheim et al. 2012).

The results in this study, along with our previous work on CB1 receptor stimulation, add more evidence to the current literature that CB1 and CB2 receptors have several distinct behavioural, neurochemical and immunological profiles, yet they overlap in some properties like antinociception and catalepsy (when higher doses are tested) (Valenzano, Tafesse et al. 2005; Rahn, Makriyannis et al. 2007).

One limitation in this study is the lack of data on stress-induced reinstatement. This aspect would be worth exploring in further studies. It is clear that neurotransmitters, such as noradrenaline and corticotrophin releasing factor, are involved in mediating stress-induced reinstatement (Zislis, Desai et al. 2007) and we cannot exclude an involvement of CB2 receptors in stress-induced reinstatement of nicotine-seeking behaviour at this point.

In conclusion, the findings in this study provide evidence that CB2 receptors are not involved in the reinforcing effects of nicotine or in reinstatement of nicotine-seeking behaviour.
induced by cues and nicotine priming in rats. In this study we used the intravenous self-administration paradigm, which has been previously used by us and several other laboratories to assess the pivotal role CB1 receptors play in the reinforcing effects of nicotine. However, in this study using the same paradigm we were not able to demonstrate a similar role of CB2 receptors on nicotine self-administration behaviour or reinstatement of nicotine seeking behaviour. Hence, we believe that ligands modulating the CB1 receptors (either directly or indirectly by modulating endocannabinoid tone) could potentially be a more useful tool than CB2 ligands in modulating the reinforcing and relapse related effects of nicotine (Scherma, Panlilio et al. 2008b; Forget, Coen et al. 2009a; Gamaleddin, Guranda et al. 2011). The findings in this study could be specific to nicotine and not generalizable to other drugs of abuse. Therefore, further studies are warranted to investigate the role of CB2 receptors on the reinforcing and relapse related effects different drugs of abuse.
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Chapter 4: The selective anandamide transport inhibitor VDM11 attenuates reinstatement of nicotine seeking behaviour, but does not affect nicotine intake

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All the animal training, animal feeding, intravenous catheterization surgeries, self administration training and drug treatment for the self administration studies were preformed by Gamaleddin I except for the experiment testing the effect of VDM11 on nicotine induced reinstatement of nicotine seeking which was performed by Guranda M. Gamaleddin I performed all the statistical analysis and wrote the manuscript. Drs. Le Foll B and Goldberg SR were involved in the study design for the all the experiments and made the necessary revisions to the manuscript.

Reprinted from British Journal of Pharmacology, Volume 164, Gamaleddin et al., “The selective anandamide transport inhibitor VDM11 attenuates reinstatement of nicotine seeking behaviour, but does not affect nicotine intake.” Copyright (2012) with permission from John Wiley and Sons ©2011 Wiley. All right reserved
ABSTRACT

The endocannabinoid system appears to play a pivotal role in mediating the rewarding and reinforcing effects of nicotine. Recent studies have shown that the inhibition of fatty acid amide hydrolase (FAAH) attenuates reinstatement of nicotine-seeking induced by nicotine priming and nicotine-associated cues. FAAH hydrolyses the endogenous endocannabinoid anandamide, as well as other non-cannabinoid ligands such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA).

Since OEA and PEA are able to attenuate both nicotine-taking and nicotine-seeking behaviour, the specific role of anandamide remains unclear. In this study, we have tested the selective anandamide uptake inhibitor, VDM11, which elevates anandamide levels without affecting levels of OEA/PEA, on nicotine-taking and nicotine-seeking behaviour.

We used a nicotine intravenous self-administration model in rats to assess the effect of VDM11, given IP, on nicotine taking using fixed and progressive ratio schedules of reinforcement as well as on reinstatement of nicotine-seeking induced by nicotine priming and nicotine-associated cues. VDM11 did not affect levels of responding for nicotine under fixed-ratio and progressive-ratio schedules of reinforcement. In contrast, VDM11 dose-dependently attenuated reinstatement of nicotine-seeking behaviour induced by nicotine-associated cues and nicotine priming. These results indicate that ligands elevating anandamide levels could have therapeutic value for preventing relapse into nicotine-seeking behaviour and should be tested in humans trying to quit smoking.
INTRODUCTION

The endocannabinoid system is involved in drug-taking behaviour and relapse for various drugs of abuse, including nicotine (De Vries and Schoffelmeer 2005; Le Foll and Goldberg 2005; Le Foll, Forget et al. 2008a). The endocannabinoid system consists of the endocannabinoids [mostly anandamide and 2-arachidonoylglycerol (2-AG)], the target receptors for those endocannabinoids (cannabinoid CB₁ and CB₂ receptors, but also non-cannabinoid targets for anandamide) (Alexander 2009), enzymatic degradation systems (through fatty acid amide hydrolase (FAAH) for anandamide and monoacylglycerol lipase for 2-AG) and a putative transport uptake system (Di Marzo 2006; Di Marzo, Melck et al. 1998; Di Marzo, De Petrocellis et al. 2001; Mechoulam, Frde et al. 1998; Sugiura and Waku 2002a; Piomelli 2003).

Preclinical and human clinical studies (Le Foll, Forget et al. 2008a) have indicated that blocking endocannabinoid transmission by CB₁ receptor antagonists/inverse agonists could be a useful strategy for the treatment of human smokers. Rimonabant, a CB₁ receptor inverse agonist used in Europe for the treatment of obesity (Scheen, Finer et al. 2006; Burch, McKenna et al. 2009; Leite, Mocelin et al. 2009), increased the smoking cessation rates in controlled trials (Le Foll, Forget et al. 2008a). Unfortunately, the use of rimonabant has been associated with increased risk of anxiety and depression (Moreira, Grieb et al. 2009; Nathan, O'Neill et al. 2011) and, hence, rimonabant was withdrawn from the European market at the end of 2008 (Le Foll, Gorelick et al. 2009).

Interestingly, we have recently discovered that elevating endogenous anandamide levels could be an alternative therapeutic strategy. We first evaluated the effects of URB 597 (a FAAH
inhibitor) (Fegley, Gaetani et al. 2005), as pharmacological blockade of FAAH activity prolonged many behavioural and neurobiological effects of anandamide. We found that URB 597 administration reversed abuse-related behavioural and neurochemical effects of nicotine in rats (Scherma, Medalie et al. 2008a; Forget, Coen et al. 2009a). URB 597 elevates not only brain levels of anandamide, but also those of the non-cannabinoid acylethanolamides, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) which act on α-type peroxisome proliferator-activated receptors (PPARα)(Fegley, Gaetani et al. 2005; Bond, P. Leff et al. 1995; Astarita, Di Giacomo et al. 2006a; Mascia, Pistis et al. 2011). As the effects of URB 597 could be mediated by either elevated levels of anandamide, by elevated levels of OEA and PEA, or by combinations of these three endogenous ligands, there is a need to explore further the effects of anandamide alone on nicotine-taking and nicotine-seeking behaviour.

In this study, we evaluated the effect of the selective anandamide uptake inhibitor, VDM11 on nicotine-taking and nicotine-seeking behaviour. VDM11 elevates anandamide levels with minimal effects on levels of OEA, PEA or 2-AG (the other endogenous cannabinoid ligand) both *in vitro* (De Petrocellis, Bisogno et al. 2001) and *in vivo* (van der Stelt, Mazzola et al. 2006). Our aim was to study the effect of selectively elevating levels of anandamide in the brain on IV nicotine self-administration under fixed-ratio (FR) and progressive-ratio (PR) schedules of reinforcement, as well as nicotine-seeking behaviour induced by nicotine-associated cues and nicotine priming. The anandamide uptake inhibitors are of potential therapeutic benefit in the treatment of pain, motor impairments and anxiety (Fernandez-Espejo, Caraballo et al. 2004). If proven to modulate reinstatement of nicotine-seeking behaviour, this class of ligands could have a potential therapeutic benefit as a smoking-cessation therapy.
MATERIALS & METHODS

Animals

All animal care and experimental procedures described in this report complied with the guidelines of the Canadian Council on Animal Care (compatible with NIH guidelines), and were reviewed and approved by the institutional Animal Care Committee. Male Long Evans rats (Charles River, Lachine, PQ, Canada), experimentally naive at the start of the study and initially weighing 250–275 g, were used. All rats were individually housed in a temperature-controlled environment on a 12 h reverse light/dark cycle (lights off from 07:00 h to 19:00 h). Before any experimental manipulation, animals were given a minimum of 7 days to habituate to the colony room, during which they were weighed, handled and received unlimited access to both food and water. After habituation, all rats were diet restricted to five pellets or 20 g daily and had free access to water.

Apparatus

Nicotine IV self-administration studies were carried out in commercially available experimental chambers (Med Associates, St Albans, VT, USA) located in sound-attenuating boxes and equipped with two levers, a house light and two cue lights, one located above each lever. For half the animals, the left lever was the active lever and for the other half, the right lever was the active lever.
Experimental procedures

Food maintained behaviour:

Techniques for initial acquisition of food-maintained behaviour and surgery were similar to those already reported (Corrigall and Coen 1989; Forget, Coen et al. 2009a; Gamaleddin, Wertheim et al. 2012). Animals learned to lever press for food reinforcement on a continuous reinforcement schedule, in which each press on the active lever resulted in the delivery of a 45 mg food pellet. During these acquisition sessions, the house light was on, with no illumination of the cue lights above the levers. Daily 1 h acquisition sessions were conducted for 5 days. Once food-maintained behaviour was acquired, IV catheters were surgically implanted.

