The effects of a neutral cannabinoid-1 receptor antagonist on intravenous nicotine self administration behaviour

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT
Introduction: Tobacco dependence is a chronic disorder that carries the risk of relapse at any
time point during abstinence. It is a major health issue in the world and current
pharmacotherapies have had limited efficacy. Therefore, development and validation of novel
treatments are required.
Objective: Investigate the novel neutral cannabinoid-1 receptor antagonist AM4113 on nicotine
(main psychoactive ingredient in tobacco)-taking behaviour in animals.
Methods: Using the nicotine intravenous- and food control- self administration paradigms, we
tested the acute and chronic (10-days) effects of AM4113 on nicotine- and food-taking
behaviour.
Results: Acute AM4113 treatments (1-, 3-, 10-mg/kg) reduced nicotine self administration.
Chronic AM4113 administration (10mg/kg) produced a sustained reduction of nicotine-taking
behaviour during the course of the treatment. In the similar food control self administration
experiments, AM4113 overall produced no effect.
Conclusion: AM4113 can attenuate nicotine-taking behaviour and its effect is sustained under
chronic treatment.
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ABBREVIATIONS

Δ⁹-tetrahydrocannabinol.................................................................THC
2-arachidonoylglycerol.................................................................2-AG
Cannabinoid -1 and -2 receptor...................................................CB1 and CB2
Fatty acid amide hydrolase.............................................................FAAH
Gamma-aminobutyric acid.............................................................GABA
Monoacylglycerol lipase...............................................................MAGL
Nicotinic acetylcholine receptors................................................nAChR
STudies with Rimonabant And Tobacco USE.....................................STRATUS
World Health Organization..........................................................WHO
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Section I: INTRODUCTION

1.1. Smoking

1.1.1. Tobacco Dependence

Tobacco smoking and its second-hand effects are a major health issue in Canada and the world as a whole. According to a 2013 World Health Organization report, five million deaths worldwide per year can be attributed directly to tobacco consumption, and by 2030 it is expected to reach eight million. An additional 600,000 deaths per year can be linked to second-hand exposure to tobacco smoke (WHO, 2013). These rising mortality numbers are not the only concern, as tobacco consumption continues to still grow on a global scale which can be primarily attributed to its high rate of growth in low- and middle-income countries. Of the one billion smokers currently in the world, about 80% live in these countries (WHO, 2013). The negative consequences of tobacco smoking are not limited to just the health of the smoker and individuals exposed to second-hand smoke. Premature death as a result of tobacco consumption has economic consequences as well, directly impacting one’s family income, increasing the nation’s health care costs, and impacting economic development particularly in the developing countries (WHO, 2013).

In Canada, although consumption has decreased in recent years, there are still currently 4.6 million current smokers (15+ years old) (Health Canada, 2012) and it is estimated that the societal cost of tobacco smoking was around $17 billion dollars in 2002 (Rehm et al., 2006). Furthermore, it is one of the highest preventable causes of morbidity and mortality in the developed world (US Department of Health and Human Services, 2012). Lung cancer, cardiovascular disease and chronic obstructive pulmonary disease are some of the leading causes of death as a result of cigarette smoking (Cohen et al., 2005b). Therefore, even in
developed, high-income nations like Canada, there still remains work to be done in managing this health crisis both in regards to individuals and the health care system. Such work will certainly entail the development of treatments for addicted individuals, and as such a better understanding of tobacco addiction will be required.

The complexity behind tobacco addiction results from a multi-faceted etiology. There are biological-, psychological- and societal- aspects (Cohen et al., 2005b) behind tobacco dependence, and all factors need to be addressed. Curing tobacco dependence will not be feasible if approaches are not taken to incorporate methods of action through cognitive therapy, pharmacological intervention and the changing of societal norms on the views of smoking.

Tobacco dependence is driven by both positive and negative reinforcement where its use promotes states of well being and reduces feeling of discomfort, respectively (Le Foll et al., 2008). Similar to other addictive drugs, it also carries the constant potential of relapse at any stage of abstinence (Cohen et al., 2005b; Forget et al., 2009; Le Foll and Goldberg, 2006), making it very difficult to treat. Although addicted individuals are aware of the negative consequences of drug taking, they continue to seek and take the drug compulsively (Le Foll et al., 2008; Lerman et al., 2007). It is believed that neuroadaptations in the central nervous system are associated with the change from initial use to the state of chronic use and drug dependence (Le Foll et al., 2008; Lerman et al., 2007; Watkins et al., 2000).

1.1.2. Current Smoking Cessation Aids

Currently there are three first-line pharmacotherapies available to assist with quitting smoking. In order of their approval, the available smoking cessation aids are nicotine-replacement therapies, the partial nicotinic agonist varenicline (Chantix/Champix, Pfizer), and the dual dopamine and norepinephrine reuptake inhibitor and nicotinic acetylcholine receptor
(nAchR) antagonist bupropion (Wellbutrin/Zyban, GlaxoSmithKline) (Le Foll et al., 2013; Lerman et al., 2007; Mundey, 2009).

Nicotine-replacement therapies demonstrate the most favourable side-effect profile of the three pharmacotherapies (Mundey, 2009), but the overall efficacy of all the options has been limited, despite being significant (Le Foll et al., 2013), as abstinence is sustained by only 20% of smokers long term (Lerman et al., 2007). It has been found that combining one of these smoking cessation aids with counseling can improve smoking cessation rates versus utilizing pharmacotherapy alone (Mundey, 2009), however the fact remains that more effective treatments are still needed. In addition, when taking into consideration that 80% of worldwide smokers live in low- and middle-income nations (WHO, 2013), treatments that are affordable as well as effective is of utmost importance (Le Foll et al., 2013). Current smoking cessation aids are priced higher compared to cigarettes themselves (Lerman et al., 2007), making the thought of quitting for some smokers financially unreasonable at least in the short term, especially when they can treat their cravings with cheaper cigarettes.

In the development of ligands for the treatment of drug dependence, the development has centred on creating compounds that act either as antagonists or partial agonists (Lerman et al., 2007). The thought behind utilizing an antagonist is that it causes blockade of the target receptor and thus a full agonist is not able to bind to its receptor to elicit its effect. On the other hand, the rationale behind using a partial agonist is that to some degree it behaves as an agonist and to some degree as an antagonist. Therefore, it is able to generate partial efficacy and in the case of nicotine addiction this could help alleviate withdrawal effects in the absence of the full agonist nicotine. In the presence of nicotine, it can behave as an antagonist that prevents nicotine from binding and causing its full effect (Lerman et al., 2007).
1.1.3. Drug Addiction

Drug addiction is characterized as a disorder where an individual compulsively seeks and uses a drug, loses control over the level of its intake despite the known negative consequences associated with its use, and carries the unfortunate reality of chronic relapse at any time point during abstinence (Kenny, 2007; Koob, 2000; Koob and Volkow 2010; Maldonado et al., 2006). If the consequences of using abused drugs are known, one might question why would people do it the first time? The fact is, although the negative consequences may be known, people try and experiment with abused drugs because it is also known these drugs can be pleasurable via the activation of the brain’s reward circuitry (Koob, 2000). The problem of addiction arises when use can no longer be controlled and one transitions into uncontrollable chronic use. Under these unfortunate circumstances, the brain reward system and its associated neurotransmitters become compromised and the system no longer functions normally (Koob, 2000).

According to Koob and Volkow (2010), the disorder of drug addiction carries concurrently the disorders of impulse control and compulsion, with positive reinforcement being linked to the former and negative reinforcement with the latter. Furthermore, it is suggested that addiction can be broken down into a repetitive 3-stage cycle consisting of (1) preoccupation and anticipation stage, (2) binge or intoxication stage and (3) withdrawal or negative affect stage (Koob and Volkow, 2010).

As such, drug addiction can be thought of as a complex mental disorder where the brain reward system becomes hijacked by the abused drug and the actions of the individual are dictated and driven by their compulsion for more of the addictive substance.
1.1.4. *Nicotine*

It was first identified in the U.S. Surgeon General 1988 report (US Department of Health and Human Services, 1988) that nicotine was the main addictive component of the 4,000 chemicals that make up tobacco smoke (Caille et al., 2012; Le Foll and Goldberg, 2006; Le Foll et al., 2008; Le Foll et al., 2013; Mansvelder and McGehee, 2002; WHO, 2013). This decision was heavily influenced by the seminal work of Goldberg and colleagues (1981) where they demonstrated nicotine could maintain high rates of responding in the drug intravenous self-administration model. Nicotine’s addictive potential can be explained in one part by its rapid accumulation in the central nervous system which is within approximately 7 seconds from point of inhalation (Rose et al., 2010). It produces both positive reinforcing effects of tobacco smoke in the form of increased energy, mild euphoria, increased arousal/concentration and reduced stress, as well as decreased symptoms of withdrawal (negative reinforcement) of both somatic (e.g., bradycardia, gastrointestinal discomfort) and affective (e.g., anxiety, dysphoria, irritability ) (see review Watkins et al., 2000). With respect to Koob and Volkow’s (2010) 3-stage addiction cycle, addiction to nicotine is driven more so by withdrawal-induced negative affect and anticipation stage compared to the binge or intoxication stage (Koob and Volkow, 2013).

At the molecular level nicotine is an agonist that binds to the ligand-gated ion channel nAchRs which exist as hetero- and homo-meric receptors comprised of 5 subunits (Caille et al., 2012; Le Foll et al., 2008; Mansvelder and McGehee, 2002). The two main nAchRs are the α4β2* and α7 nAchR, but it has been identified that the α4β2* nAchR is the crucial player in nicotine addiction (Cohen et al., 2005b; Le Foll et al., 2008; Picciotto et al., 1998).
1.1.5. *Nicotine’s Reinforcing Effects and the Mesocorticolimbic System*

Nicotine’s rewarding properties and validation as the active ingredient behind tobacco addiction have been verified using electrical intracranial self-stimulation (ICSS) in animals. This groundbreaking paradigm was first demonstrated to be rewarding in animals by Olds and Milner (1959). In the paradigm, animals are able to self-stimulate their own medial forebrain bundle with an electrical pulse triggering the brain’s reward circuitry. Furthermore, the effects of drugs of abuse on the paradigm can also be tested (Kenny, 2007). Kenny and Markou (2006) demonstrated nicotine lowers the ICSS threshold, therefore implying nicotine potentiates the rewarding effects of ICSS and the animal no longer needs to self-stimulate as much to reach the same threshold it reached in the absence of nicotine. This indicates nicotine carries rewarding properties of its own.

Addiction to nicotine, similar to other drugs of abuse, occurs through the mesocorticolimbic system, also known as the reward pathway for addictive drugs and natural rewards (e.g., food) (Le Foll et al., 2008). The system is located in the ventral midbrain, and dopamine is the main neurotransmitter of the system. Dopamine neurons project from the ventral tegmental area to the nucleus accumbens, olfactory tubercle, amygdala, and hippocampus (Maldonado et al., 2006; Mansvelder and McGehee, 2002; Wise, 2004). The reinforcing effects of drugs of abuse are believed to be a result of dopamine release and the resulting increased level of extracellular dopamine in the nucleus accumbens (Le Foll et al., 2008; Lupica and Reigel, 2005; Mansvelder and McGehee, 2002; Serrano and Parsons, 2011). This has been supported by studies showing abused drugs excite ventral tegmental area dopaminergic neurons and increase dopamine concentrations in the nucleus accumbens (Cohen et al., 2002; Pidoplichko et al., 1997), and, if dopamine is blocked, there is an attenuation of the reward (Le Foll et al., 2008). In addition to its role in reward, dopamine is also involved
cognition, motivation and learning (Le Foll et al., 2008; Lupica and Reigel, 2005; Wise 2004). Dopamine however is not the only neurotransmitter in the mesocorticolimbic system. Gamma-aminobutyric acid (GABA) and glutamate neurons play important modulatory roles (inhibition and stimulation, respectively) of dopamine neurons and dopamine levels (Watkins et al., 2000).

Nicotine interacts with the mesocorticolimbic system via the nAchR and through two connected mechanisms that ultimately promote dopamine release in the nucleus accumbens (Figure 1). The first mechanism, the direct one, is that nicotine binds to the α4β2* nAchR located on dopamine neurons in the ventral tegmental area, which depolarizes them as a result and thereby promotes dopamine release in the nucleus accumbens (Cheers et al., 2007; Le Foll et al., 2008; Pidoplichko et al., 1997). The indirect mechanism is via the GABA and glutamate afferents which modulate dopamine neurons. The α4β2* nAchR is also located on GABA neurons and upon nicotine binding GABA is released to inhibit further dopamine release in the nucleus accumbens. However, nicotine also binds to the α7 nAchR located on glutamate afferents, which help to stimulate dopamine release. Therefore, there is a delicate balance of modulation originating from GABA and glutamate neurons onto dopamine neuron activity, and thus the rewarding effects of nicotine (Cohen et al., 2005b; Mansvelder and McGehee, 2002). Initial rewarding effects of nicotine probably proceed through the α4β2* nAchR located on dopamine neurons (Cheer et al., 2007) with a balance of potentiation from glutamate neurons (via α7 nAchR) and inhibition by GABA neurons (via α4β2* nAchR) (Cohen et al., 2005b; Mansvelder and McGehee, 2002). However, α4β2* nAchRs desensitize more rapidly in comparison to the α7 nAchR. Therefore, following desensitization of the α4β2* nAchR on dopamine and GABA neurons, glutamate neurons remain active longer and prolong stimulation of dopamine neurons, thus potentiating dopamine release in the nucleus accumbens.
and ultimately prolonging nicotine’s reinforcing effects (Cheer et al., 2007; Cohen et al., 2005b; Mansvelder and McGehee, 2002).

**Figure 1. Nicotine’s effect on the mesocorticolimbic reward pathway.** Nicotine’s initial rewarding effects are a result of stimulating ventral tegmental area $\alpha 4\beta 2^* nAchR$ located on dopamine neurons to release dopamine in the nucleus accumbens with a balance of potentiation from glutamate neurons (via $\alpha 7 nAchR$) and inhibition by GABA neurons (via $\alpha 4\beta 2^* nAchR$). The $\alpha 4\beta 2^* nAchRs$ desensitize more rapidly in comparison to the $\alpha 7 nAchR$, and therefore, glutamate neurons remain active longer. This prolongs stimulation of dopamine neurons, thus potentiating dopamine release in the nucleus accumbens and ultimately prolonging nicotine’s reinforcing effects.

PFC: prefrontal cortex; NAcc: nucleus accumbens; VTA: ventral tegmental area. Figure adapted from Cohen et al., 2005b, Maldonado et al., 2006 and Picciotto, 2003.

1.1.6. **Modeling Nicotine Addiction at the Preclinical Level**

Currently there is no preclinical model that fully emulates the human experience of tobacco smoking (Koob and Volkow, 2010), that is, there is no established rodent model of voluntary cigarette smoke inhalation at this time. The best available model to researchers, and
Currently, the gold standard model of nicotine addiction in animals, is the drug intravenous self-administration paradigm (Caille et al., 2012).

In the intravenous self-administration model, animals learn to press on a lever or nose-poke a required number of times (depending on the schedule of reinforcement) to voluntarily self-administer drug intravenously through a surgically implanted catheter that is externally attached to a syringe pump filled with drug (Lerman et al., 2007). The model is able to measure and study the reinforcing effects of the drug in question by the amount of presses and ultimately the number of infusions that the animal voluntarily achieves (Lerman et al., 2007). Furthermore, this model can then be taken advantage of to study the effects of therapeutic agents on the addictive drug’s reinforcing effects (Cohen et al., 2002; Lerman et al., 2007), modeling how effective a drug can possibly be in modulating drug-taking behaviour in the human condition.

Self-administration paradigms with nicotine have been established in rats (Corrigall and Coen, 1989; Donny et al., 1995), mice (Martin-Garcia et al., 2009; Fowler and Kenny, 2011; Yan et al., 2012b) and non-human primates (Le Foll et al., 2007), despite nicotine’s weak reinforcing effects when compared to highly rewarding drugs like cocaine (Caille et al., 2012; Manzardo et al., 2002). Due to nicotine’s weak reinforcing effects, more consideration needs to be put into the design and parameters for nicotine self-administration. The first of these is species. As mentioned, mice, rats and non-human primates have been used, but rats are the most commonly utilized. However, choice of strain is also important for establishing nicotine self-administration with the Long Evans, Wistar, and Sprague-Dawley strains shown to be effective (Caille et al., 2012; Shoaib et al., 1997). Another unique aspect of nicotine intravenous self-administration, again due to nicotine’s weak reinforcing properties, is that the rats need to be food-restricted during the course of the experiment for optimal performance.
Also, they need to first learn operant responding for food (in the absence of any associative environmental stimuli) before nicotine exposure in order to establish nicotine intravenous self-administration (Caille et al., 2012).

A key component of the nicotine self-administration model is the presence of environmental stimuli (e.g., cue light, audible tone) presented concurrently with the infusion of nicotine. It has been reported that the average number of nicotine infusions and number of rats who acquire nicotine intravenous self-administration can be increased by the inclusion of such concurrent cues (Caggiula et al., 2002; Cohen et al., 2005a), similar to the human condition where conditioned associations to environmental stimuli through Pavlovian conditioning also increase drug use (Lerman et al., 2007). The choice of using an operant box with nose-hole versus levers is also an important choice. The work of Clemens and colleagues (2010) suggests nose-hole poking as being more efficient at establishing nicotine intravenous self-administration. In terms of choosing an appropriate dose of nicotine for use in the paradigm, a 30- or 60- µg/kg/infusion is most used, as higher doses produce aversive effects while lower doses are not reinforcing (Donny et al., 1998; Caille et al., 2012). The choice of schedule of reinforcement, which is the response requirement that the animal needs to achieve in order to earn a reinforcement (e.g., nicotine infusion), is also a parameter to consider. The two most commonly used are the fixed-ratio and progressive ratio schedules of reinforcement. Under the fixed ratio, for example fixed ratio-5, the animal needs to respond five times in order to earn a reinforcer. Under the progressive ratio schedule of reinforcement, the response requirement increases progressively for each subsequent reinforcer (Gamaleddin et al., 2012a; Roberts and Bennett, 1993). Lastly, limited access [e.g., 1-hr duration sessions] (Corrigall and Coen, 1989) or extended access [e.g., 23-hr duration sessions] (O’Dell et al., 2007) to nicotine can be used for nicotine self-administration experiments (Caille et al., 2012).
The nicotine self-administration paradigm can also be used to model relapse and test therapeutic compounds for the prevention of relapse. This model is known as reinstatement of nicotine-seeking (Caille et al., 2012; Shaham et al., 2003). In the reinstatement models, animals first acquire stable nicotine self-administration before undergoing extinction sessions (i.e., no nicotine infusions, no cues) to extinguish operant responding for nicotine. Once extinguished, reinstatement tests can be initiated to see if stimuli (e.g., nicotine-associated cue light, nicotine priming injection, foot shock-induced stress) can prompt the animal to once again seek nicotine (i.e., by lever press or nose poke) (Caille et al., 2012). In the cue-induced reinstatement model, the cue is usually a light or tone originally paired with nicotine infusions during acquisition. For cue-induced reinstatement studies, these cues are able to sustain drug-seeking behaviour in the absence of nicotine (Cohen et al., 2005a) as they acquire conditioned reinforcing properties of their own during nicotine intravenous self-administration acquisition through Pavlovian conditioning (Cohen et al., 2005b; Le Foll and Goldberg, 2009). As a result, these cues can elicit responding of its own in the absence of nicotine reinforcement when presented to extinguished animals, and promote nicotine-seeking behaviour. Therefore, this animal paradigm is supposed to model the human relapse phenomena, where a discrete cue (i.e., another individual smoking a cigarette) can stimulate craving and trigger relapse (Caggiula et al., 2001; Caille et al., 2012; Forget et al., 2009; Shaham et al., 2003). In the nicotine priming injection-induced reinstatement, the animal is injected subcutaneously with nicotine prior to a reinstatement session, and the systemic presence of nicotine is in theory supposed to promote responding in the operant chamber though nicotine IV remains unavailable (Caille et al., 2012; Chiamulera et al., 1996). This paradigm models when an abstinent smoker smokes a single cigarette which in turn leads to a full blown relapse (i.e., returns to prior daily smoking habits). The last reinstatement model for nicotine-seeking is stress in the form of electric foot shock.
With humans, this represents when abstinent smokers relapse in response to the build up of stress (e.g., financial problems and accumulating debt) or a stressful situation (e.g., final examination) (Caille et al., 2012). In all reinstatement models described, a therapeutic agent can be administered prior to the reinstatement session to test for its efficacy in reducing nicotine-seeking behaviour in the presence of a cue, nicotine injection or stress.

1.2. Neutral CB1 Antagonist AM4113

1.2.1. Endocannabinoid System and Nicotine Addiction

The influential work of De Vries et al. (2001) served as the catalyst to many studies that have now shown the endocannabinoid system to be critically involved in drug addiction, including nicotine (Le Foll et al., 2013). The endocannabinoid system consists of endogenous cannabinoid transmitters (endocannabinoids), 2-arachidonoylglycerol (2-AG) and anandamide being the primary ones, their receptors notably cannabinoid -1 and -2 (CB1 and CB2, respectively), the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) responsible for degrading anandamide and 2-AG, respectively, and lastly a transport reuptake system (Di Marzo et al., 1998, 2001; Mechoulam et al., 1998; Piomelli, 2003, 2005; Piomelli et al., 2000; Serrano and Parsons, 2011; Sugiura and Waku, 2002; Le Foll et al., 2013). Endocannabinoids are rapidly synthesized on-demand and their degradation proceeds quickly as well (Janero and Makriyannis, 2009; Serrano and Parsons, 2011). They have been identified as retrograde messengers that bind to CB1 and CB2 receptors (Lupica and Riegel, 2005; Wilson and Nicoll, 2001). Anandamide is a partial agonist with high affinity for the CB1 receptor, while 2-AG is a full agonist at both CB receptors that is synthesized in higher levels compared to anandamide, but its affinity for the receptors is lower than that of anandamide.
The CB receptors are G\textsubscript{i/o}-protein coupled receptors (GPCR) that result in the ultimate downstream effect of neurotransmitter release inhibition via inhibition of adenylate cyclase, voltage-gated calcium channels, and activation of the inward-rectifying potassium channels (Janero and Makriyannis, 2009; Serrano and Parsons, 2011). The CB1 receptor is mainly expressed in the central nervous system, including the ventral tegmental area and nucleus accumbens (Cohen et al., 2005b; Herkenham et al., 1991; Maldonado et al., 2006), and in fact is the most widely expressed G-protein coupled receptor in the central nervous system (Janero and Makriyannis, 2009; Maldonado et al., 2006). CB1 receptors are also expressed peripherally in areas such as the gastrointestinal tract (Cohen et al., 2005b; Pertwee et al., 1996). They have been implicated in cognitive, motor, and sensory function, and gastrointestinal physiology (Janero and Makriyannis, 2009). CB2 receptor expression is primarily limited to the periphery, but it has been detected in the brain as well (Cohen et al., 2005b; Janero and Makriyannis, 2009; Serrano and Parsons, 2011).

With respect to endocannabinoid system interaction with systems involved in addiction (i.e., mesocorticolimbic system), endocannabinoids are synthesized on demand and are released Ca\textsuperscript{2+} -dependently from depolarized dopamine neurons (Janero and Makriyannis, 2009; Melis et al., 2004; Riegel and Lupica, 2005). CB1 receptors interestingly do not appear to be expressed on dopamine neurons (Herkenham et al., 1991; Lupica and Reigel, 2005), but are expressed on GABAergic (ventral tegmental area) and glutamatergic (ventral tegmental area and nucleus accumbens) afferents in the mesocorticolimbic system (Maldonado et al., 2006). Therefore, endocannabinoids are able to modulate dopamine transmission indirectly through interaction with GABA and glutamate neurons.
1.2.2. Targeting the CB1 Receptor: Rimonabant and Nicotine Addiction

Targeting the CB1 receptor for the treatment of nicotine addiction has garnered the greatest attention out of all the possible targets in the endocannabinoid system. This is mainly due to the early success and promise of rimonabant (SR141716; Acomplia, Sanofi-Aventis) as a smoking cessation aid. Rimonabant, an inverse agonist for the CB1 receptor, was initially approved as an obesity treatment in over 50 countries worldwide, including the European Union in June 2006, making it the first CB1 ligand to make it to market. In addition to being approved for obesity, it was also undergoing evaluation as a possible effective smoking cessation aid (Cahill and Ussher, 2011; Le Foll et al., 2013).