Implantation of intravenous catheters:

Surgical procedures for implantation of chronic IV catheters were similar to those already reported (Corrigall and Coen 1989; Forget, Coen et al. 2009a; Gamaleddin, Wertheim et al. 2012). Briefly, catheters were implanted into the jugular vein, exiting between the scapulae. Surgery was performed under anaesthesia induced by xylazine (10 mg/kg, IP) and ketamine hydrochloride (90 mg/kg, IP). Incision sites were infiltrated SC with a local anaesthetic, marcaine (0.125%). Buprenorphine was given for post-operative analgesia (0.03 mg/kg, SC), and a single dose of penicillin (30 000 units, i.m.) was administered at the completion of surgical procedures. Animals were allowed to recover for 1 week before drug self-administration sessions were begun.
Self-administration procedures:

Acquisition of nicotine self-administration was performed under an FR schedule of reinforcement at a unit dose of 30 µg/kg/infusion of nicotine base. Session duration was 60 min. The start of each 60 min session was signalled by illumination of the house light. In the presence of the illuminated house light, 1–5 active lever presses resulted in the delivery of a nicotine infusion. Each infusion was followed by a time out period of 60 s, during which the house light was dimmed, the cue light above the active lever illuminated and lever press responses had no programmed consequences. During the first week of acquisition, response requirements were FR1 (i.e. each active lever press during the time-in period resulted in the delivery of a nicotine infusion). Response requirements were then gradually increased to reach a final value of FR5, by which time self-administration behaviour was stable and the animals had a 15–20 day history of nicotine self-administration. Self-administration sessions occurred mostly 5 days a week.

Testing under the FR5 schedule of reinforcement:

Animals were considered to have acquired stable nicotine self-administration when they pressed the active lever more than twice the number of times they pressed the inactive lever, received a minimum of 10 infusions per 60 min session and had less than 20% variation in the number of infusions earned per session during two consecutive sessions.

Once stability was reached, the animals were given IP injections of vehicle (tocrisolve) to habituate them to the injection procedure for an additional 3 days. Rats (n = 18) were then tested
using IP injections of vehicle or VDM11 at doses of 1, 3 and 10 mg/kg given 30 min before the start of the session, in a counterbalanced, within-subject design.

**Testing under the PR schedule of reinforcement:**

The same group of animals (n = 14) (four animals were excluded due to blocked catheters) was used to self-administer nicotine (30 µg/kg per infusion) under the FR schedule and then was directly switched to a PR schedule where the response requirement increased with each successive injection. The response requirement progression was based on the formula $5e^{0.25 \times [\text{inj. number} + 3]}$, with the first two values replaced by 5 and 10 (modified from Roberts and Bennett 1993). Thus, the response requirements for successive injections were 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. The break point was defined as the highest ratio completed prior to the first 30 min period without a response on the active lever. PR sessions lasted a maximum of 4 h. The animals were allowed 10 days of nicotine self-administration under the PR schedule before testing with the pharmacological compounds began. Testing of VDM11 (1–10 mg/kg, 30 min before the session) was performed using a counterbalanced within-subject design.

**Reinstatement**

Reinstatement testing was performed on a new group of animals (this experiment started with 18 rats, but only 14 rats completed testing on all doses of VDM11 on cue-induced reinstatement due to attrition). After acquisition of nicotine self-administration as described previously, an extinction phase was conducted by withholding nicotine and its associated cues
(house light stayed on and cue lights stayed off throughout the session). Responses on the active and inactive lever were recorded, but had no programmed consequences. An extinction criterion was established for each animal individually and was defined as total active lever responses being less than 20 presses. The extinction criteria had to be maintained for two consecutive days in order to conduct testing. All animals reached extinction criteria within an average of eight extinction sessions. Both extinction and reinstatement sessions lasted for 60 min.

**Effects of VDM11 on cue-induced reinstatement of nicotine-seeking behaviour**

All tests were carried out in a counterbalanced, within-subject design. After each test, extinction was re-established until extinction criteria were obtained for at least two consecutive days. Rats were pretreated 30 min before the session with vehicle or 1, 3 and 10 mg/kg VDM11 in a counterbalanced order to measure the effects of VDM11 on cue-induced reinstatement of nicotine-seeking behaviour. Cue-induced reinstatement tests were conducted under conditions identical to that of self-administration, except that responses on the active lever (on an FR5 schedule) resulted in contingent presentation of the cues (light above the active lever on and house light off for 60 s) without nicotine availability (no infusions). Responses on the inactive lever were recorded but had no programmed consequences. The testing sessions lasted 1 h.

**Effects of VDM11 on nicotine-induced reinstatement of nicotine-seeking behaviour.**

A new group of animals (n = 7) were given same training and extinction and were subsequently used to determine the effects of VDM11 (1, 3, and 10 mg/kg IP 30 min before the session) on nicotine-induced reinstatement. Nicotine priming was performed as described by
Forget, Pushparaj et al. (2010a) and Forget, Wertheim et al. (2010b) by administering 0.15 mg/kg nicotine SC, 10 min before the test session. During the extinction and nicotine-induced reinstatement testing sessions, the cue light above the active lever was always off.

Data analysis

The number of active and inactive lever presses or nicotine infusions was recorded and analysed. To analyse the difference in responses between the active and inactive levers along time in the acquisition of nicotine self-administration under FR and PR schedules and during extinction, we used a two-way ANOVA. To analyse the effects of VDM11 on the number of nicotine infusions earned under the FR and the PR schedule of reinforcement, a one-way ANOVA was performed. For reinstatement studies, a one-way repeated measures ANOVA was used to assess the reinstatement effect and the effects of VDM11 on reinstatement induced by nicotine priming and nicotine-associated cues.

Drugs

(-) Nicotine hydrogen tartrate (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline, the pH was adjusted to 7.0 (±0.2) and the solution was filtered through a 0.22 mm syringe filter (Fisher Scientific, Pittsburgh, PA, USA) for sterilization purposes. All nicotine doses are reported as free base concentrations. Nicotine was administered IV in a volume of 100 µl/kg per injection for self-administration studies or was administered SC at the dose of 0.15 mg/kg for reinstatement studies.
VDM11 [(5Z, 8Z, 11Z, 14Z)-N-(4-hydroxy-2-methylphenyl)-5, 8, 11, 14-eicosatetraenamide] (Tocris Bioscience, Ellisville, MO, USA) was dissolved in Tocrisolve and injected IP at a volume of 1 ml/kg 30 min before the start of the session.

RESULTS

Acquisition of nicotine self-administration behaviour under FR schedule of reinforcement

During the first week of acquisition, responding on the active lever, which had previously been reinforced by food and was now reinforced by nicotine infusion, decreased to relatively low levels. When response requirement was then increased to reach a final value of FR5 over the next 2 weeks, responding on the active lever that was reinforced by nicotine infusion increased to the high levels previously maintained by food, while responding on the inactive lever remained low (Figure 15a). ANOVA revealed a significant effect of time ($F_{14, 644} = 13.35; P < 0.001$), a significant effect of lever (i.e. active vs. inactive; $F_{1, 46} = 44.16; P < 0.0001$) and a significant interaction between time and lever ($F_{14, 644} = 7.77; P < 0.001$).

Acquisition of nicotine self-administration behaviour under PR schedule of reinforcement

Animals achieved stable self-administration under the PR schedule of reinforcement within 9 days, after which subsequent testing with VDM11 started. ANOVA performed on acquisition of nicotine self-administration under PR schedule of reinforcement showed a non-significant effect of time ($F_{8, 208} = 1.08; P = 0.37$), a significant effect of lever (i.e. active vs. inactive levers, $F_{1, 26} = 27.36; P < 0.0001$) and no significant interaction between time and lever ($F_{8, 208} = 1.17; P = 0.31$) (Figure 15b).
Extinction

The data presented in Fig 15c, reflect the extinction pattern for the group of animals (n = 16) used in the cue-induced reinstatement testing (only 14 animals completed testing on cue-induced reinstatement). Animals were subjected to extinction sessions that lasted for 8 days; during which most of the animals reached extinction criteria and testing with VDM11 on reinstatement was started (extinction training was pursued for the remaining rats until they reach the extinction criteria). ANOVA performed on the 8 days of extinction showed a significant effect of time (F_{8,240} = 48.19; P < 0.0001), a significant effect of lever (F_{1,30} = 27.10; P < 0.0001) and a significant interaction between time and lever (F_{8,240} = 27.10; P < 0.0001). LSD post hoc analysis showed no significant difference between responses on the active and inactive levers after the third day of extinction (P= 0.65)
(a) Acquisition of nicotine (30 mg·kg⁻¹ per infusion) self-administration under an FR schedule of reinforcement. A: the number of active and inactive lever presses (mean ± SEM) received in each session under the different schedules of reinforcement (FR-1, FR-2, FR-5) ANOVA showed a significant effect of time ($P < 0.0001$) and active versus inactive lever ($P < 0.0001$), and a significant interaction between lever presses and time ($P < 0.0001$).

(b) Acquisition of nicotine (30 mg·kg⁻¹/infusion) self-administration under PR schedule of reinforcement. B: the number of active and inactive lever presses (mean ± SEM) received in each session under PR schedule of reinforcement. ANOVA showed no significant effect of time ($P = 0.37$), a significant difference between active and inactive levers ($P < 0.0001$), and no significant interaction between lever presses and time ($P = 0.31$).

(c) Extinction of nicotine self-administration behaviour before reinstatement testing. C: the number of active and inactive lever presses (mean ± SEM) received in each extinction session. ANOVA performed on the 8 days of extinction showed a significant effect of time ($P < 0.0001$) and lever ($P < 0.0001$), and a significant interaction between lever presses and time ($P < 0.0001$). LSD post hoc analysis showed no significant difference between responses on the active and inactive levers after the 3rd day of extinction ($P = 0.65$); $*P < 0.05; **P < 0.01$. 