Smoking cessation related preclinical studies were initiated by Cohen and colleagues (2002), as they were the first to demonstrate rimonabant’s potential. Rimonabant was able to reduce nicotine-taking behaviour under a fixed ratio-4 schedule of reinforcement and they showed it also effectively blocked nicotine-induced dopamine release in the nucleus accumbens shell and the bed nucleus of the stria terminalis. Furthermore, in their drug discrimination studies they demonstrated that rimonabant was not producing its effect by substituting for nicotine and it did not antagonize the nicotine cue (Cohen et al., 2002). Using the progressive ratio schedule of reinforcement, Forget et al. (2009) found rimonabant to be similarly effective at reducing the number of nicotine infusions earned. Rimonabant’s efficacy was not limited to just nicotine-taking behaviour, but also nicotine-seeking behaviour. In cue-induced reinstatement experiments, rimonabant effectively suppressed nicotine-seeking behaviour (Cohen et al., 2005a; Forget et al., 2009), and the same was true for nicotine-priming induced reinstatement (Forget et al., 2009). Lastly, Diergaarde and colleagues (2008) demonstrated that rimonabant was efficacious in context-induced reinstatement, as rimonabant reduced nicotine-seeking in this model as well. The efficacy and consistency of rimonabant
was not just centred on the nicotine intravenous self-administration model either. In the conditioned place preference paradigm, rimonabant reduced the preference for the nicotine-paired side (Forget et al., 2005; Le Foll and Goldberg, 2004; Le Foll et al., 2013). Therefore, all the studies (Cohen et al., 2002; Cohen et al., 2005a; Forget et al., 2005; 2009; Le Foll and Goldberg, 2004) have consistently demonstrated that rimonabant is effective at attenuating the motivational and reinforcing effects of nicotine, suggesting strong promise of its capability to be a novel smoking cessation aid.

Rimonabant’s success was not limited to the preclinical level. In phase III clinical trials, it also demonstrated efficacy in promoting smoking cessation. In two randomized controlled trials [STRATUS (STudies with Rimonabant And Tobacco USe)-EU 2006 and STRATUS-US 2006] investigating rimonabant’s effectiveness in promoting quitting, it was found that the probability of smoking cessation was 1.5 fold greater with 20mg rimonabant treatment compared to placebo (Cahill and Ussher, 2011). However, as quickly as the promise of rimonabant as a smoking cessation aid and obesity treatment came, it also rapidly deteriorated due to the occurrence of side effects. In clinical trials, side effects included depressive disorders, anxiety, insomnia, and thoughts of suicide in addition to the very common undesirable effects of upper respiratory tract infections and nausea (Cahill and Ussher, 2011; European Medicines Agency, 2008; Janero and Makriyannis, 2009; Sanofi Aventis, 2007). Due to these side effects, and the fact that in some select groups of patients the risk for these side effects doubled, the United States Food and Drug Administration (FDA) did not recommend rimonabant for obesity nor smoking cessation indication (Janero and Makriyannis, 2009). Subsequently, on June 29, 2007, this left Sanofi-Aventis no choice but to withdraw its New Drug Application (Sanofi Aventis, 2007; Janero and Makriyannis, 2009). Furthermore, in the European Union, where it was approved for obesity treatment, psychiatric and psychotic
disorders continued to be reported and nervous disorders such as convulsions as well were reported during the post-marketing phase (European Medicines Agency, 2008). Therefore, due to growing complications of psychiatric safety resulting from rimonabant use both in phase III clinical trials and in the market, the European Medicines Agency invoked the withdrawal of rimonabant from the European Union market in October of 2008 (Cahill and Ussher, 2011; Le Foll et al., 2013). The demise of rimonabant was furthered by Sanofi Aventis, the developers and makers of the drug, when they announced on November 5, 2008 that rimonabant was to be removed entirely from the world market, and all ongoing clinical studies involving rimonabant for other indications (including smoking cessation) were halted (Janero and Makriyannis, 2009; Le Foll et al., 2013; Sanofi Aventis, 2008). The domino effect then followed with the other manufacturers of CB1 inverse agonists, Merck (taranabant) and Pfizer (otenabant), halting their development and testing programs for their CB1 inverse agonists (Janero and Makriyannis, 2009; Le Foll et al., 2013).

The undesirable side effects that occurred during rimonabant’s clinical trials and during its time on the market did not all come as a surprise. Nausea was identified at the preclinical level (Parker et al., 2003; Salamone et al., 2007), as well as the presentation of anxiety (Navarro et al., 1997). Furthermore, there was more preclinical evidence that supported the findings seen in clinical trials and in the market. This appeared after the withdrawal of rimonabant from the world market. Under chronic administration paradigms, rimonabant displayed anxiety-like (O’Brien et al., 2013) and depressive-like (Beyer et al., 2010) phenotypes in rats.

1.2.3. Targeting the CB1 Receptor: Supportive Evidence for Use In Nicotine Addiction

The premise of the CB1 receptor as being a viable target for use in nicotine addiction does not solely rely on the efficacy of rimonabant. Other CB1 inverse agonists have been
developed on the background of rimonabant. AM251 and SLV330 are two examples, and they also have demonstrated effectiveness on nicotine-taking and nicotine-seeking behaviour (Shoaib, 2008; de Bruin et al., 2011).

To date, there is only one study to show contrary results in terms of CB1 receptor involvement in nicotine addiction. Cossu et al. (2001) compared CB1 wild-type and knockout mice in nicotine self-administration. They found no difference between the two genotypes, as both acquired nicotine self-administration. Therefore, these results imply either CB1 receptors are not necessary for the acquisition of nicotine intravenous self-administration or that there are compensatory mechanisms involved in the absence of CB1 receptors that facilitate the acquisition (Cohen et al., 2005b).

Studies investigating CB1 receptor stimulation on nicotine addiction have been conducted as well, to demonstrate what effect the alternative to blocking the receptor has on nicotine’s reinforcing effects. Gamaleddin and colleagues (2012a) examined the effect of WIN 55,212-2, a CB1/2 agonist on nicotine-taking and nicotine-seeking behaviour. They found that under the progressive ratio schedule of reinforcement WIN 55,212-2 increased the reinforcing effects and the motivation to press for nicotine compared to baseline levels. Furthermore, in the cue-induced and nicotine priming injection-induced reinstatement models, WIN 55,212-2 potentiated nicotine-seeking behaviour. Since WIN 55,212-2 is a CB1 and CB2 agonist, they cleverly challenged WIN 55,212-2 administration with the CB1 inverse agonist, rimonabant, and CB2 antagonist, AM630 to decipher which receptor was indeed behind the effects being seen in nicotine-seeking. Rimonabant was able to effectively attenuate WIN 55,212-2 induced nicotine-seeking, while AM630 demonstrated no effect (Gamaleddin et al., 2012a). Thus, this would help to support the notion that the CB1 receptor, the main cannabinoid receptor located
in the central nervous system, to be the target of interest for nicotine addiction treatments and not the CB2 receptor.

To further solidify CB2 receptor’s non-involvement in nicotine addiction, Gamaleddin et al. (2012b) tested different CB2 ligands on nicotine self-administration and nicotine-seeking behaviour. Both the CB2 agonist, AM1241 and the CB2 antagonist, AM630, demonstrated no effect on nicotine self-administration under fixed ratio-5 and progressive ratio schedules of reinforcement. Similarly, both CB2 ligands had no effect on cue-induced and nicotine-priming reinstatement of nicotine-seeking (Gamaleddin et al., 2012b). Thus, the results lean towards the opinion of CB2 receptors not being crucial to nicotine’s reinforcing effects.

Recently, however, evidence has arisen to challenge the notion that CB2 receptors are not involved in nicotine’s reinforcing effects. Navarrette et al. (2013) conducted a comprehensive study incorporating both pharmacological (AM630) and genetic manipulation (knockout) of CB2 receptors. Firstly, CB2 knockout mice acquired less nicotine infusions in comparison to wild-type mice. When the wild-type mice were treated with AM630, nicotine self-administration was also attenuated. Secondly, in observations of mecamylamine-precipitated nicotine withdrawal syndrome, CB2 knockout mice demonstrated less somatic signs of withdrawal in comparison to wild-type littermates. Again, when wild-type mice were treated with the CB2 antagonist, AM630, they too showed similar reduced expression of withdrawal somatic signs, as was seen with the CB2 knockout mice (Navarrette et al., 2013). Therefore, due to there being limited studies on CB2 compared to CB1 with respect to nicotine addiction, it is currently inconclusive to as what role, if any, CB2 receptors play in nicotine’s reinforcing effects. Interestingly, it also important to note how the contradicting studies for both CB1 and CB2 were done in mice and that they involved genetic manipulation. Maybe in
rats the CB1 receptor and not the CB2 receptor is vital in nicotine reinforcing effects, while in mice it is vice versa. This however, is another topic of discussion, but interesting, nonetheless.

1.2.4. Alternative endocannabinoid system targets in nicotine addiction

The initial momentum and partial success of rimonabant helped to fuel further research into manipulating the endocannabinoid system in the hopes of identifying a new aid for smoking cessation, as the targets were not limited to just the cannabinoid receptors. There are a wealth of targets including: MAGL, FAAH, and the endocannabinoid re-uptake enzyme, with our group having studied the latter two.

In the study by Forget et al. (2009), in addition to investigating rimonabant, they also tested the irreversible and selective inhibitor of FAAH, URB597. FAAH is responsible for degrading anandamide, and when blocked by URB597, brain levels of anandamide can increase 3 to 5 fold (Fegley et al., 2005; Kathuria et al., 2003; Piomelli et al., 2006). As previously mentioned, nicotine is also a stimulus for increasing endocannabinoid levels in the brain (Gonzalez et al., 2002), and therefore the co-administration of nicotine and URB597 would likely have an additive effect on anandamide levels. For Forget et al. (2009), this effect however did not alter the motivational or reinforcing effects of nicotine, as they witnessed no significant effect of URB597 on nicotine self-administration under the progressive ratio schedule of reinforcement. In cue-induced and nicotine-induced reinstatement, however, Forget and colleagues (2009) did find URB597 efficacious at reducing nicotine-seeking. There is evidence to support the idea that URB597 should be effective in attenuating nicotine-taking and –seeking behaviour because it, like rimonabant, decreases nicotine-induced dopamine release in the nucleus accumbens (Melis et al., 2008; Scherma et al., 2008). These findings however seem contrary to what would be expected from URB597. If anything it should produce results similar to WIN 55,212-2, a CB1/2 agonist, as URB597 increases the
endogenous CB1 agonist anandamide. Therefore, URB597 should potentiate nicotine’s reinforcing effects. A study by Melis and colleagues (2008) gives insight into this conundrum. They demonstrate that the decrease in nicotine-induced dopamine neuronal activity in the mesocorticolimbic system via URB597 administration proceeds through a non-anandamide related pathway (Forget et al., 2009; Melis et al., 2008). URB597, in addition to increasing anandamide levels, also increases the levels of oleoylthanolamide and palmitoylethanolamide, which are non-cannabinoid fatty acid ethanolamides (Forget et al., 2009; Sun et al., 2007). These ethanolamides bind to peroxisome proliferator-activated nuclear receptor α (PPARα), which, when activated, decreases dopamine release that is stimulated by nicotine (Forget et al., 2009; Melis et al., 2008). Therefore, although URB597 targets the endocannabinoid system, its effect on nicotine’s reinforcing effects seems to occur via a non-endocannabinoid pathway.