*
Effects of VDM11 on nicotine self-administration under the FR5 schedule

ANOVA showed no effect of VDM11 pretreatment on the number of nicotine infusions ($F_{3, 51}=0.07087$, $P=0.97$), and pair wise comparisons with baseline level indicated that pre-treatment with VDM11 (1, 3 and 10 mg/kg) did not affect the number of nicotine infusions received during the session (Figure 16a).

Effects of VDM11 on nicotine self-administration under the PR schedule

ANOVA showed no effect of VDM11 pretreatment on the number of nicotine infusions ($F_{3, 39}=1.843$, $P>0.05$). VDM11 (1, 3 and 10 mg/kg) at the various doses tested failed to produce any change in the break point values, compared with vehicle (Figure 16b).
Figure 16

Effects of VDM11 on nicotine self-administration under FR5 and PR schedules of reinforcement

(a) Effects of pre-treatment with VDM11 (1, 3 and 10 mg·kg⁻¹, IP) on nicotine (30 mg·kg⁻¹ per infusion) self-administration under the FR5 schedule. Data are expressed as mean ± SEM of the number of infusions obtained during the 60 min session. VDM11 did not affect responding versus vehicle pre-treatment (n = 18); P = 0.97.

(b) Effects of pre-treatment with VDM11 (1, 3 and 10 mg·kg⁻¹, IP) on nicotine (30 mg·kg⁻¹ per infusion) self-administration under the PR schedule. Data are expressed as mean ± SEM of the number of infusions obtained during the 4 h sessions. VDM11 did not affect BP P > 0.05 versus vehicle pre-treatment (n = 14); P = 0.15.
Effects of VDM11 on reinstatement of nicotine seeking induced by nicotine-associated cues

ANOVA performed on active lever presses indicated a main effect of cues per se on reinstatement of nicotine seeking compared with baseline conditions (P < 0.001). ANOVA performed on the active lever presses indicated a main effect of VDM11 (F_{4,52} = 14.22; P < 0.05) and Newman–Keuls post hoc analysis showed that pre-treatment with 3 and 10 mg/kg VDM11, 30 min before the start of the session, attenuated the reinstatement induced by nicotine-associated cues (P < 0.05), as compared with reinstatement under vehicle pre-treatment. Neither presentation of nicotine-associated cues nor VDM11 administration had a significant effect on responding on the inactive lever (F_{4,52} = 1.019; P = 0.40) (Figure 17a).

Effects of VDM11 on reinstatement of nicotine seeking induced by nicotine priming

ANOVA performed on active lever presses indicated a main effect of priming with nicotine (0.15 mg/kg) on nicotine-seeking behaviour, as compared with baseline conditions (P < 0.001: Figure 12b). ANOVA analysis of the active lever presses indicated a main effect of VDM11 (F_{4,24} = 9.963; P < 0.05) and Newman–Keuls post hoc analysis showed that pre-treatment with 3 and 10 mg/kg VDM11, 30 min before the start of the session, attenuated the effect of nicotine priming (P < 0.05), compared with reinstatement under vehicle pretreatment. Neither priming injections of nicotine nor VDM11 (1, 3 and 10 mg/kg) treatment had any significant effect on responding on the inactive lever (F_{4,24} = 0.947; P = 0.43) (Figure 17b).
Figure 17 Effects of VDM11 on reinstatement of nicotine-seeking behaviour induced by presentation of nicotine associated cues and by priming doses of nicotine. (a) Effects of pre-treatment with VDM11 (1, 3 and 10 mg·kg⁻¹, IP) on cue-induced reinstatement of nicotine-seeking behaviour. A significant reinstatement of nicotine-seeking behaviour was produced by presentation of nicotine-associated cues alone (*P < 0.001). Newman–Keuls post hoc analysis after significant ANOVA showed that pre-treatment with VDM11 (3 and 10 mg·kg⁻¹, IP) significantly reduced cue-induced reinstatement of nicotine-seeking behaviour (#P < 0.05), n = 14. (b) A significant reinstatement of nicotine-seeking was also produced by pre-treatment with nicotine (0.15 mg·kg⁻¹) (*P < 0.001). Newman–Keuls post hoc analysis after significant ANOVA showed that VDM11 (3 and 10 mg·kg⁻¹, IP) significantly reduced the reinstatement of nicotine-seeking behaviour induced by a priming injection of 0.15 mg·kg⁻¹ nicotine administered 10 min before the session (#P < 0.05). Data are expressed as mean ± SEM of the number of lever presses: BSL, during extinction; vehicle (Veh) pre-treatment (visual cues); n = 7.
DISCUSSION

Anandamide transport inhibitors represent a novel class of ligands that modulate endocannabinoid neurotransmission. Our results indicate that elevation of anandamide levels by the selective uptake inhibitor VDM11 reduced reinstatement of nicotine-seeking behaviour induced by either presentation of nicotine-associated cues or by nicotine priming. In contrast, VDM11 did not have an effect on responding for nicotine under FR or PR schedules of reinforcement.

Our findings with VDM11 are in agreement the previous results with URB597. URB597 attenuated nicotine-induced reinstatement of nicotine seeking using both IV nicotine self-administration and conditioned place preference paradigms (Forget, Coen et al. 2009a; Scherma, Panlilio et al. 2008b). URB597 also attenuated acquisition of nicotine self-administration under an FR schedule (Scherma, Panlilio et al. 2008b). In contrast, URB597 did not alter nicotine self-administration behaviour under a PR schedule (Forget, Coen et al. 2009a). The present findings obtained with VDM11 indicates that this ligand has a similar profile; that is, it is able to disrupt nicotine seeking induced by both cues and priming, but does not affect established nicotine-taking behaviour. Further studies will be required to evaluate whether VDM11 can affect acquisition of nicotine self-administration behaviour, as it has been reported that URB597 significantly decreases acquisition of nicotine self-administration (Scherma, Panlilio et al. 2008b).

As mentioned earlier, inhibition of FAAH increases levels of several endogenous substances in the brain, including the endocannabinoid anandamide and the non-cannabinoid
fatty acid acylethanolamides OEA and PEA, which are ligands for PPARα (Scherma, Panlilio et al. 2008b). Recently, it has been shown that ligands acting selectively as PPARα agonists can reduce reward-related effects of nicotine (Mascia, Pistis et al. 2011). In those studies, PPARα agonists dose-dependently decreased nicotine self-administration and nicotine-induced reinstatement, but did not alter food- or cocaine-reinforced operant behaviour (Mascia, Pistis et al. 2011). Therefore, the effects observed with URB597 could have been mediated by only PPARα activation by OEA and PEA. It is interesting to note that VDM11 exhibited a different profile from that of the PPARα agonists, being unable in the present experiments to affect established nicotine self-administration behaviour.

One possible limitation of our findings is the fact that the effects could be modulated by non-specific effects on motor response. Beltramo and colleagues have previously reported that intracerebroventricular injection of AM404 (a VDM11 analogue) induced mild hypokinesia as shown by the increase in immobility time and decreased motor behaviour stimulated by dopamine D2 receptor agonists (Beltramo, de Fonseca et al. 2000). However, such effects are unlikely to mediate the effects observed here with VDM11 as we found no significant effect on the ability of the animals to lever press for nicotine under both FR and PR schedules (Figure 16 a &b).

Another limitation of our study is that we tested only one anandamide uptake inhibitor. Further studies evaluating the ability of other anandamide uptake inhibitors, such as AM404, which also elevate anandamide levels selectively, are needed to validate our findings. Interestingly, it has been reported that AM404 administration reduces alcohol self-administration
under an FR schedule of reinforcement but it did not affect reinstatement of alcohol-seeking, suggesting that either there may be differences between AM404 and VDM11, or that anandamide may cause differential effects on drug-taking and drug-seeking depending on the substance under study (Cippitelli, Bilbao et al. 2007).

It is possible that VDM11 could have an inhibitory effect on FAAH enzymatic activity in vitro (De Petrocellis, Melck et al. 2000), however, such an effect has not been reported consistently. Any potential inhibitory effects of VDM11 on FAAH activity could be assay-related and also dependent on the source of the compound or the pH used in the assay, as well as the concentration of fatty acid-free BSA used in the experiment (Fowler, Tiger et al. 2004).

Anandamide and 2-AG are the two main endocannabinoids in the brain (Bisogno, Melck et al. 2000; Hanus, Abu-Lafi et al. 2001; Huang, Bisogno et al. 2002; Porter, Sauer et al. 2002; Di Marzo 2006). Anandamide not only binds to cannabinoid CB1 receptors with high affinity (K_i= 52 nM) (Devane, Hanus et al. 1992; Felder, Briley et al. 1993; Childers, Sexton et al. 1994; Terranova, Michaud et al. 1995) but also acts on CB2 receptors and may also have non-cannabinoid-mediated effects, notably through the transient receptor potential vanilloid (TRPV) ion channels (Di Marzo, Berrendero et al. 2000). The present experiments do not provide information on the downstream mechanisms by which VDM11 acts to produce its effects on nicotine reinstatement. Activation at CB1, CB2 and TRPV receptors are possible mechanisms. Interestingly, like anandamide, 2-AG also binds to cannabinoid CB1 receptors, but with lower affinity (K_i= 15 µM). 2-AG acts as a full agonist at CB1 receptors (Devane, Hanus et al. 1992; Felder, Briley et al. 1993; Childers, Sexton et al. 1994; Terranova, Michaud et al. 1995).
whereas anandamide acts as a partial agonist (Sugiura and Waku 2002a). Because 2-AG is a full agonist and anandamide is a partial agonist, it is possible that anandamide will oppose some effects of 2-AG (Maccarrone, Rossi et al. 2008). It may appear surprising that an experimental approach that increases endocannabinoid tone produces opposing effects. However, the administration of a full cannabinoid agonist, such as WIN 55,212–2, has been shown to increase motivation for nicotine and produce reinstatement of nicotine-seeking behaviour (Gamaleddin, Wertheim et al. 2012). Moreover, such opposite effects have also been described in experiments utilizing nicotine full agonists and partial agonists, with nicotine (full agonist) being able to induce nicotine-seeking (Scherm, Panlilio et al. 2008b), while varenicline (a nicotinic partial agonist) reduces the motivation to self-administer nicotine and decreases cue-induced reinstatement of nicotine-seeking (Le Foll B 2011)(abstract SRNT 2011). These hypotheses will be evaluated through further ongoing studies that will investigate the separate roles of anandamide and 2-AG on nicotine-seeking behaviour.

Our results indicate that elevation of anandamide levels reduced reinstatement to nicotine seeking induced by nicotine-associated cues as well as by exposure to a priming dose of nicotine. As the ligands elevating anandamide levels are likely to be devoid of the psychotropic side effects of the CB₁ inverse agonist rimonabant (Le Foll, Gorelick et al. 2009), this class of ligands may have some therapeutic potential to prevent relapse in abstinent smokers.
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Chapter 5: GENERAL DISCUSSION

7.1 RESULTS OF THE PRESENT STUDIES

The main objective of the work presented here was to investigate the effects of modulating cannabinoid receptors and the endogenous cannabinoid anandamide on nicotine self administration and reinstatement of nicotine seeking behaviour. To this end we used the following stepwise approach:

Step 1- Evaluating the effect of systemic activation of CB receptors on nicotine self administration under fixed and progressive ratio schedules of reinforcement and on the reinstatement of nicotine seeking behaviour induced by nicotine associated cues. We also investigated whether the effects observed with a CB agonist would be reversed by selective blockade of CB1 receptors.

Step 2- Evaluating the effect of systemic, selective CB2 receptor activation and blockade on nicotine self administration under fixed and progressive ratio schedules of reinforcement as well as on the reinstatement of nicotine seeking induced by nicotine priming or nicotine associated cues.

Step 3- Evaluating the effect of selectively elevating anandamide levels, through inhibition of its reuptake into the postsynaptic neuron, on nicotine self-administration under fixed and
progressive ratio schedules of reinforcement as well as on the reinstatement of nicotine seeking induced by nicotine priming or nicotine associated cues.

Throughout these studies the effects of cannabinoid ligands on the reinforcing property of nicotine were examined using an operant self-administration paradigm. The effects of these ligands on nicotine seeking were examined using the reinstatement procedure by examining the effect of re-exposure to cue previously associated with nicotine self-administration or by priming injection with nicotine following extinction of nicotine self-administration behaviour. The reinstatement procedure has been shown to be a reliable paradigm to assess relapse to drug with good predictive and face validity. In our implementation of this paradigm, rats were trained to lever press for nicotine under a fixed ratio (FR) schedule of reinforcement that was gradually increased (FR1 to FR5). Upon completion of schedule requirements, the rats received an infusion of nicotine (30µg/kg/infusion in a volume of 28-45µl according to the body weight of the rat), paired with illumination of a cue light above the lever. Such pairing imposes some reinforcing properties upon cue light, which is originally neutral itself. Thereby, the cue light gains secondary motivational salience and develops the ability to reinstate extinguished nicotine seeking behaviour despite the absence of the primary reinforcer, nicotine (Shaham, Shalev et al. 2003).

Self-administration training was carried out for a minimum of 15 sessions in almost all experiments. Throughout these sessions the rats obtained 15-20 infusions of nicotine per session. This length of training and number of infusions/cue presentations strengthened the nicotine-cue
association allowing for robust reinstatement of nicotine seeking behaviour upon the reintroduction of the cues after extinction.

In the first study, we used a non-selective CB agonist (WIN 55,212-2) (due to the lack of selective CB1 agonists at the time when the study was conducted) to assess the effect of activation of CB receptors on nicotine self-administration under fixed and progressive ratio schedules of reinforcement. WIN 55,212-2 (1mg/kg), significantly reduced the number of nicotine infusions obtained under a FR5 schedule of reinforcement. However, the same dose increased the number of nicotine infusions obtained under a progressive ratio schedule of reinforcement. Furthermore, we tested the ability of a priming doses of WIN 55,212-2 to reinstate nicotine seeking behaviour. Systemic administration of WIN 55,212-2 dose dependently reinstated nicotine seeking behaviour. To determine whether the effects we observed with WIN 55,212-2 were CB1 or CB2 receptor mediated, we attempted to block WIN 55,212-2 induced reinstatement using the selective CB1 inverse agonist rimonabant or the selective CB2 antagonist AM630. We found that WIN 55,212-2 induced reinstatement was blocked by rimonabant but not by AM630, indicating that WIN 55,212-2 induced reinstatement is mostly CB1 receptor mediated. To validate our findings we tested the effect of administration of WIN 55, 212-2 on cue induced reinstatement of nicotine seeking. Our results show that WIN 55, 212-2 dose dependently enhanced cue induced reinstatement and this enhancement was blocked by rimonabant indicating again that it is CB1 receptor mediated. As a control, we tested the effect of WIN 55,212-2 on food self-administration under fixed and progressive ratio schedule of reinforcement. As anticipated, WIN 55,212-2 dose dependently increased food self-administration under fixed and progressive ratio schedules of reinforcement. These findings
suggest that the highest dose of WIN 55,212-2 used in our studies (1mg/kg) does not decrease operant responding per se.

To have a more complete understanding of the role of CB receptors on the reinforcing effects of nicotine, we assessed the role of selective activation and blockade of CB2 receptors, using the compounds AM1241 and AM630 respectively, on nicotine self administration and on the reinstatement of nicotine seeking behaviour induced by nicotine associated cues or nicotine priming.

Our results indicated that neither selective activation nor blockade of CB2 receptors modulated nicotine self-administration under fixed or progressive ratio schedules of reinforcement. Moreover, activation and blockade of CB2 receptors failed to alter the reinstatement of nicotine seeking behaviour induced by nicotine-associated cues or nicotine priming.

We have previously explored the effect of increasing the endogenous cannabinoid anandamide through the inhibition of FAAH (the enzyme responsible for its degradation) on the reinstatement of nicotine seeking behaviour induced by nicotine priming (data not shown). Interestingly, FAAH inhibition using the compound URB 597 significantly attenuated nicotine priming induced reinstatement of nicotine seeking behaviour (Scherma, Panlilio et al. 2008b). URB 597 has been shown to elevate brain levels of anandamide, but also brain levels of the non cannabinoid acylethanolamides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). These ligands act on alpha-type peroxisome proliferator-activated receptors (Bond, P. Leff et al. 1995; Fegley, Gaetani et al. 2005; Astarita, Di Giacomo et al. 2006a; Mascia, Pistis et al. 2011).
The effects of URB 597 could thereby be mediated by either elevated levels of anandamide, by elevated levels of OEA and PEA, or by combinations of these three endogenous ligands. We were interested in delineating the specific role of anandamide in the effects observed with compound URB597 on nicotine-induced reinstatement. Our approach was to inhibit the transport system that re-uptakes anandamide into cells for further FAAH-mediated hydrolysis (Freund, Katona et al. 2003; Piomelli 2003; Di Marzo, Bifulco et al. 2004; Glaser, Kaczocha et al. 2005; Moore, Nomikos et al. 2005). For this approach, we administered the selective anandamide transport inhibitor VDM11. We tested the effect of VDM11 on nicotine self administration under fixed and progressive ratio schedules of reinforcement, and on the reinstatement of nicotine seeking behaviour induced by nicotine associated cues and nicotine priming. One important advantage of VDM11 is that it does not bind to vanilloid receptors as do other compounds in its class such as AM404 (Zygmunt, Chuang et al. 2000). Our results showed that VDM11 dose dependently attenuated reinstatement of nicotine seeking behaviour induced by both nicotine-associated cues and nicotine priming but failed to modify nicotine self-administration behaviour under fixed and progressive ratio schedules of reinforcement. Hence, our results point to a pivotal role of anandamide in the effects observed with URB597 which similarly attenuated the reinstatement of nicotine seeking without modifying stable nicotine self administration (Scherma, Panlilio et al. 2008b; Scherma, Fadda et al. 2009).
In the present experiments, nicotine, at a dose of 30 µg/kg/infusion, supported robust self administration under a fixed ratio schedule of reinforcement in male Long Evans rats; which is comparable to previous reported studies (Corrigall 1999; Donny, Caggiula et al. 1995; Paterson, Froestl et al. 2004; Paterson and Markou 2003; Shoaib, Schindler et al. 1997).

The major finding in this experiment is that the highest dose of the cannabinoid agonist WIN 55,212-2 decreased nicotine self-administration under an FR5 schedule of reinforcement while increasing the break point under a PR schedule (Fig. 4b). It has been demonstrated that changing the unit dose of nicotine available under the FR schedule strongly affects rates of responding and the number of infusions earned by the animals (Donny, Caggiula et al. 1998; Le Foll, Wertheim et al. 2007). The 30 µg/kg/infusion dose of nicotine has been previously demonstrated to produce the highest level of responding (Corrigall and Coen 1989). It has been previously noted that, changes in responding under FR schedule can be difficult to interpret, as an increase or a decrease in responding could be as result of an increase or decrease in the reinforcing efficacy of the drug (Arnold and Roberts 1997).