The alternative to targeting the enzymes that degrade endocannabinoids, is to target the enzymes that re-uptake the endocannabinoids for degradation. AM404, a synthetic anandamide and 2-AG reuptake inhibitor ligand (Bisogno et al., 2001; Gamaleddin et al., 2013) provided the avenue for such testing. Interestingly, AM404 produced similar results to URB597, as it had no effect on nicotine-taking under either schedule of reinforcement, and was only shown to be effective at attenuating the reinstatement nicotine-seeking behaviour following cue-presentation and nicotine priming injection (Gamaleddin et al., 2013). Furthermore, an analog of AM404, VDM11, which is strictly an anandamide re-uptake inhibitor, was also tested in the same set of experiments, and produced the same results as URB597 and AM404 (Gamaleddin et al., 2011). Therefore, according to the results with the most selective ligand tested of the two, VDM11, it appears increasing anandamide levels promotes the reduction of nicotine-seeking behaviour. At first, this too may seem opposite to what is expected from an endogenous agonist based on the results obtained with WIN 55,212-2, a CB1/2 agonist, and
rimonabant which blocks the CB1 receptor as an inverse agonist. However, anandamide is in fact a partial agonist of CB1 receptors and it binds to them with high affinity (Janero and Makriyannis, 2009; Serrano and Parsons, 2011). Thus, anandamide blocks the CB1 receptor to some degree as it prevents 2-AG (full agonist) (Janero and Makriyannis, 2009; Serrano and Parsons, 2011) from binding to the receptor. Thus the stronger potentiating effects of 2-AG on nicotine-induced dopamine release are blocked.

1.2.5. Neutral CB1 Antagonist: AM4113

The endocannabinoid system has been targeted from different avenues in an attempt to identify a new ligand capable of becoming a novel therapeutic smoking cessation aid. The most promising target in the endocannabinoid system to date, however, still remains the CB1 receptor. Firstly, results that have been gathered from targeting the CB2 receptor are inconclusive at this stage. Secondly, the different compounds that have blocked enzyme degradation systems and reuptake systems have only been partially successful. These ligands only appear to be effective at reducing nicotine-seeking, but not nicotine-taking. Therefore, if hypothetically extrapolated to humans, if approved these drugs would likely only be beneficial in preventing relapse, but would provide no assistance to individuals who are current smokers and desire to quit. CB1 inverse agonists have demonstrated the most consistent results and are the only class of drugs that show promise as an agent that would assist quitting and prevent relapse. Therefore, targeting the CB1 receptor via blockade presents itself as the best viable target. It is crucial though, that the blockade of the receptor is neutral with no intrinsic activity, as current opinion has the side effects of rimonabant being due to its inverse agonist properties, although this remains to be proven (Kangas et al., 2013).

The novel neutral CB1 antagonist, AM4113, developed by Dr. Alexandros Makriyannis’ laboratory, is one ligand that meets the criteria mentioned above, and may hold
great promise as a smoking cessation aid. AM4113 is a pyrazole-3-carboxamide analog of rimonabant, but, unlike rimonabant, carries no intrinsic activity at the CB1 receptor (Sink et al., 2008). In forskolin-stimulated cAMP assays in CB1-transfected HEK cells, AM4113 demonstrated no effect on cAMP accumulation, while the CB1 inverse agonists (rimonabant and AM251) increased cAMP accumulation (Chambers et al., 2007; Sink et al., 2008). This result is indicative of AM4113’s neutral antagonistic properties, and differentiation from its predecessor, rimonabant. With respect to its selectivity for the CB1 receptor, AM4113 has demonstrated a 100-fold selectivity for the CB1 receptor versus CB2 receptor, similar to rimonabant (143-fold selectivity) (Chambers et al., 2007; Sink et al., 2008). To verify that AM4113 crosses the blood-brain barrier, thus being able to function in the central nervous system where the mesocorticolimbic reward pathway resides, Chambers et al. (2007) utilized the CB1 agonist (CP55, 940) induced hypothermia paradigm, where the hypothermic effect is a direct result of activated CB1 receptors in the anterior hypothalamus. AM4113 was able to efficaciously block this effect, therefore confirming its ability to cross the blood brain barrier (Chambers et al., 2007).

1.2.6. AM4113 and Food

AM4113, similar to its predecessor rimonabant, was developed and is being tested as a possible obesity treatment as its first indication of interest. This is evident with the great majority of literature testing AM4113 effects in vivo on food-related behaviour. However, these studies can give insight into AM4113’s possible effect on nicotine-related behavior as rimonabant was found efficacious in both behavioural paradigms. AM4113 has been observed to be efficacious at reducing both food-operant responding (Sink et al., 2008, 2009a) and food intake levels (Chambers et al., 2007; Hodge et al., 2008; Sink et al., 2008). Furthermore, an initial study by Chambers and colleagues (2007) treating animals with AM4113 for 5 days,
demonstrated AM4113 to be effective on food intake under chronic administration as well. However, a more recent study by Cluny et al. (2011), where AM4113 was administered daily for 14 days showed AM4113 to have a transient effect on food intake, with AM4113 losing its attenuation effect on food intake by the fifth day onwards when compared to control food intake levels. This outcome potentially has grave implications for the application of AM4113 in nicotine-related behaviours, and furthermore for its clinical potential as a treatment for obesity or smoking cessation, as it would need to be effective long-term due to the nature of the disorders. In addition, this reduction in food intake is believed to be also responsible for the reduced rate of body weight gain in these animals compared to vehicle controls (Chambers et al., 2007; Cluny et al., 2011).

1.2.7. AM4113 and Oral Bioavailability

A novel therapeutic agent’s potential to reach clinical studies, and then the market, is not solely based on its effectiveness, but also its practicality. That is, the drug needs to be user-friendly, and the ideal route for drug administration when considering ease of use is oral. Sink and colleagues (2009a) were the first to attempt oral administration of AM4113 in animals to test its bioavailability and efficacy following this route of administration. Shockingly, oral AM4113 in 1 part Tween 80 and 9 parts saline vehicle failed to affect food responding even at the highest dose tested of 32mg/kg, while the 1mg/kg dose was effective when administered intraperitoneally (Sink et al., 2009a). However, Cluny et al. (2011) later attempted chronic (7-days) AM4113 oral administration using a 4% dimethylsulfoxide and an extra light olive oil vehicle of a 50mg/kg dose, and found it to be effective on food intake on almost all treatment days (days 2-5, 7). However, it should be noted, that comparing the results of the two studies would be difficult as many variables were different (e.g., food-restriction vs. no restriction, measuring food operant responding vs. food intake, acute vs. chronic treatment, vehicle and
dose differences). Nonetheless, under the correct parameters and vehicle composition, it is probable AM4113 has oral bioavailability and efficacy.

1.2.8. **AM4113 and Food: Centrally or Peripherally Mediated?**

Based on Chambers and colleagues (2007) initial study demonstrating that AM4113 does indeed cross the blood brain barrier, it was assumed that AM4113 mediated its effects on food-motivated behaviours within the central nervous system. Sink et al.’s (2009b) study involving intracerebroventricular administration of AM4113, however, dispelled that assumption. They showed intracerebroventricular AM4113 to have no effect on food motivated operant responding, and concluded that AM4113’s effect on food related behaviours may be mediated by CB1 receptors in the lower brainstem or in the periphery (e.g., gastrointestinal tract) (Sink et al., 2009b).

1.2.9. **AM4113 and Side Effect Profile**

With most drugs, the expected therapeutic potential of the drugs is what garners the most anticipation. However, in the case of AM4113, due to its negative connection to its predecessor rimonabant, researchers have been particularly concerned with AM4113’s side effect profile. It was hoped that AM4113 would have the same therapeutic effectiveness as rimonabant, but that the side effects would not be present, since the inverse agonist properties of rimonabant were no longer present. It seems that these hopes may be justified.

With respect to gastrointestinal disorders, rimonabant is known to cause nausea and emesis in humans (European Medicines Agency, 2008). AM4113 has thus far returned no negative indicators for gastrointestinal disorders. Chambers et al. (2007) used morphine-6-glucoronide to induce vomiting episodes in ferrets and they found that AM4113 does not potentiate this effect. Sink and colleagues (2008) observed that AM4113 administration in rats
does not cause conditioned gaping, a marker for nausea in the species, that was seen with the CB1 inverse agonist AM251 (McLaughlin et al., 2005). Therefore, all indications are that AM4113 will unlikely cause nausea or emesis, and that the effect was a consequence of rimonabant's inverse agonist properties. Furthermore, it also helps to support the notion that AM4113’s reduction of food intake is not a direct result of it promoting nausea in the animal (Chambers et al., 2007).

Preliminary results for the appearance of psychiatric disorders in animal models have also looked favourable for AM4113. In the elevated plus maze, a behavioural test used to identify anxiety-like behaviour, the anxiogenic compound FG-7142 and the CB1 inverse agonist AM251 displayed an anxiety-like phenotype, while this was not observed for AM4113 treated rats (Sink et al., 2010). In addition, in the depression related forced swim test, AM4113 demonstrated no effect (Jutkiewicz et al., 2010). Thus, the preliminary profile of AM4113 with respect to promoting psychiatric side effects is promising and appears safer as compared to rimonabant; however, due to the severity and risk associated with anxiety and depression (i.e., suicidal thoughts), these studies should be replicated and verified by other groups. AM4113 should also be tested under chronic administration conditions as was done with rimonabant (Beyer et al., 2010). This will provide a more accurate and complete examination of the AM4113 side effect profile.

Unfortunately, AM4113 is not completely free of side effects. Preclinical studies have identified that AM4113 administration is associated with increased scratching and grooming (Hodge et al., 2008; Jarbe et al., 2008; Sink et al., 2010). This is of concern because itching (pruritus) in humans was a common side effect of the CB1 inverse agonists rimonabant (European Medicines Agency, 2008) and taranabant (Kirkham, 2008). Furthermore, increased scratching and grooming was witnessed with rimonabant (Jarbe et al., 2002; Salamone et al.,
2007) prior to clinical study initiation. Based on this fact alone, it is likely that AM4113’s promotion of grooming and scratching in animals will translate to itching (pruritus) in humans, but to what degree remains to be determined.

1.3. **Statement of Problem, and Purpose and Rationale of the Study:**

Tobacco dependence is a serious worldwide health concern with staggering morbidity and mortality rates (WHO, 2013). Current medications, however, have had limited efficacy and more novel effective therapeutic agents are required to help curb tobacco dependence (Le Foll et al., 2013; Lerman et al., 2007). Rimonabant, a CB1 inverse agonist, was once a promising therapeutic ligand for smoking cessation as it was shown to be effective both at the preclinical (Cohen et al., 2002; 2005a; Diergaarde et al., 2008; Forget et al., 2009) and clinical level (Cahill and Ussher, 2011). However, due the elevated frequency of psychiatric side effects associated with its use, such as anxiety, depression and thoughts of suicide (Cahill and Ussher, 2011; European Medicines Agency, 2008), rimonabant did not succeed. AM4113, a neutral CB1 antagonist and pyrazole-3-carboxamide analog of rimonabant is being considered as the next therapeutic ligand. It is hypothesized that AM4113 will retain the same therapeutic efficacy as rimonabant with respect to smoking cessation, but will be free of the side effects of rimonabant as it is also free of rimonabant’s inverse agonist properties (Le Foll et al., 2013). The purpose of this study was to begin preclinical testing of AM4113 in nicotine addiction behavioural related paradigms. Using the nicotine self-administration paradigm, which is recognized as the gold standard animal model of nicotine addiction, this study plans to test AM4113’s effects on nicotine-taking behaviour.
1.4. Statement of Research Hypothesis

The neutral cannabinoid-1 receptor antagonist AM4113 will attenuate nicotine-taking behaviour in the nicotine intravenous self-administration paradigm.
Section II: **MATERIALS AND METHODS**

**Animals**

Experimentally naïve, male Long Evans rats initially weighing 250-350g (Charles River, Lachine, QC, Canada) were used for all experiments unless stated otherwise. Upon arrival to the facility they were habituated to the housing conditions (temperature controlled room; reversed light cycle: lights off approximately from 730 to 1930h) for approximately 5 days under pair housing and ad libitum food and water. After this period, they were split into single housing and restricted to 4 food pellets (approximately 20g; 5001 Rodent Diet, LabDiet, St. Louis, MO, USA) per day and unrestricted water access for the remainder of the experiment unless stated otherwise. Animals were weighed before every session. All animal procedures and protocols were reviewed and approved by the Centre for Addiction and Mental Health (CAMH) Animal Care Committee in accordance with Canadian Council on Animal Care (CCAC) guidelines for animal research.