To assess if the decrease in responding induced by WIN 55, 212-2 was due to a decrease or an increase in the reinforcing effects of nicotine, we studied the effects of WIN 55, 212-2 on responding maintained by a lower and a higher unit dose of nicotine, 10 and 60µg/kg/infusion respectively (Fig. 4a). While changing the unit dose of nicotine (10 & 60 µg/kg/infusion)
decreased the level of responding of the animals for nicotine, pre-treatment with WIN 55,212-2 did not modify the responding of the animals under these doses. This could be attributed to a floor effect as the responding of the animals at these doses was at a significantly lower level than that observed for the 30 µg/kg/infusion dose. Given that there is a more linear relationship between the unit dose of nicotine and the motivation to self-administer nicotine when a PR schedule is used (Donny, Caggiula et al. 1999; Le Foll, Wertheim et al. 2007), we studied the effects of WIN 55, 212-2 on responding for nicotine (30 µg/kg/infusion) under a PR schedule (Fig. 5). WIN 55, 212-2 significantly increased the motivation for nicotine under this schedule, as reflected by the average breaking point values of 56 versus 95 obtained under vehicle and 1 mg/kg WIN 55, 212-2, respectively (Fig. 5a). The apparent opposite results obtained under the FR and PR schedule may initially appear to be difficult to reconcile. The decrease in responding for nicotine under the FR schedule did not appear to be due to motor-disruptive effects of WIN 55, 212-2 administered alone since responding for food was increased by WIN 55, 212-2 using FR5 and PR schedules (Fig. 6). This conclusion is in agreement with the findings reported by (Wegener, Kuhnert et al. 2008) on locomotion using the open field paradigm. The increase in responding for food could be attributed to the appetite stimulant effect of CB receptor activation and is consistent with previous reports (Higgs, Barber et al. 2005; Solinas and Goldberg 2005a).

The fact that administration of WIN 55,212-2 decreased responding for nicotine under the FR schedule could likely be explained by an increase in reinforcing effects of nicotine leading the animals to reduce their responding. This explanation is supported by the increase in break point elicited by the same dose of WIN 55, 212-2 under PR schedule. Another less likely explanation for the decrease in responding under FR schedule is the synergistic disruption in
locomotor activity resulting from of WIN 55, 212-2 and higher levels nicotine infusions. The absence of disruption noted with WIN 55, 212-2 under the PR schedule could be attributed to the lower intake of nicotine under this schedule (7.1 ± 0.8 infusions received in 4 hours under the PR schedule compared to 23 ± 2.2 infusions in 1 hour under the FR schedule).

Therefore, it appears that WIN 55, 212-2 significantly increased the motivation for nicotine under the PR schedule, and that this effect of WIN 55, 212-2 could not be elicited under the FR5 schedule.

Our findings with nicotine are in apparent agreement with a previous report which showed that systemic pre-treatment with 3mg/kg of the CB agonist Δ9THC significantly decreased heroin self administration under fixed ratio schedule of reinforcement (Solinas & Goldberg 2005). On the other hand, 1mg/kg dose of Δ9THC increased heroin self-administration under progressive ratio schedule of reinforcement. Moreover, similar to our findings, the same study demonstrated that pre-treatment with 1mg/kg of WIN 55, 212-2 increased the motivation for heroin under PR schedule of reinforcement. Findings reported by Solinas and Goldberg (2005), in addition to our findings, highlight the need to use different schedules of reinforcement to evaluate the effects of any compound on the reinforcing effects of drugs of abuse as different outcomes and interpretations can be derived with the use of only one schedule. It should be noted that WIN 55,212-2 administration significantly decreases cocaine self-administration under an FR schedule (Fattore, Martellota et al. 1999), an effect that has been proposed by the authors as reflecting a blockade of the reinforcing effects of cocaine rather than a locomotor impairment. Further experiments comparing the effects of WIN 55,212-2 on responding maintained by
cocaine under different schedules of reinforcement are needed to delineate the possible involvement of non-specific motor effects.

The paradoxical findings observed under fixed and progressive ratio schedules are interesting, and indeed highlight the significance of using both FR and PR schedules to study the reinforcing effects of different ligands. Our findings suggest that each of the two schedules measures behaviours that are mediated through separate, though overlapping, neurobiological pathways. While FR schedule measures the qualitative positive reinforcing effects of nicotine, PR schedule is useful in measuring its reinforcing efficacy.

7.3 SYSTEMIC ADMINISTRATION OF THE CB AGONIST WIN 55,212-2 DOSE DEPENDENTLY REINSTATED NICOTINE SEEKING BEHAVIOUR THROUGH A CB1 RECEPTOR MECHANISM

This study was the first to demonstrate that stimulation of CB1 receptors induces the reinstatement of nicotine seeking (Fig. 4). These findings are consistent with the findings of De Vries et al. (2001) that activation of CB receptors reinstates cocaine seeking (De Vries et al. 2001). Mounting evidence has shown that blockade of CB1 receptors attenuates the reinstatement of drug-seeking behaviour and that this effect is confirmed with a large variety of drugs of abuse (De Vries & Schoffelmeer 2005; Le Foll & Goldberg 2005a). It appears clear that the effects observed with WIN 55,212-2 are produced through CB1 receptors, as a CB1 inverse agonist reversed the effects of WIN 55,212-2, while a CB2 antagonist could not (see Fig. 4b).
Therefore, our results support the critical role of CB1 receptors in the reinstatement of drug seeking across various drugs of abuse.

**7.4 SYSTEMIC ACTIVATION OF CB RECEPTORS DOSE DEPENDENTLY ENHANCED CUE INDUCED REINSTATMENT OF NICOTINE SEEKING THROUGH A CB1 RECEPTOR MECHANISM**

Administration of WIN 55,212-2 also potentiated the ability of the presentation of nicotine-associated cues to reinstate nicotine seeking (Fig. 5). However, WIN 55,212-2 also directly induced nicotine seeking; hence we cannot exclude the possibility that some of those effects were mediated through potentiating the effects of nicotine-associated cues. An important role of CB1 receptors in the reactivity to drug associated cues has been previously proposed (De Vries and Schoffelmeer 2005; Le Foll and Goldberg 2005a). Notably, this influence has been previously reported by our collaborators and others on reactivity to nicotine-associated stimuli using the nicotine-induced place preference paradigm (Forget, Hamon et al. 2005; Le Foll and Goldberg 2005a) and the reinstatement paradigm (Forget, Coen et al. 2009a). This is further supported by a study which showed that pre-treatment with delta-9-tetrahydrocannabinol ($\Delta 9$THC) enhanced cue induced reinstatement of amphetamine seeking (Anggadiredja, Nakamichi et al. 2004).

On the other hand it has been previously shown that exposure to anxiogenic stimuli such as foot-shock can produce reinstatement of nicotine seeking (Buczek, Le et al. 1999). One possible limitation of the current study is a possible anxiogenic effect of WIN 55,212-2 that could lead to
an increase the responding on the active lever. However, studies with mice have shown that similar doses of WIN 55,212-2 produced anxiolytic effects (Haller, Varga et al. 2004). In agreement, cannabinoids have been shown to decrease amphetamine induced anxiety-like behaviours (Hayase, Yamamoto et al. 2005).

7.5 SELECTIVE ACTIVATION OF CB2 RECEPTORS DID NOT MODIFY NICOTINE SELF ADMINISTRATION AND REINSTATEMENT OF NICOTINE SEEKING BEHAVIOUR.

We demonstrated that selective activation of CB2 receptors failed to modify nicotine self administration under fixed ratio-5 and progressive ratio schedules of reinforcement. Our findings with AM1241 are in agreement with results reported by Ishiguro and colleagues (2007) on alcohol intake using the selective CB2 agonist JWH015. In that study, selective activation of CB2 receptors did not affect alcohol intake in C57Bl/6 mice under fixed ratio schedule of reinforcement. However, the same study using the same strain of mice showed that JWH015 was able to increase alcohol intake in mice subjected to chronic mild stress, which is a paradigm not utilized in our study. These findings were later replicated by the same group which also reported that blockade of CB 2 receptors decreased food consumption in C57Bl/6 mice but failed to produce significant changes in food intake for Balb/c and DBA/2 mice (Onaivi, Ishiguro et al. 2008). Moreover, the study by Ishiguro et al. (2007) demonstrated that mice with a high alcohol preference showed reduced CB2 receptor gene expression while mice with little preference to alcohol showed no changes in CB2 receptor gene expression.
In contrast, Xi et al. (2011) demonstrated that systemic intranasal and local intra accumbal activation of CB2 receptors using the selective CB2 receptor agonist JWH133 produced a dose dependent decrease in cocaine self administration under fixed and progressive ratio schedules of reinforcement. Moreover, the same study showed that CB2 receptor activation attenuated cocaine induced increases in locomotion and cocaine induced increases in extracellular dopamine in the nucleus accumbens in wild type and CB1 receptor knockout mice, but not in CB2 knockout mice, confirming that the effects observed were CB2 receptor mediated (Xi, Peng et al. 2011). The discrepancy between our findings with AM1241 on nicotine self-administration under fixed ratio schedule and the findings of Xi and colleagues (2011) could be attributed to the difference in substance of abuse (nicotine vs. cocaine), the strain of animals (rats vs. mice), the difference the CB2 agonist used (JWH133 vs. AM1241) and finally the difference in schedule used (FR5 vs. FR1).