**Apparatus**

The food- and nicotine- self-administration experiments were conducted in commercially available chambers housed in sound-attenuating boxes from Med Associates Inc. (St. Albans, VT, USA). The chambers included a house-light, and opposite to it two retractable levers and two cue-lights situated above each lever. For the food self-administration experiments, the chamber also included a food receptacle in between the two levers and a food-pellet dispenser situated outside the chamber, but inside the box. For the nicotine self-administration chambers, there was an external pump that infused nicotine solution via tygon lines that traversed through the box and chamber and directly connected to the animal’s catheter. In all experiments, the start of a session was signaled by the illumination of the house-light and the extension of the
two retractable levers simultaneously. The end of the session was marked by the retraction of the two retractable levers and the house-light going off simultaneously. Each animal had a designated active lever, defined as the lever that is reinforced with a reward (i.e., nicotine infusion or food pellet) if the schedule of reinforcement response requirement is reached, and an inactive lever, defined as the lever that presses on resulted in no programmed consequences. The designation of active and inactive lever was counterbalanced, such that some animals would have the left lever as active while the others would have the right lever designated as active. The operant chambers and designated experimental programs were run through a Med Associates Inc. microcomputer controller box connected to a computer installed with MED-PC software program from Med Associates Inc. (East Fairfield, VT, USA).

**Experiments**

Procedures and techniques were conducted similarly to previously published work by Corrigall and Coen (1989), Forget et al. (2009), Gamaleddin et al. (2011; 2012a,b; 2013), Le Foll et al. (2012), Pushparaj et al. (2013) and Yan et al. (2012a).

*Food-operant training:*

Animals to be used for nicotine intravenous self-administration were first trained to lever press for food pellets (45mg Dustless Precision Pellets, BioServ, Frenchtown, NJ, USA) in an operant food chamber (as described above). During training, the house-light was on at all times and animals had to press the active lever once in order to receive delivery of 1 food pellet into the food receptacle with no presentation of contingent cues (i.e., cue light). Trials included a 5 second time-out period during which lever presses were recorded, but had no consequences. All active lever presses and reinforcement counts were recorded during the session. In addition, presses on the inactive lever at any time had no consequences, but were recorded. Operant
training sessions terminated at the 60-min time point or earlier whenever 100 food pellets had been obtained. Animals received a minimum of 5 sessions of training and by the 5th session animals had to achieve 100 food pellets within 20-min in order to continue on to nicotine intravenous self-administration experiments. In cases when animals did not reach criteria, they were trained for a maximum of 4 additional training sessions, or less if criteria was reached prior to, and were continued onto nicotine intravenous self-administration experiments.

Surgery:
Before surgery, derapen (30,000 U of penicillin G) was first injected subcutaneously at most 72 hours and at least 24 hours prior to surgery, and was continued on a Monday, Wednesday, Friday schedule and stopped 7 days post-operation. To anaesthetize the animal, a mixture solution of ketamine (75mg/kg), xylazine (10mg/kg) and saline was injected intraperitoneally. Once anaesthetized, the animal was prepped (shaved and cleansed at the sites of incision) and was subcutaneously treated with the local anesthetic marcaine (0.125%) at the incision sites. Ketoprofen (5mg/kg, subcutaneously) was also administered pre-surgery and 24 hours after for analgesia. The animal was then implanted with either a lab modified purchased polyurethane catheter (RJVR-10, Strategic Applications Inc., USA; n=3) or a commercially available pre-constructed silastic catheter (IVSA28, CamCaths, Cambridgeshire, United Kingdom; n= 21) which entered into the jugular vein and exited between the scapulae. Animals were given a minimum of seven days of recovery prior to initiating self-administration studies. Catheter patency was maintained with daily heparinized saline (3 units/rat/day) flushes. Catheter patency was verified at time points of suspected catheter failure and at the conclusion of the experiments by first via pulling back blood through the catheter intravenously, and if unsuccessful, by thiopental (1.5mg/rat) or methohexital (3mg/rat) administration intravenously. An animal was considered to be patent if blood was pulled back, and/or if the animal
responded to the thiopental/methohexital challenge by demonstrating either hyperactivity, loss of co-ordination and/or muscle tone.

*Nicotine intravenous self-administration acquisition:*

Animals were trained in 60-min sessions first under fixed ratio-1 (fixed ratio-1) schedule of reinforcement, where 1 press on the active lever resulted in the delivery of a nicotine infusion (30ug/kg/infusion of nicotine base), with presentation of the associated cue light (time lit = time-out period) above the active lever and a time-out period (during which active lever presses were recorded, but had no consequence) of 10 seconds or 60 seconds depending on the experiment (described in detail below). The house-light in the operant box was on at all times except when the schedule of reinforcement count was reached and the cue-light was on. All active lever presses and reinforcement counts were recorded during the session. Presses on the inactive lever at any time had no consequences, but were recorded. Animals were trained on fixed ratio-1 for 5 days and fixed ratio-2 (i.e., 2 presses on the active lever were required to achieve a nicotine infusion) for 3 days. Animals being tested under the fixed ratio-5 schedule of reinforcement underwent 7 days of fixed ratio-5 acquisition training, while those being tested under the progressive ratio schedule of reinforcement underwent 2 days of fixed ratio-5 acquisition training.

*Effects of acute AM4113 on fixed ratio-5 nicotine-taking behaviour:*

Animals (N= 8) were continued on a fixed ratio-5 nicotine intravenous self-administration (60-min session) for the entirety of the experiment. The first 3 sessions following acquisition, animals were injected with saline to habituate them to the injection procedure, and saline injections were continued onwards unless an AM4113 treatment was to be injected. AM4113 treatments were initiated in a counter-balanced within subject design and were only injected
once the animal displayed stability for taking nicotine under saline injections for 2 consecutive sessions which was considered to be the reinforcement count being within 20% of the previous session count and with the active lever press count being twice greater than the inactive lever press count. The experiment was mainly conducted Monday to Friday, with Monday having to be a saline injection session. The time-out period for this experiment was 60 seconds.

Effects of acute AM4113 on progressive ratio nicotine-taking behaviour:

For this experiment, the time-out period during fixed ratio acquisition was 10 seconds, but the duration of the sessions remained the same at 60-min. Following fixed ratio-5 acquisition training, animals were switched to 7 sessions on the progressive ratio schedule of reinforcement which also had a 10 second time-out period, however the session duration was 240-min or automatically terminated following 30-min of no registered active lever presses. In order to achieve each subsequent infusion, the active lever response requirement increased according to the formula \(5e^{(0.25\times\text{inf number})-5}\) (Roberts & Bennett, 1993) except with the modification that the first values were 5 and 10. The next 3 sessions following progressive ratio acquisition, animals (N=9) were injected with saline to habituate them to the injection procedure, and saline injections were continued onwards unless an AM4113 treatment was to be injected. AM4113 treatments were initiated in a counter-balanced within subject design and were only injected once the individual animal displayed stability criteria similar to those described for the fixed ratio-5 experiment. The experiment was mainly conducted Monday to Saturday, with Monday being a saline injection session.

Effects of chronic AM4113 on fixed ratio-5 nicotine-taking behaviour:

Animals (N=7) were continued on fixed ratio-5 nicotine intravenous self-administration (60-min session; 60-seconds time-out) for the entirety of the experiment. The first 3 sessions
following acquisition, animals were injected with saline to habituate them to injection procedure. They were then subjected to: (1) 3 sessions of vehicle treatment, (2) 10 sessions of 10 mg/kg AM4113 treatment, (3) 3 sessions of vehicle treatment, and finally (4) 3 sessions of saline treatment. Sessions were conducted seven-days per week starting from the fifth session of fixed ratio-5 acquisition until the completion of the experiment.

**Food control self-administration experiments:**

**Acute:** A group of 10 rats was used for all acute effects of AM4113 in food control self-administration experiments. An initial pilot study using a 30-min AM4113 pretreatment time was tested, after which a 60-min pretreatment time was used for all other food- and nicotine-related experiments. Both pretreatment times were tested under both schedules of reinforcement (fixed ratio-5 and progressive ratio). Food control self-administration experiments were conducted using the same parameters and procedures as the nicotine self-administration experiments, except: (1) food-operant responding training was not required prior to acquisition training, (2) no intravenous catheterization was conducted on the animals, and (3) a 45mg food pellet served as the reinforcer instead of a nicotine infusion when the animal successfully achieved the response requirement for delivery of a reinforcer.

**Chronic:** A group of 11 rats was used to test the chronic effects of 10mg/kg AM4113 in food control self-administration experiments under both schedules of reinforcement (fixed ratio-5 and progressive ratio). This experiment was conducted similarly to the chronic AM4113 on nicotine-taking experiment except that: (1) food-operant responding training was not required prior to acquisition training, (2) no intravenous catheterization was conducted on the animals, and (3) a 45mg food pellet served as the reinforcer instead of a nicotine infusion when the animal successfully achieved the response requirement for delivery of a reinforcer.
Drugs

For self-administration experiments (-)-nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and the pH of the solution was adjusted to 7.35 +/- 0.15. The solution was sterilized by filtering it through a syringe PURADISC™ 25 AS disposable filter device (G.E. Healthcare, Buckinghamshire, UK). The nicotine dose used was 30ug/kg/infusion (base) with a delivery volume of 100ul/kg/infusion administered as in previously published work by Corrigall and Coen (1989), Forget et al. (2009), Gamaleddin et al. (2011; 2012a,b; 2013), Le Foll et al. (2012), Pushparaj et al. (2013) and Yan et al. (2012a).

The neutral CB1 antagonist AM4113 (N-piperidin-1-yl-2,4-dichlorophenyl-1H-pyrazole-3-carboxamide) was developed and synthesized in Dr. Alexandros Makriyannis’ laboratory (Center for Drug Discovery, Northeastern University). AM4113 was suspended in the previously used vehicle combination of 1 part dimethylsulfoxide: 1 part Tween 80 : 8 parts saline (Hodge et al., 2008; Sink et al., 2008; 2010), and this combination also served as the vehicle control solution for the studies. Doses of AM4113 tested were 0-, 0.3-, 1-, 3- and 10-mg/kg (depending on the individual experiment) using a counterbalanced within subject design where each animal receives each dose in a randomized order. Drug was administered intraperitoneally 60-min prior to the start of the session (unless stated otherwise) with a delivery volume of 1ml/kg. Dose range, route of administration, and delivery volume were based on previously published studies with AM4113 (Cluny et al., 2011; Sink et al., 2008; 2009a; 2010).

Data Analysis

To analyze the acute effects of AM4113 in both nicotine- and food-taking experiments, a within-subject one-way repeated measures analysis of variance (ANOVA) was conducted on reinforcement count which was followed by Bonferroni post-tests (when appropriate)
comparing the doses of AM4113 to baseline saline (BSL) and vehicle control (VEH). For the analysis of the chronic effects of AM4113 in both nicotine- and food-taking experiments a within-subject one way repeated measures ANOVA was performed on reinforcement count followed by Bonferroni multiple comparison tests (when appropriate) comparing last day of saline treatment (Saline-3= session 6) and last day of vehicle treatment (VEH-3= session 9) to sessions 10 through 25. Statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to be statistically significant.
Section III: RESULTS

3.1. Pilot Study: Acute effects of AM4113 on food-taking behaviour with 30-min pretreatment time:

Food self-administration experiments serve as control experiments to drug self-administration experiments and are, in general, conducted before drug self-administration experiments. They serve to identify whether the ligand, in this study AM4113, has any non-specific effects (e.g., motor/locomotory effect) that may affect operant responding, and therefore lead to a false positive in drug self-administration experiments. Therefore, it is ideal for the ligand to have no effect in the food self-administration experiments as this permits for a direct interpretation of the specific effects of the ligand during drug self-administration.

AM4113, however, presents a unique circumstance for the food self-administration control experiments. It, similar to rimonabant, was designed as an anti-obesity agent, and studies (Hodge et al., 2008; Sink et al., 2008; Sink et al., 2009) have shown it to have an effect on food-responding behaviour, as was to be expected. However, this had to be verified in our paradigm prior to initiating nicotine self-administration experiments.
Figure 2. AM4113 administered 30-min prior to food self-administration session reduces food-taking behaviour. Acute effects of AM4113 (30 min pretreatment time) on mean (±SEM) number of food pellets earned under: (A) fixed ratio-5 (Session: 1-hr), and (B) progressive ratio (Session: ≤4-hr) schedules of reinforcement (N=10). * P<0.05 vs. Saline BSL; # P<0.05 vs. VEH (One-way repeated measures ANOVA followed by Bonferroni Post-test)

Figure 2-A and -B display the acute effect of the higher doses of AM4113 (pretreatment time=30min) in reducing food-taking behaviour under fixed ratio-5- and progressive ratio- schedules of reinforcement, respectively. For the acute effects of AM4113 on the fixed ratio-5 schedule of reinforcement (Fig 1. A), a one way repeated measures ANOVA resulted in a main effect of AM4113 treatment [F(4,9)= 5.758, P= 0.0011], with Bonferroni post-hoc tests revealing 3- and 10-mg/kg AM4113 being statistically significant (P<0.05) in reducing the number of food pellets earned as compared to saline-baseline levels. In addition, 10mg/kg AM4113 was also significant (P<0.05) compared to vehicle. 1mg/kg was significantly (P<0.05) different from 3- and 10-mg/kg AM4113. Saline-baseline and vehicle control were not significantly different (P>0.05)

For the progressive ratio schedule of reinforcement equivalent experiment (Fig. 2B), Bonferroni post-hoc tests only detected 10mg/kg AM4113 to be significantly (P<0.05) effective at reducing the number of food pellets earned compared to saline-baseline levels following significant one-way repeated measures ANOVA [F(4, 9)= 2.706, P= 0.0455]. Saline-
baseline and vehicle control were not significantly different from each other, and no significant differences between doses was detected (P>0.05).

3.2. Acute effects of AM4113 on food-taking behaviour:

Following the significant effects of the higher doses of AM4113 on food-taking behaviour, the above experiments were then repeated using the same animals and a 60-min pretreatment time. The longer pretreatment time choice was based on the fact that the effect of 3mg/kg dose disappeared, and the effect of the 10mg/kg lessened from the fixed ratio-5 to progressive ratio experiment. It was hypothesized that this was due to the extended run time of the progressive ratio experiment (fixed ratio-5: 1-hr session vs. progressive ratio: maximum 4-hr session), and that the added run time of the progressive ratio experiment probably contributed to the lesser effect of AM4113 on the food intake paradigm. Since it is ideal for AM4113 to have no effect in the control food self-administration experiment prior to initiating nicotine self-administration experiments, the experiments were repeated using the same group of animals with the longer pretreatment time.

Figure 3. AM4113 administered 60-min prior to the food self-administration session has no effect on food-taking behaviour. Acute effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of food pellets earned under: (A) fixed ratio-5 (Session= 1-hr), and (B) progressive ratio (Session= ≤4-hr) schedules of reinforcement (N=10). One-way repeated measures ANOVA P>0.05.
As seen in figures 3-A and -B, AM4113 had no effect on the number of food pellets earned and this was verified by non-significant one-way repeated measures ANOVAs [F(4, 9) = 2.214, P = 0.0869; F(4, 9) = 1.180, P = 0.3361] for both fixed ratio-5 and progressive ratio experiments, respectively.

Therefore, 60 min was chosen as the pretreatment time for all following experiments.

3.3. Effect of AM4113 chronically administered on food self administration behaviour

To fully validate a potential therapeutic agent, testing it under chronic conditions better simulates how a drug is utilized by humans. It also demonstrates whether tolerance or sensitization arises to the drug under such a regimen. Therefore, a chronic experiment was first conducted in the control food self-administration experiments under both schedules of reinforcement to determine whether any non-specific (i.e., motor) effects resulted from chronic AM4113 (10mg/kg, intraperitoneal) administration.

**Figure 4. Chronic administration of AM4113 causes minor disturbances on fixed ratio-5 food-taking behaviour.** Chronic effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of food pellets earned under fixed ratio-5 schedule of reinforcement (N = 11). * P<0.05 vs. Saline-3; ** and ## P<0.01 vs. Saline-3 (Session 6) and VEH-3 (Session 9), respectively. (One-way RM ANOVA followed by Bonferroni post-hoc tests)
For the chronic effects of AM4113 on number of food pellets earned under fixed ratio-5 schedule of reinforcement, Bonferroni post-hoc tests detected session 13 to be significant (P<0.05) compared to saline-3 (session 6), and the final day of 10mg/kg AM4113 treatment (session 19) to be significantly different (P<0.01) from saline-3 (session 6) and vehicle-3 (session 9) for food pellets earned, following a significant main effect in the one-way repeated measures ANOVA [F(24, 10)= 3.145, P< 0.0001] as seen in figure 4.

A one-way repeated measures ANOVA on the chronic effects of AM4113 on food-taking behaviour utilizing a progressive ratio schedule of reinforcement was also significant [F (24, 10) = 3.409, P< 0.0001]. As depicted in figure 5, Bonferroni post-hoc tests showed both saline-3 (session 6) and vehicle-3 (session 9) food pellets earned to be significantly (P<0.001) different from that of session 15.

Figure 5. Chronic administration of AM4113 causes minor disturbance on progressive ratio food-taking behaviour. Chronic effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of food pellets earned under progressive ratio schedule of reinforcement (N=11). *** and ### P<0.001 vs. Saline-3 (Session 6) and VEH-3 (Session 9), respectively. (One-way RM ANOVA followed by Bonferroni post-hoc tests)
3.4. Acute effects of AM4113 on nicotine-taking behaviour under fixed ratio-5 schedule of reinforcement:

This experiment was conducted to determine whether AM4113 could effectively reduce the reinforcing effects of nicotine in the intravenous self-administration paradigm following acute treatment, and to identify the dose-response relationship.

A one-way repeated measures ANOVA resulted in a main effect of treatment \([F (4, 7) = 13.68, P<0.0001]\), with Bonferroni post-hoc tests resulting in 1- (P<0.001), 3- (P<0.001) and 10- (P<0.001) mg/kg AM4113 to be significant at reducing the number of nicotine infusions earned per session compared to saline-baseline control (Figure 6). Furthermore, compared to vehicle control, Bonferroni post-hoc test showed 1- (P< 0.05), 3- (P< 0.001) and 10- (P< 0.01) mg/kg AM4113 to be significant as well. Saline-baseline and vehicle control were not significantly different (P>0.05), and no significant differences between doses was detected (P>0.05).

**Figure 6. AM4113 reduces nicotine-taking under fixed ratio-5 schedule of reinforcement.** Acute effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of nicotine infusions earned under fixed ratio-5 (Session: 1-hr) schedule of reinforcement (N=8). *** P<0.001 vs. Saline BSL; # P<0.05 vs. VEH; ## P<0.01 vs. VEH; ### P<0.001 vs. VEH (One-way repeated measures ANOVA followed by Bonferroni post-hoc tests)
3.5. Acute effects of AM4113 on nicotine-taking behaviour under progressive ratio schedule of reinforcement:

Challenging AM4113 on nicotine intravenous self-administration under a progressive ratio schedule of reinforcement serves a similar purpose to the fixed ratio-5 experiment equivalent, however it provides additional information. Under the progressive ratio schedule, the effort the animals are willing to exert for each successive infusion can be equated to how much they value the reinforcing effects of nicotine. The “breakpoint” is the number of active lever presses required for the last infusion that an animal earns in a progressive ratio session. Thus, the breakpoint is able to quantify the specific reinforcing value of nicotine for each particular animal. Changes in this break point following pretreatment with an experimental ligand provide evidence suggesting the ligand is capable of modifying the reinforcing value of nicotine. Therefore, the results of this experiment also demonstrate how AM4113 is able to reduce the perceived reinforcing value of nicotine. In addition, since all doses were effective in reducing the number of infusions earned in the fixed ratio-5 experiment an additional dose of 0.3 mg/kg was added to demonstrate an ineffective dose.

As evident in figure 7, the doses that were effective under the fixed ratio-5 experiment, also demonstrated effectiveness in reducing the number of nicotine infusions earned under a progressive ratio schedule of reinforcement. One-way repeated measures ANOVA showed the treatment effect \[F (5, 8) = 10.77, P<0.0001\] to be significant. Bonferroni post-hoc tests demonstrated that 1- (P<0.001 and P<0.01), 3- (P<0.001 and P<0.001) and 10- (P<0.001 and P<0.01) mg/kg AM4113 significantly reduced the number of nicotine infusions earned relative to saline-baseline and vehicle controls, respectively. The lowest dose of AM4113 (0.3 mg/kg), although it reduced the reinforcement count, was not significantly different (P>0.05) compared to saline-baseline and vehicle controls. Saline-baseline and vehicle controls were not
significantly different (P>0.05) from each other, and between the doses only 0.3mg/kg versus 3mg/kg was significant (P<0.05).

Figure 7. AM4113 reduces nicotine-taking under progressive ratio schedule of reinforcement. Acute effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of nicotine infusions earned under progressive ratio (Session: ≤ 4-hr) schedule of reinforcement (N=9). *** P<0.001 vs. Saline BSL; ## P<0.01 vs. VEH; ### P<0.001 vs. VEH (One-way repeated measures ANOVA followed by Bonferroni post-hoc tests)

3.6. Effect of AM4113 chronically administered on nicotine intravenous self-administration behaviour under fixed ratio-5 schedule of reinforcement

This experiment was conducted to identify whether chronic treatment of AM4113 (10mg/kg, intraperitoneally) on nicotine taking has a sustained, transient or sensitized effect during the course of the daily treatment. In addition, the post-AM4113 treatment period demonstrates whether the effect is sustained or whether the absence of the treatment gives way to the re-establishment of nicotine taking behaviour similar to the original saline treatment baseline levels.

The chronic effects of AM4113 on the number of nicotine infusions earned (Figure 8) resulted in the main effect being significant following a one-way repeated measures ANOVA [F(24, 6)= 15.78, P< 0.0001]. Bonferonni multiple comparison test resulted in an AM4113
treatment and post-AM4113 treatment effect with sessions 10-21 (P<0.001), 22 (P<0.05) and 23-25 (P<0.001) being significant compared to saline-3 (session 6). Furthermore, compared to vehicle-3 (session 9) Bonferroni multiple comparison tests showed sessions 10-17 (P<0.001), 18-20 (P<0.01), 23 (P<0.001) and 25 (P<0.05) to be significantly different. Saline-3 (session 6) and vehicle-3 (session 9) were not significantly different from each other.

Figure 8. Chronic administration of AM4113 attenuates fixed ratio-5 nicotine-taking behaviour. Chronic effects of AM4113 (60 min pretreatment time) on mean (±SEM) number of nicotine infusions earned under a fixed ratio-5 schedule of reinforcement (N=7). * and #, P<0.05 vs. Saline-3 (Session 6) and VEH-3 (Session 9), respectively; ## P<0.01 vs. VEH-3 (Session 9); ### and #### P<0.001 vs. Saline-3 (Session 6) and VEH-3 (Session 9), respectively. (One-way RM ANOVA followed by Bonferroni post-hoc test)
Section IV: GENERAL DISCUSSION, CONCLUSIONS, RECOMMENDATIONS FOR FUTURE RESEARCH

GENERAL DISCUSSION

The neutral CB1 antagonist AM4113 was able to effectively attenuate nicotine self-administration behaviour under both the fixed ratio-5 and progressive ratio schedules of reinforcement in acute experiments, while demonstrating no significant effect in similar food control self-administration experiments. Under the chronic administration paradigm of AM4113 on nicotine-taking behaviour (fixed ratio-5), it replicated and sustained its effect, as with the acute studies, for the course of the treatment period. In addition, it had a carry-over effect into the post-treatment days where the number of nicotine infusions earned never returned to baseline levels. In the similar food control self-administration experiments (fixed ratio-5 and progressive ratio), chronic AM4113 demonstrated minor disturbances in food-taking behaviour, but not consistently as was seen with the nicotine self-administration experiment.

4.1. EFFECT OF AM4113 ON FOOD-TAKING BEHAVIOUR

4.1.1. Acute effects of AM4113 on food self-administration

AM4113, from previous studies (Hodge et al., 2008; Sink et al., 2008; Sink et al., 2009a), is known to affect food-taking behaviour which is thought to be due to its ability to suppress appetite through direct actions in the periphery (e.g., gastrointestinal tract) (Sink et al., 2009b). Food self-administration experiments serve as an important control to nicotine self-administration experiments, as they permit the identification of possible non-specific motor effects that may affect operant responding. Therefore, testing AM4113 under the food control self-administration paradigm presented a possible confounding factor. However, due to
methodological differences in previous studies (e.g., 60-min vs. 30-min session [Sink et al., 2008; Sink et al., 2009a] and Long Evans vs. Sprague Dawley rats [Hodge et al., 2008; Sink et al., 2008; Sink et al., 2009a]), it was important to verify published results utilizing our particular paradigm. Using the same pretreatment time (30-min) and food-restricting the animals, we found similar results to Sink et al. (2008; 2009a) with the higher doses of AM4113 affecting food-taking behaviour under the same fixed ratio-5 schedule of reinforcement. However, in addition, we conducted an experiment with the progressive ratio schedule of reinforcement, which permits the animal to run a maximum session of 4 hours given there is no 30-min period of inactivity before then. Under this schedule of reinforcement, only the highest dose of 10mg/kg was significant compared to the saline-baseline control.