7.6 SELECTIVE BLOCKADE OF CB2 RECEPTORS DID NOT MODIFY NICOTINE SELF ADMINISTRATION OR REINSTATMENT OF NICOTINE SEEKING

Similar to our findings with CB2 activation, systemic administration of CB2 antagonist AM630 did not affect the responding of the animals for nicotine self-administration under the fixed ratio-5 or the progressive ratio schedule of reinforcement. Our findings with AM630 on nicotine self administration under a fixed ratio schedule are in agreement with results reported by Ishiguro and colleagues (2007) that demonstrated that CB2 receptor blockade did not modify alcohol consumption in C57Bl/6 mice. However, AM630 was able to reduce alcohol
consumption in the same strain of mice subjected to chronic mild stress (Ishiguro, Iwasaki et al. 2007). Moreover, Xi et al. (2012) showed that neither systemic administration of AM630 nor AM251 (selective CB1 inverse agonist) modified cocaine self-administration in mice. However, in the same study, systemic administration of AM630 blocked a JWH133 induced decrease in cocaine self-administration. Furthermore, the same study demonstrated that intra-accumbens administration of AM630 increased extracellular levels of dopamine and locomotion in wild type and CB1−/− mice, but not in CB2−/− mice. Additionally, administration of AM630 in the nucleus accumbens also blocked the reduction in cocaine self-administration and extracellular dopamine produced by systemic administration of JWH133 (Xi, Peng et al. 2011).

It should be noted that data on the behavioural properties of AM1241 are relatively scarce and are mainly limited to studying its effects on locomotion, catalepsy, immotility and antinociception. We selected a dose range that had potent antinociceptive effects, yet no locomotor, cataleptic or ambulatory side effects (Yamamoto, Mikami et al. 2008). The doses used in our studies are similar to doses used by several previous studies (Malan, Ibrahim et al. 2002; Ibrahim, Porreca et al. 2005; Rahn, Makriyannis et al. 2007). Similarly, AM630 has been seldom tested in drug dependence paradigms and thus we used a relatively wide dose range of AM630 based on several prior reports (Garcia-Gutierrez, Perez-Ortiz et al. 2010; Sticht, Long et al. 2011).

Our results have thus indicated that, compared to CB1 receptors, CB2 receptor activation and/or blockade does not modulate the reinforcing effects of nicotine. It appears logical to speculate that this apparent difference in function is due to differences in receptor density and
co-localization between the two receptors in areas related to memory and reward. Indeed immunohistochemical studies have shown that localization of CB2 receptors partially overlaps with CB1 receptor distribution in areas of the brain such as the hippocampus and the substantia nigra reticulata (Brusco, Tagliaferro et al. 2008b; Onaivi, Ishiguro et al. 2008). However, several bodies of evidence have showed that the distribution of both receptors is also distinct in other areas of the brain related to reward and memory, such as the internal segment of the globus pallidus, caudate nucleus and putamen (Graybiel 2005). Interestingly, it has been shown that even in areas of the brain where CB1 and CB2 receptors overlap, such as in the hippocampus and the substantia nigra reticulata, the CB1 receptors are located presynaptically while CB2 receptors are located postsynaptically (Brusco, Tagliaferro et al. 2008b). Moreover, CB2 receptors in the hippocampus and the substantia nigra reticulata are present in a specific manner, mostly in the cytoplasm of neuronal cell bodies and dendrites. These areas are also shown to be heavily enriched with CB1 receptors that are mainly presynaptic and expressed mostly in GABAergic neurons. The type of neurons expressing CB2 receptors remains to be elucidated (Brusco, Tagliaferro et al. 2008b). While the neurobiological and behavioural implications of the presynaptic versus postsynaptic localization of CB1 and CB2 receptors respectively is still unknown, it is tempting to speculate that this distinction could explain the distinct functional effects observed following their modulation.
7.7 SYSTEMIC ADMINISTRATION OF THE ANANDAMIDE REUPTAKE INHIBITOR VDM11 ATTENUATED REINSTATEMENT OF NICOTINESEEKING BEHAVIOUR BUT FAILED TO MODIFY NICOTINE SELF ADMINISTRATION

Systemic administration of VDM11 did not modify nicotine self administration under fixed ratio-5 or progressive ratio schedules of reinforcement. Systemic administration of VDM11 dose dependently attenuated the reinstatement of nicotine seeking induced by nicotine associated cues and nicotine priming.

One important reason for the choice of the compound VDM11 is that unlike its analogue AM404 it does not activate vanilloid receptor TRPV1 (De Petrocellis, Bisogno et al. 2000; Zygmunt, Chuang et al. 2000). This allows us to conclude that the effects observed with VDM11 are mostly due to elevation of the levels of anandamide and not due to non-specific binding to TRPV1 receptors. One possible limitation of our findings is that this class of compounds has also been shown to decrease reuptake of the other main endocannabinoid in the brain, 2-arachidonoylglycerol (2-AG) (Bisogno, MacCarrone et al. 2001; Hajos, Kathuria et al. 2004). However, it has been reported by, de Lago and colleagues (2005) that VDM11 has shown to be equally potent at increasing both AEA and 2-AG levels, but at different times – 1hr and 5hrs, respectively (de Lago, Petrosino et al. 2005). Given the design of our reinstatement paradigm (30-min pre-treatment and 1-hr testing session) the role of 2-AG in resulting from inhibition of uptake should be minimal.

Our results with VDM11 do not provide information on the specific downstream mechanisms by which it produces its effects on reinstatement of nicotine seeking. However,
activation at CB1, CB2 and TRPV receptors by anandamide are possible mechanisms. It may appear rather hard to reconcile that an approach that increases levels of anandamide produces an opposite effect when compared to the administration of WIN 55,212-2, which has been shown to increase motivation for nicotine and produce reinstatement of nicotine-seeking (Gamaleddin, Wertheim et al. 2012). These contrasting findings could be partially explained by the fact that anandamide acts as a partial agonist at the CB1 receptors compared to WIN 55, 212-2 which acts as a full agonist at the same receptors. Such apparently opposite effects are also observed with other full and partial agonists e.g. nicotine vs. varenicline (full vs. partial agonist, respectively). Nicotine is able to induce nicotine seeking (see Fig.12b) and (Scherma, Medalie et al. 2008a), while varenicline is able to reduce the motivation to self-administer nicotine and attenuates cue-induced reinstatement of nicotine-seeking (Le Foll et al., 2011).

Like anandamide, 2-AG also binds to cannabinoid CB1 receptors, but with lower affinity (Ki=15 μM versus 252 nM for anandamide). 2-AG acts as a full agonist at CB1 receptors (Devane, Hanus et al. 1992; Felder, Briley et al. 1993; Childers, Sexton et al. 1994; Terranova, Michaud et al. 1995), whereas anandamide acts as a partial agonist (Sugiura and Waku 2002a). Since 2-AG is a full agonist and anandamide a partial agonist, and since the affinity of anandamide to CB1 receptors is more than 2-AG, it is possible that anandamide may counteract some of the effects of 2-AG that mediate nicotine-seeking behaviour (Maccarrone, Rossi et al. 2008). This hypothesis could reconcile the effects observed here with the findings that CB1 receptor stimulation produces nicotine-seeking (Gamaleddin, Guranda et al. 2011).

Another hypothesis is that supramaximal activation of CB1 receptors, such as that achieved by the exogenous agonist WIN 55, 212-2, might block anandamide or 2AG mediated short or
long term synaptic plasticity. This blockade may in turn affect glutamatergic afferents to
dopaminergic neurons (short term suppression of excitation) by which dopaminergic neurons are
sensitized to drug priming or drug associated cues. These hypotheses need to be addressed in
further ongoing studies that would delineate the respective role of anandamide versus 2-AG on
nicotine-seeking behaviour.

Although the enzymes for AEA and 2-AG hydrolysis e.g. FAAH (fatty acid amide
hydrolase) (see Ueda, Yamanaka et al. 2001 for a review) and the monoacylglycerol lipase
(Dinh, Carpenter et al. 2002) have been cloned, the proteins responsible for the membrane
transport of endocannabinoids have not. The lack of molecular characterization and cloning of
endocannabinoid proteins may in fact suggest that endocannabinoid transport across the plasma
membrane occurs uniquely through passive diffusion. If intracellular transport does in fact rely
on a concentration gradient, this should depend heavily on the rate of intracellular metabolism by
FAAH, monoacylglycerol lipase or other enzymes (Bisogno, MacCarrone et al. 2001; Deutsch,
Glaser et al. 2001; Glaser, Abumrad et al. 2003). This hypothesis is supported by studies
showing that FAAH inhibitors are able to interfere with AEA cellular uptake (Jarrahian, Manna
et al. 2000). Furthermore, data using crystallographic techniques have suggested that FAAH,
although normally located on intracellular membranes, may undergo conformational changes
giving it potential access to the inner layer of a plasma membrane (Bracey, Hanson et al. 2002).

On the other hand, several lines of evidence and indirect observations support the existence
of an endocannabinoid membrane transport protein that is independent from the intracellular
metabolism mediated by degradation enzymes: (i) There are selective compounds that are able to
inhibit the cellular uptake of AEA without affecting the activity of FAAH enzyme (Di Marzo,
Griffin et al. 2002; Ortar, Ligresti et al. 2003; Lopez-Rodriguez, Viso et al. 2003), (ii) AEA accumulation inside the cells is inhibited by AEA uptake inhibitors and increased by some FAAH inhibitors (Kathuria, Gaetani et al. 2003), (iii) AEA uptake can still be demonstrated in cells lacking the expression of FAAH (Deutsch, Glaser et al. 2001; Di Marzo, Bisogno et al. 1999) and (iv) Endocannabinoids such as NADA and noladin are still rapidly taken up by cells, and yet they are refractory to enzymatic hydrolysis (Fezza, Bisogno et al. 2002; Huang, Bisogno et al. 2002).