Therefore, it was hypothesized that the additional session run time possible under a progressive ratio schedule of reinforcement permitted more time for compensatory mechanisms to override the effect of AM4113 in the system and ultimately reduced the drug’s effect on food self-administration behaviour. One possible compensatory mechanism could be the bidirectional signaling that occurs along the vagus nerve which connects the brain (i.e., hypothalamus) and the gastrointestinal tract, which has been appropriately termed the brain-gut axis (Cluny et al., 2012). The brain-gut axis, and in particular, the gastrointestinal tract, are believed to be significantly involved in the regulation of short-term food intake (Cluny et al., 2012), which would be applicable to our food control self-administration experiments. The hypothalamus receives the energy (i.e., hunger) status in the periphery from the gastrointestinal tract, liver and pancreas, and is responsible for initiating appropriate responses with respect to maintaining proper energy and nutrient status. Orexigens such as anandamide and ghrelin are released to stimulate feeding, whereas anorexigens (e.g., cholecystokinin and peptide YY) are released to suppress feeding (Cluny et al., 2012). Therefore, with respect to AM4113 and its
effect on our food-restricted animals in food control self administration experiments, AM4113 is probably able to attenuate the endocannabinoid signal promoting feeding; however, with increased time compensatory signaling via ghrelin and the lack of anorexigen signaling probably results in the stimulation of feeding behaviour. This hypothesis with respect to our results is further supported by the fact that Hodge and colleagues (2008) had demonstrated with a 30-min pretreatment time and 50-min session that AM4113 treatment reduced feeding counts during the first 20-min of the session as compared to vehicle treated rats, but that in the remaining time the feeding counts were comparable between AM4113 treatment and vehicle treatment groups. With a 60-min pretreatment time, we then repeated the experiments, and, as predicted in our hypothesis, AM4113 treatment no longer significantly attenuated food self-administration under either schedule of reinforcement.

Another possible explanation that can be advanced for the lack of effect seen with the 60-min pretreatment time relative to the 30-min pretreatment is tolerance. The same rats were used to first conduct experiments with the 30-min pretreatment time and then with the 60-min pretreatment time. Therefore, it is possible after first exposure to AM4113 (during fixed ratio-5, 30-min pretreatment time experiments) the animals began to develop tolerance, which is why there was a loss of effects in the subsequent progressive ratio (pretreatment time = 30-min) experiment and both 60-min pretreatment time experiments. This alternate possibility is supported by Cluny and colleagues’ (2011) finding that AM4113 has a transient effect on food intake levels. However, this result was obtained under chronic daily administration of AM4113 making it difficult to fully extrapolate to our current study. Therefore, at this time, it seemed possible the lack of effect obtained under 60-min pretreatment time was a result of compensatory mechanisms overriding the effect of AM4113 or development of tolerance to AM4113 due to prior exposure, or lastly, the combination of both explanations.
4.1.2. Chronic effects of AM4113 on food self-administration

With the possibility that the AM4113 effect might be transient in our acute experiments and with Cluny et al. (2011) demonstrating that the drug had a transient effect on food intake during the course of chronic treatment, there was a possibility of this transient effect appearing during nicotine self-administration. Therefore, to address this concern, and to conduct a control for our chronic AM4113 on nicotine self-administration experiments, we initiated chronic studies with AM4113 on food control self-administration using our parameters. This set of experiments would also allow us to detect the development of possible non-specific motor effects as a result of repeated (vs. acute) administration that may affect interpretation of chronic AM4113 on nicotine self-administration.

For the chronic AM4113 treatment on food self-administration (fixed ratio-5), after being trained and stabilized with food operant responding saline habituation injections were initiated followed by three days of vehicle treatment to see if repeated vehicle (which contains the irritants dimethylsulfoxide and Tween 80) injections could disrupt food-taking behaviour. Both controls (saline and vehicle injections) failed to have an effect and so treatment with the highest dose of AM4113 (10mg/kg) was administered for ten days. AM4113 was only able to minorly disrupt food-taking behaviour over the course of the treatment, significantly decreasing number of food pellets obtained on only 2 of the 10 treatment days. Once vehicle and saline injections were re-initiated the number of food pellets earned returned to baseline levels. The experiment was then repeated in the same group of animals under progressive ratio schedule of reinforcement and once again AM4113 was only able to minorly disrupt the number of food pellets earned (significant decrease for 1 out of 10 treatment days) during the course of AM4113 treatment. In behavioural experiments, many factors can affect responding (e.g., noise, lighting, feeding time, home cage weekly changes), and despite the best efforts by
experimenters to control for them, sometimes they can have an effect on responding. Due to the chronic nature of the experiment, it is possible that one or a combination of these factors along with the AM4113 treatment may have disrupted responding during those days of significance. Although two treatment days during the fixed ratio-5 experiment and one treatment day during the progressive ratio experiment showed significant effects, the overall results demonstrate that AM4113 only minorly disrupts food-taking behaviour.

Chamber and colleagues (2007), and Cluny and colleagues (2011) both conducted studies examining the chronic effects of AM4113 on food-intake and both found significant effects. Chambers et al. (2007) conducted five days of treatment with the last three being significant, and Cluny et al. (2011) treated their rats with 14 days of AM4113, and found the first four days significant and then food intake levels returned back to baseline levels (transient effect). There are differences between our study and theirs (Chambers et al., 2007; Cluny et al., 2011) that can help to explain the difference in results. Firstly, we used male Long Evans rats, while they utilized male Sprague-Dawley rats. It is possible that the two strains have a different tolerance rate when it comes to AM4113. Secondly, and a more likely explanation for the discrepancy, is the parameters of the experiment and the hunger status of the animals. For the entirety of our experiments animals were food-restricted, while the other studies do not mention in their methods any food restriction. Also, our session testing length (1-hr for fixed ratio-5 and max 4-hr for progressive ratio) was much shorter than theirs (18-hr). Lastly, they were measuring the amount of strawberry- and chocolate-flavoured Ensure plus liquid diet (Chambers et al., 2007 and Cluny et al., 2011, respectively) their animals consumed in response to treatment, while we measured the number of 45mg food pellets the animals earned in response to treatment. Therefore, all these different factors may have accounted for the discrepancy between our results.
4.1. CONCLUSION

In interpreting the overall food control results, it seems likely the effects of AM4113 in our acute and chronic paradigms are a result of the reduced effect of AM4113 on food-taking behaviour due to the increased pretreatment time permitting compensatory mechanisms, possibly via the brain-gut axis, to supersede AM4113’s effect. In addition, according to the results (no significant disruption in food self-administration under 60-min pretreatment time), AM4113 did not display any non-specific motor effects that could affect operant responding. However, although this may be the case in terms of the quantitative results, increased grooming and scratching were noticed in the animals in response to AM4113 treatment, but this was not directly measured by our group. Furthermore, this was witnessed more when the animals were in their transport cage immediately following injection versus when they were in the test chamber, but this could be due to the fact that it is more difficult to easily see such behaviour when the animal is in the testing chamber.

4.2. EFFECT OF AM4113 ON NICOTINE-TAKING BEHAVIOUR

4.2.1. Acute effects of AM4113 on nicotine self-administration

The neutral CB1 antagonist AM4113 was effective in attenuating nicotine-taking behaviour in the intravenous self-administration paradigm under both schedules of reinforcement. In the fixed ratio-5 experiment all doses tested (1-, 3- and 10-mg/kg) significantly attenuated the number of nicotine infusions earned compared to baseline-saline and vehicle treatment. The strong effect of the CB1 neutral antagonist under the fixed ratio schedule of reinforcement is in agreement with previously published works involving its predecessor, CB1 inverse agonists (rimonabant, AM251 and SLV330). Cohen and colleagues (2002) found rimonabant to be effective at reducing the number of nicotine infusions earned
with the two highest doses they tested (0.3- and 1.0-mg/kg), while SLV330 was effective at the highest-dose tested (10mg/kg) only (de Bruin et al., 2011). Only one contrasting study that tested with fixed ratio schedule of reinforcement and CB1 manipulation, produced results different from those presented here. Cossu and colleagues (2001) using CB1 receptor knockout mice and a fixed ratio schedule of reinforcement did not find any difference in nicotine self-administration between the wild-type mice and knockout mice. However, there are significant differences between this study and the present study. The first of these is the species (mice versus rats). Secondly, the manipulation of CB1 receptors in the Cossu et al. (2001) study was not through reversible pharmacological means. One possibility that could help to explain the lack of attenuation seen in Cossu et al. (2001) study is that the CB1 receptor is not required in acquisition of nicotine self-administration and/or there are compensatory pathways that can permit the acquisition of nicotine self-administration to occur in the absence of CB1 receptors (Cohen et al., 2005b). Nevertheless, pharmacological blockade of CB1 receptors, either with a neutral CB1 antagonist or inverse agonists, reduces the number of nicotine infusions earned by animals.

Interpreting the effect of a compound on nicotine self-administration behaviour under the fixed ratio schedule of reinforcement does have a caveat, as it has been found that the dose-response curve for nicotine follows an inverted U shape (Forget et al., 2009; Gamaleddin et al., 2012a). Therefore, if a drug is able to reduce the number of nicotine infusions earned under fixed ratio schedule of reinforcement, it is sometimes difficult to interpret whether it is increasing or decreasing the reinforcing effects of nicotine (Arnold & Roberts, 1997; Forget et al., 2009; Gamaleddin et al., 2012a). This is why supporting experiments need to be conducted alongside those utilizing the fixed ratio schedule of reinforcement. For this study, we conducted a similar experiment on nicotine-taking except utilizing the progressive ratio
schedule of reinforcement because it has been documented that the dose-response relationship for nicotine under the progressive ratio schedule is a linear one (Corrigall et al., 2001; Donny et al., 1999; Forget et al., 2009; Gamaleddin et al., 2012a; Le Foll et al., 2007; Paterson et al., 2004). Due to the escalating response requirement for every subsequent infusion, the progressive ratio schedule is thought to measure both the motivational (i.e., how willing is the animal to put in more effort to receive the next infusion and therefore how much the drug is “worth” to the animal) and reinforcing effects of the drug, in this case nicotine (Arnold and Roberts, 1997; Forget et al., 2009; Markou et al., 1993; Paterson and Markou, 2005).

Under the progressive ratio schedule of reinforcement, nicotine self-administration was reduced similarly, with the same doses being effective. An additional dose of 0.3mg/kg was included to see if a lower dose would have an effect on nicotine-taking behaviour, and, although 0.3mg/kg AM4113 displayed a reduction in the number of nicotine infusions earned compared to saline-baseline and vehicle, the effect was not significant.

Our results are in agreement with the previous findings of Forget et al. (2009) with rimonabant on nicotine self-administration under a progressive ratio schedule of reinforcement. Firstly, Forget and colleagues’ (2009) two highest doses (1- and 3-mg/kg) of rimonabant were significantly effective at reducing the number of nicotine infusions earned, and secondly, their lowest dose of 0.3mg/kg, like ours, demonstrated a non-significant decrease in reinforcement counts.

Upon viewing the dose-response curves of AM4113 under both schedules of reinforcement, one can see a possible U-shaped dose response curve, with 3-mg/kg dose being the most effective at reducing the reinforcement count of nicotine. Furthermore, looking at Cohen and colleagues (2002) dose-response curve of rimonabant (Figure 1, bottom panel, first day of treatment only), one can see a similar U-shape dose response curve with 0.3-mg/kg
being at the base of the curve. Only Forget and colleagues (2009) study does not display a U-
shape dose-response curve, but that may be due to their not testing a higher dose, such as
10mg/kg of rimonabant. Ultimately, it would be interesting to add an additional dose of 20
mg/kg to our own study (30 mg/kg may pose difficulty getting into suspension) to see whether
AM4113 actually produces a U-shaped dose-response curve. Without the added dose, the
current data are inconclusive with respect to the U-shaped dose-response curve.