Several experimental techniques have been used to indirectly validate the hypothesis supporting the functional existence of a membrane protein. These techniques include; (i) Using different cell types and assay conditions in which FAAH has minimal activity, (ii) Using synaptosomes from brains of mice that lack FAAH (Cravatt, Giang et al. 1996), so that there would be no expected interference from enzyme with the uptake process, (iii) Verifying the results obtained through using several AEA analogues that have a similar chemical structure to AEA on AEA cellular uptake, and hydrolysis by the same cell type and (iv) The study of the efflux from cells of de novo biosynthesized AEA, a process which cannot be facilitated by FAAH and was suggested to be mediated by the same protein responsible for AEA uptake (Hillard, Edgemond et al. 1997).

There is evidence that AEA and 2-AG share the same membrane transport mechanism, to the point that it is described by several authors as endocannabinoid uptake (or transport) rather than simply AEA uptake. Thus, it is possible that 2-AG will have partially contributed to the effects we observed with VDM11. Moreover, AEA binds to cannabinoid CB1 receptors with high affinity, but also acts on CB2 receptors and may have non-cannabinoid mediated effects,
notably through the transient receptor potential vanilloid type 1 (TPRV1) (Zygmunt, Chuang et al. 2000). Finally, anandamide has shown to produce nonspecific effects upon calcium signalling and cell proliferation (Kelley and Thayer 2004).

Our differential findings observed with CB receptor activation using the exogenous cannabinoid agonist WIN 55,212-2 and anandamide reuptake inhibitor VDM11 on reinstatement of nicotine seeking behaviour are consistent with studies using the conditioned place preference (CPP) paradigm. Scherma et al. (2012) reported that systemic administration of THC, (an exogenous CB agonist) potentiated the rewarding effects of nicotine, while AM404 (anandamide reuptake inhibitor) attenuated THC induced reinstatement of extinguished nicotine CPP. Our findings using the self administration paradigm and the findings of Scherma, et al. (2012) using the CPP paradigm could be explained by the fact that the effects of AEA transport inhibition are functionally selective, potentiating the actions of AEA only when and where there is a demand for it (Piomelli, 2003; Pertwee, 2005; Solinas, Goldberg et al. 2008) unlike the exogenous CB agonist. The selectivity observed with VDM11 could also be attributed to the fact that it enhances only AEA signal, resulting in a functional selectivity, which may in turn result in regional differences, with specific regions of the brain accumulating more AEA than others (Bortolato, Campolongo et al. 2006).

Although we did not explore the mechanism by which VDM11 exerts its effects on the rewarding effects of nicotine, yet it has been previously shown that administration on nicotine selectively increases levels of anandamide in areas of the brain that are key in mediating reward e.g. the limbic forebrain (Gonzalez, Cascio et al. 2002). Thus protecting anandamide from degradation could activate presynaptic cannabinoid receptors in glutamatergic neurons in the
VTA. This subsequently leads to a reduction in glutamate release and hence reducing the activity of dopaminergic neurons in the VTA and release of dopamine from their terminals in the shell of the nucleus accumbens. This hypothesis is supported by the finding that AM404 reduced nicotine induced elevation in dopamine levels in the nucleus accumbens shell (Scherma, Justinova et al. 2012) which is considered a key region for mediating the reinforcing effects of several drugs of abuse including nicotine (Pontieri, Tanda et al. 1996; Koob 2000; Di Chiara 2002; Wise 2004). However, this hypothesis falls short of explaining why VDM11 attenuates reinstatement of nicotine seeking yet does not modulate nicotine taking under fixed and progressive ratio schedules.

We hypothesize that the effects observed with VDM11 on reinstatement of nicotine seeking could be fully explained by disruption of memory. The NMDA subtype of glutamatergic receptors have shown to involved in memory reconsolidation. Antagonism of NMDA receptors has shown to be amnesic when administered prior to a memory retrieval task. These findings suggest that NMDA receptors are involved in reconsolidation of spatial memories (Przybyslawski and Sara 1997), sucrose-associated memories (Lee and Everitt 2008), fear-associated memories (Lee, Milton et al. 2006), and drug-conditioned place preference memories (Kelley, Anderson et al. 2007; Sadler, Herzig et al. 2007). In addition, it has been shown that enhancing endocannabinoid signalling through anandamide reuptake inhibitor AM404 during extinction training facilitated the extinction of startle elicited by a shock-associated context (Chhatwal, Davis et al. 2005; Bitencourt, Pamplona et al. 2008; Pamplona, Bitencourt et al. 2008). This effect has been blocked by the CB1 inverse agonist rimonabant and hence is proposed to be CB1 receptor dependent (Bitencourt, Pamplona et al. 2008). Acquisition of
extinction was also slowed by blockade of the cannabinoid CB1 receptor (Marsicano, Wotjak et al. 2002; Varvel, Anum et al. 2005b) and accelerated by CB1 agonists or cannabinoid reuptake inhibitors (Chhatwal, Davis et al. 2005; Pamplona, Prediger et al. 2006). Increasing levels of the endogenous cannabinoid anandamide appears to accelerate extinction of both fear and spatial memories (Varvel, Wise et al. 2007).

Given the above information we hypothesize that once the animals are subjected to drug or conditioned stimuli this leads to activation of glutamatergic neurotransmission and activation of NMDA receptors. This in turn leads to the release of anandamide in the post synaptic neuron and its transport in a retrograde manner to activate cannabinoids receptors located primarily in presynaptic neurons. Once activated, CB1 receptors attenuate the release of glutamate and could subsequently lead to deactivation of NMDA receptors in a process known as depolarization induced suppression of excitation. Once, the NMDA receptors are deactivated, this further facilitates the extinction of conditioned stimulus that was developed by repeated exposure to the drug and drug associated cues and hence prevents reinstatement of drug seeking. In conclusion, we propose a model that involves an anandamide mediated facilitation of extinction learning that in turn blocks NMDA subtype of glutamatergic neurons, which are key in reconsolidation, and retrieval of extinguished memory. We further hypothesize that this deactivation of NMDA receptors occurs in areas of the brain related to memory and learning such as the extended amygdala and ventro medial prefrontal cortex. In agreement with the findings observed with anandamide reuptake inhibitors, low doses of WIN 55,212-2 (0.25mg/kg) have also been shown to facilitate extinction of contextual fear. This facilitation was reversed using the selective CB1 receptor antagonist SR141716A (0.2mg/kg) indicating that the effects observed with WIN
55,212-2 are CB1 receptor mediated (Pamplona, Prediger et al. 2006). However, higher doses of WIN 55,212-2 (1.25-2.5mg/kg; IP), that are close to the doses of WIN 55,212-2 that we used in our nicotine self administration and reinstatement experiments, have been shown to have no facilitatory role on extinction of contextual fear (Pamplona, Prediger et al. 2006). This further supports our findings that activation of CB receptors through exogenous CB agonists using higher doses reinstates drug seeking behaviour while enhancement of endogenous cannabinoid signalling attenuates reinstatement of extinguished drug seeking.

It should be stated that further behavioural, electrophysiological and molecular studies are warranted to dissect and verify this hypothesis and delineate any further down stream substrates that could be implicated in this hypothesis and could in turn play a co-facilitator role in the behavioural outcomes observed in our studies.

The effects observed with VDM11 on reinstatement of nicotine seeking behaviour do not appear to be as a result of a motor depressant effect as a similar dose of AM404 (VDM11 analogue) did not reduce activity of animals in the open field paradigm. In addition AM404 did not reduce the time spent in the centre of the field, a measure of anxiety (Scherma, Justinova et al. 2012).

There are several hypotheses (that need further validation) that could explain the rather contradictory yet interesting findings in our different studies.

1. WIN 55,212-2 is a full CB agonist while anandamide is a partial agonist and partial agonists are used and recognized widely as being non reinforcing yet reduce cravings for certain substances of abuse e.g. nicotine and varenicline.
2. The approach used in our experiments increases levels of anandamide in a regionally selective manner i.e. in areas where it has been already produced in the brain, while systemic administration of WIN, 55,212-2 activates almost all the central and peripheral CB receptors in the body i.e. in a non-selective manner.

3. Increasing levels of anandamide in the brain, increases the metabolism of the other endogenous cannabinoid full agonist 2-AG (Maccarrone, Rossi et al. 2008) and hence anandamide could possibly be neutralizing the effect of 2 AG. Moreover, since anandamide has a greater affinity for CB receptors compared to 2 AG, increasing levels of anandamide in certain areas of the brain where it is produced could potentially displace 2 AG from the CB receptors in these areas.

4. Activation of CB1 receptors is implicated in attenuating reconsolidation of extinction memory. This attenuation is proposed to be mediated through inhibition of NMDA subtype of glutamatergic receptors in the basolateral amygdala (BLA) and ventromedial prefrontal cortex (vmPFC). Hence enhancement of endogenous cannabinoid signalling through AEA reuptake inhibitors and low doses of CB agonists could possibly facilitate extinction of conditioned stimuli associated with drug exposure and ultimately lead to attenuation of reinstatement of drug seeking behaviour. Our experiments using the anandamide reuptake inhibitor VDM11 support this hypothesis. However, our experiments using the CB agonist WIN 55, 212-2 show contrasting findings compared to VDM11. The fact that lower doses of WIN 55, 212-2 enhance extinction learning while higher doses of WIN55,212-2 (1.25-2.5 mg/kg) do not facilitate extinction learning
(Pamplona, Prediger et al. 2006) could explain the contrasting findings observed with both drugs on reinstatement of nicotine seeking.

To summarize, given their low side effect profile and the set of characteristics observed with the anandamide reuptake inhibitor VDM11 on reinstatement of nicotine seeking behaviour, this class of compounds could provide a promising direction in the development of medications for the prevention of relapse in abstinent smokers.

**CONCLUSION**

The current body of work is the first to demonstrate that activation of CB receptors through an exogenous agonist increases the motivation for of nicotine and leads to reinstatement of nicotine seeking behaviour and enhances cue induced reinstatement of nicotine seeking after systemic administration. These findings appear to be CB1 receptor induced as the selective CB1 antagonist rimonabant, but not the CB2 antagonist AM630 reversed it. Furthermore, studies conducted to test the effect of CB2 receptor activation and blockade in modulating the reinforcing effects of nicotine revealed negative results indicating that CB2 receptors are not involved in the reward related pathways involved in nicotine addiction. However, these findings could be strain and drug specific and CB2 receptors could be involved in mediating the reinforcing effects with other drugs of abuse (Onaivi, Ishiguro et al. 2008; Xi, Peng et al. 2011).

Interestingly, we found that activation of CB receptors through increasing levels of endogenous cannabinoid ligands produced an effect that is in apparent contradiction with our findings with exogenous full cannabinoid agonist WIN 55,212-2.

We previously demonstrated that elevation of levels of the endogenous cannabinoid ligand anandamide through inhibition of its degradation by the enzyme FAAH attenuated reinstatement
of nicotine seeking induced by nicotine priming (data not shown). We later validated these findings using a more selective approach that increases levels of anandamide without affecting levels of other non-cannabinoid ligands OEA and PEA.

The main findings of the current body of work can be summarized in the following main points:

1. Direct activation of CB receptors through administration of CB agonist WIN 55,212-2 significantly increased motivation for nicotine.

2. Direct activation of CB receptors using WIN 55,212-2 significantly reinstated nicotine-seeking behaviour per se and enhanced cue induced reinstatement of nicotine seeking behaviour.

3. CB2 receptor activation and/or blockade did not modulate nicotine self-administration or reinstatement of nicotine seeking behaviour induced by nicotine associated cues or nicotine priming.

4. Indirect activation of CB receptors through increasing levels of endogenous cannabinoid and non-cannabinoid attenuates reinstatement of nicotine seeking induced by nicotine priming.

5. Selective elevation of levels of anandamide through inhibition of its reuptake significantly attenuated reinstatement of nicotine seeking induced by nicotine associated cues and nicotine priming without affecting nicotine taking or motivation for nicotine.

Taken together, the above findings suggest that CB receptor activation could produce two apparently opposite effects depending on the mode of activation (direct administration of CB full agonist versus elevation endogenous ligand indirectly through inhibition of its degradation). Our
studies also suggest that CB1 rather than CB2 receptors are the key player in effects presented in our studies.

**FUTURE DIRECTIONS**

A number of questions have arisen from the experimental work presented here and elsewhere regarding the mechanism by which anandamide attenuates the reinstatement of drug seeking. Here, we propose several experimental steps in an attempt to delineate the regional and temporal mechanism by which the endogenous cannabinoids produce their paradoxical effects on reinstatement of nicotine seeking compared to exogenous cannabinoids.

1) It would be interesting to delineate the brain regions involved in mediating the effects observed with increasing levels of anandamide. Therefore, we propose that intraregional injection of VDM11 in areas of the brain that may be involved in mediating extinction memory such as the extended amygdala and vmPFC. In addition, we also propose that VDM11 would be administered in areas related to reward such as the shell of nucleus accumbens as a control.

2) Despite the fact that anandamide is an endogenous cannabinoid ligand, it has been hypothesized to also bind to TRPV1 receptors. In order to pinpoint whether the effects produced by anandamide are CB receptor mediated or TRPV1 receptor mediated or through both receptors. To test this, we propose the following two experiments. In the first experiment will attempt to reverse the effects of VDM11 on reinstatement of nicotine seeking through co administration of CB1 receptor antagonist rimonabant. In the second experiment, we will attempt the reversal using the TRPV1 antagonist SB-705498.
3) In the work presented here we explored the potential role of anandamide on reinstatement of nicotine seeking. However, to date, the effects of the endogenous cannabinoid ligand 2-AG on nicotine self administration and reinstatement of nicotine seeking have not been explored. It would be interesting to test the effects of different doses of JZL184 on nicotine self-administration and reinstatement of nicotine seeking behaviour. JZL184 increases levels of 2-AG through inhibition of monoacylglycerol lipase (MAGL) the enzyme responsible for degradation of 2-AG.
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APPENDIX

Standard Operating Procedure
Intravenous Catheterization Surgery

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

LAB. LOCATION: T618

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

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

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

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

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

**SURGICAL PACKS:**

Each surgical pack consists of the following:

- Surgical Draping, folded appropriately: 1 big square sheet (bottom liner), 1 smaller square sheet (for moving the rat around), and 1 rectangular sheet folded length-wise in 2 (for the catheter).
- Cotton-tipped Applicators (2-3)
- Gauze
- Suture thread (silk 4.0) around 45 cm (may vary by user) divided in 2.

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

**PREPARATION:**

**A) Catheter and surgical area preparation:**

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

**B) Rat Preparation:**

- Rats should be assessed on the day before the surgery to ensure their state of health can endure the surgical stressor.
- Rats are brought up to the surgical suite, weighed and the weight recorded on the surgical data sheet before the procedure is started. It is recommended that the rat weighs at least 300 grams.
• Adequacy of anesthesia can be confirmed by pinching the distal portion of the tail or the toes of the hind limb and noting absence of a withdrawal reflex.

• Shave the incision areas: 1- the right ventral region of the neck and 2- the dorsal area between the scapulae.

• Administer the preoperative medications as follows:
  - Marcaine (1.25%) subcutaneously along the incision line (0.1 ml/site).
  - Ketoprofen (Anafen) subcutaneously. Please see SOP for preparation and dose.
  - Ringer lactate can be given for rehydration following the formula 10ml/kg/hr. For surgeries < 45min. it’s up to the user to decide whether or not rehydration is needed.

• Apply eye ointment (Lacrilube) bilaterally to avoid corneal dryness.

• Clean the incision areas with betadine scrub (scrub 3 times in a circular motion starting from the inside to the outside), rinse with 95% alcohol and wipe in the same way, then apply Betadine on the top.

• Move the rat to the surgical table and place on the prepared draping in dorsal recumbency.

C) Surgeon Preparation:

• Hands should be washed thoroughly.

• Sterile gloves should be opened and donned at the surgical table, immediately prior to the beginning of the surgery. Care should be taken to minimize touching non-sterile surfaces including the body of the rat. Handling the rat thereafter should be done using the surgical draping.

SURGICAL PROCEDURE:

• Make an oblique incision of the skin in the ventral region of the right side of the neck. Clear the superficial muscle layer by blunt dissection.

• Locate the jugular vein and using finely serrated forceps, strip it of all fascia. Run a locating suture beneath the vein to facilitate later retrieval.

• Place the rat in ventral recumbence and make a transverse incision between the scapulae. By blunt dissection, clear a subcutaneous pocket in the tissue surrounding the incision. It must be large enough to accommodate both the mesh assembly and the excess catheter tubing.

• Insert the needle driver into the dorsal incision and direct it behind the right forearm and towards the ventral incision. In order to make this “tunnel” as superficial as possible, the tips of the driver should be pointed upwards during this process. Punch through the connective tissue to emerge at the ventral incision. Open the jaws of the driver and securely grab the trocar. Relock the jaws and pull the trocar to the dorsal incision by gently twisting the needle driver. Once the trocar has been “threaded” through both incisions, the catheter may be passed through the trocar. It must be fed in a dorsal-ventral direction; i.e. the insertion tip is introduced into the trocar from the dorsal end. Once a suitable length of catheter is visible at the ventral site, the trocar may be removed simply by pulling it out through the ventral
incision. This procedure, while cumbersome, ensures that there is no stress exerted on the catheter, thereby protecting its integrity.

- Orient the catheter such that the silastic runs medial to the PE and all tubing will ultimately lie flat. Attach a 1 cc syringe with a 22 blunted needle to the catheter and flush with sterile saline. Ensure that there are no bubbles in the line.
- Lift the jugular and using micro-scissors, make an incision 2/3 of the way through the vein. Release the vein.
- Grasp the silastic tip of the catheter and insert into the jugular incision. The tubing should feed in freely, all the way to the heat shrink. It may be necessary to adjust the amount of PE tubing present at the incision site; pulling on the scapular end may do this. Once inserted, the entire assembly should lie flat and neat.
- Tie off the catheter to the jugular with two sutures, one at each end of the heat shrink connection. It is vital that the suture does not slip off onto the silastic, as it will occlude the tubing.
- Anchor the PE 10 tubing to deep muscle with a single suture. The heat shrink is then secured to underlying tissue by a single drop of Krazy glue on its underside.
- Verify the patency of the catheter by drawing back on the syringe and obtaining blood in the PE tubing. Flush back into the rat with a small amount of saline (0.1 ml)
- Close the superficial muscle layer with 1-2 sutures. This serves as additional protection should the rat scratch at his incision. Close the skin with interrupted sutures.
- Turn the rat over and disconnect the syringe from the catheter. Cap the catheter with a filled silastic plug.
- Feed the excess PE 20 tubing into the subcutaneous pocket in a looping fashion. It is quite normal for it to encircle the incision. Insert the mesh assembly and work with it until it lies flat, centered between the scapulae, and superficial to all tubing.
- Suture the skin around the nylon bolt, making sure neither to purse the skin nor catch the mesh in the sutures.
- Move the rat to the recovery area for immediate post surgical care.

IMMEDIATE POST-OPERATIVE CARE:

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