4.2.2. Chronic effects of AM4113 on nicotine self-administration

Acutely AM4113 was demonstrated to be effective at attenuating the number of
nicotine reinforcements earned. This data prompted us to further validate the drug via chronic
administration. The importance of testing a novel drug chronically cannot be overstated as in
the human experience drugs are generally taken on a regular (e.g., daily) basis. In addition, in
the human condition, smoking (i.e., tobacco dependence), is characterized as a disorder that is
chronic, and thus would theoretically be best challenged with chronic treatment (Forget et al.
2009). Lastly, for scientific validity, chronic treatment is the best way to promote rigidity in
ones’ results, and thus avoid false-positives and/or –negatives from acute testing (Forget et al.,
2009).

When administered chronically, 10mg/kg AM4113 significantly attenuated nicotine-
taking during the course of the ten day treatment period. In addition, even when AM4113
treatment stopped and animals were re-treated with vehicle and then saline, the number of
reinforcement counts still remained significantly below saline treatment-baseline levels.
Compared to similar, but shorter (3 days), chronic treatment experiments utilizing the
predecessor CB1 inverse agonist ligands (Forget et al., 2009; Shoaib, 2008), our results are in
general agreement. Shoaib (2008) tested the CB1 inverse agonist AM251, and with the highest
dose of 10mg/kg under fixed ratio-3, AM251 significantly reduced responding for the three
days of treatment, but no significant effects on responding post-AM251 treatment were seen after this short exposure. Forget et al. (2009) chose to use their middle dose of 1mg/kg of rimonabant, which was effective acutely, to conduct chronic administration (3 days) experiments. Interestingly, they chose their middle dose to avoid the possibility of the drug accumulating within the animal’s system during daily treatment (Forget et al., 2009). They found it to be significantly effective at all time points at reducing the number of nicotine infusions earned under a progressive ratio schedule of reinforcement. They, however, did not study the post-treatment effects. Indeed, it would be interesting to test AM4113 chronically at the other doses, in particular 3mg/kg, which was the strongest in both acute fixed ratio-5 and progressive ratio experiments, in addition to also conducting the same experiments under the progressive ratio schedule of reinforcement, but it was beyond the scope of this study. We were however able to show that AM4113 has the same efficacy chronically as it did acutely, and as the predecessor CB1 inverse agonists had in previous chronic experiments.

The post-chronic AM4113 treatment effect seen in our study is very interesting. It might lead one to hypothesize that there is a sustained or carry-over effect of AM4113 post-treatment. If it is a sustained effect that is occurring post-AM4113 treatment, one possibility is that there is a change at the level of the mesocorticolimbic reward neurocircuitry that is a response to chronic AM4113 treatment. It is possible that the system has become dampened and desensitized to the presence of nicotine, and therefore no longer elicits the same strong reinforcing effects as it did prior to AM4113 treatment. The alternative hypothesis is there was a carry-over effect of AM4113. For the chronic study, we chose to use our highest dose of AM4113, 10mg/kg. Therefore, it is possible that at this high dose AM4113 just accumulated within the animals’ system, and had a carry-over effect on the post-AM4113 treatment sessions. This alternative hypothesis however, is not likely, as preliminary studies
(unpublished) have shown AM4113 to have a 2 hour half-life; therefore, it would be eliminated within approximately 10 hours from time of injection, and not be present in the animals’ system during the next session. Thus, it is likely that neuroadaptations in the neurocircuitry of the mesocorticolimbic system are responsible for the post-AM4113 treatment effect on nicotine-taking behaviour.

With relation to the studies utilizing chronic CB1 inverse agonist treatment on nicotine-taking behaviour, in Shoaib’s (2008) case, it is possible he did not see an effect post-treatment because he only treated for three days, and of course used a different compound, albeit one that acts on the same target. Unfortunately, we cannot compare our results to Forget and colleagues (2009) study since they did not do post-treatment testing.

The design of a chronic administration paradigm on drug self-administration is crucial in interpreting the results. In the author’s opinion, the best and most clean design for such a study would involve: (1) established nicotine self-administration under baseline conditions (with saline treatment) for at least 3 consecutive sessions, and (2) if stable, initiate daily drug treatment for at least 7 days (3) followed by a return back to drug self-administration under baseline conditions (for at least 3 days, and preferably more if the drug is having a post-drug treatment effect as long as the catheter patency permits this). Under this schedule, the vehicle and doses of the drug should be tested using a between subjects design similar to the experiments conducted by Vlachou and colleagues (2011). There are however, logistic issues associated with running such a study. The animal numbers and the amount of drug required increase the resources (e.g., equipment, personnel and housing rooms) required to run such a large study. Therefore, due to animal number restrictions, and restrictions in personnel, drug and housing, the present study had to be conducted with a within-subject design, where drug
treatment could be compared to both the saline and vehicle controls before and after the drug treatment. Such a design was the best alternative to running the full between subjects design.

4.2. CONCLUSION

AM4113 was demonstrated to be effective acutely at reducing the reinforcing effects of nicotine under both schedules of reinforcement. Furthermore, when the neutral CB1 antagonist was administered chronically on nicotine intravenous self-administration, it was also efficacious at reducing the number of nicotine infusions earned for the entire duration of treatment, as well as at impacting post-treatment nicotine infusion levels. These results indicate that AM4113 is effective at attenuating nicotine-taking behaviour.

4.3. DISCUSSION OF AM4113’s EFFECTS AND MECHANISM

4.3.1. Detailed Discussion of AM4113’s effects on nicotine-taking

There are two main ways by which a drug might produce such an effect on nicotine taking behaviour: (1) either through non-specific effects that affect operant responding, or (2) by reducing the reinforcing effects of an addictive substance, with the latter being the optimal mechanism for a drug to have any potential of reaching the market. In the case of AM4113, there is evidence that it can affect locomotor activity (Jarbe et al., 2008; Sink et al., 2008; Sink et al., 2010) and also increase grooming and induce scratching (Jarbe et al., 2008; Hodge et al., 2008). Both of these behaviours could potentially reduce operant responding. In our current study, neither locomotor activity nor grooming/scratching were quantitatively or qualitatively measured, as such measurements were beyond the scope of our study. It was however noticed that rats treated with AM4113 did tend to groom and scratch more, as compared to saline injected rats. It should be also mentioned that this behaviour was more noticed when the rat was placed back into its transport cage post-injection as compared to when they were in the
intravenous self-administration chamber. This could be explained either by: (1) it being a response that fades with time (since the rats are placed in the chamber 60-min post-injection) or (2) simply because it is much more difficult to view the behaviour when rats are in the intravenous self-administration chamber. Indeed, it would be interesting to measure these behaviours within our paradigms. This, however, would require us to run the animals in opened sound attenuating chambers, which in itself increases the risk for affecting operant responding and which would require more personnel to conduct such a study with a reasonable sample size.

For the locomotor activity studies, Jarbe and colleagues (2008) and Sink and colleagues (2010) both used a 30-min pretreatment time and test sessions of 10-min or less; therefore, all their measurements were conducted at what would be equivalent to 40-min into our pretreatment time period. Sink et al. (2008) however, did use a 60-min pretreatment time and an 18-min test session which would be more relevant to our study. At 4mg/kg (only dose tested), they did indeed find AM4113 to be significant at decreasing locomotor activity (Sink et al., 2008). This result, however, is not fully applicable to our study as their test session length accounts for less than 1/3 of our fixed ratio-5 session duration. Secondly, it is important not to draw the direct conclusion that decreased locomotor activity results in decreased motivation for food (Cousins et al., 1993; Salamone et al., 2007; Sink et al., 2008) or drug. Lastly, in our case, with a 60-min pretreatment time, we did not find AM4113 to affect food-taking behaviour, so therefore it is unlikely that the effect of AM4113 on nicotine self-administration we witnessed was due to non-specific motor effects or decreased locomotion.

This leads to the next question of why at 60-min pretreatment time AM4113 is effective at reducing nicotine-taking behaviour, while demonstrating no effect in the food control self-administration experiments. Both, nicotine and food are considered to be rewarding (Le Foll et
al., 2008), but AM4113 seems to only affect the reinforcing effects of nicotine, but not that of food. One argument that might naturally come to mind is that for food-deprived animals, food pellets carry a higher motivational value and greater reinforcing effects as compared to that of nicotine. This would imply the animals would exert more effort and be more motivated to press for food versus nicotine in the presence of AM4113 in their system. This would also imply they should have greater motivation for food pellets under control conditions as well. However, in our study, comparing the food control- and nicotine- self-administration experiments under a progressive ratio schedule of reinforcement, the results of which are considered to represent the reinforcing value of the drug or food, both resulted in a breaking point of 10 reinforcement counts (break-point= 124 active lever presses) under saline-baseline conditions. Therefore, at least in our experiments, a difference in the baseline motivational and reinforcing value of nicotine and food pellets was not completely responsible for the difference in response to AM4113.

The next possible theory that could explain the differential effect would be that it was due to site-specific activity of AM4113 on food- and nicotine-taking behaviour. It is quite possible AM4113 is acting in the central nervous system for its effects on nicotine-taking behaviour, while when it acts on food-taking behaviour it is mainly acting peripherally (e.g., gastrointestinal tract). Sink and colleagues’ (2009b) study investigating intracerebroventricular administration of AM4113 helps to support such a theory. In their study, they demonstrated AM4113, when administered intracerebroventricularly, to have no effect on food operant responding. However, in the same dose range, intracerebroventricular administration of AM4113 was able to reverse the effects of CB1 agonist (AM411) -induced hypo-locomotion, a behavioural response that is believed to be mediated via CB1 receptors in the forebrain and midbrain, including those in the nucleus accumbens (Sink et al., 2009b).
To further support the theory of the neutral CB1 antagonist exerting its appetite suppressing effects via peripheral CB1 receptors, it is important to look at the findings related to AM6545 (Cluny et al., 2010; Randall et al., 2010), a neutral CB1 antagonist that is peripherally restricted, and does not cross the blood-brain barrier. Acutely, AM6545 has demonstrated similar effects to AM4113 in operant food responding (Randall et al., 2010). Chronically (7-day treatment experiment) it has also had a similar effect on food intake (Cluny et al., 2010). Thus, according to these findings (Cluny et al. 2010; Randall et al., 2010; Sink et al. 2009b), the evidence suggests that the effect of AM4113 in our food control self-administration experiments is likely through its effect on CB1 receptors in the periphery, such as in the gastrointestinal tract while effects on nicotine-taking behaviour are centrally mediated, probably in the mesocorticolimbic system. It would be interesting to conduct intracerebroventricular administration of AM4113, and AM6545 treatment (IP) on nicotine-taking behaviour as possible future control experiments to further test this theory.

If this theory of site-specific activity of AM4113 is taken to be the likely cause of its effects on food- and nicotine- reinforced behaviours, it also has implications with respect to compensatory mechanisms. As previously mentioned, with a 60-min pretreatment time there are probably compensatory mechanisms that override AM4113’s inhibitory effect on food-motivated behaviour. However, with respect to nicotine-motivated behaviour, this would imply there are no compensatory mechanisms (which likely would have to be in the central nervous system) that can supersede AM4113’s effect on nicotine’s reinforcing effects. This would explain why there is an effect with a 60-min pretreatment time on nicotine-taking behaviour, and not on food-taking behaviour.

With respect to the increased grooming and scratching that were previously mentioned, according to Hodge et al.’s (2008) study where they used a 30-min pretreatment time and a 50-
min test session, it is possible that these responses could affect our nicotine intravenous self-administration results. Such an effect would relate to the response competition hypothesis (Hodge et al., 2008; Sink et al., 2010; Tallet et al., 2007a, b). According to this hypothesis, which has been applied to food intake studies, the increased grooming and scratching would compete with normal behaviour, distract the animal, and disrupt its operant feeding behaviour (Hodge et al., 2008; Sink et al., 2010; Tallet et al., 2007a,b). Interestingly, Hodge et al. (2008) decided to run a control yoked experiment to test the theory with AM4113, and found that response competition did not significantly alter food intake. They concluded that the response competition theory with respect to AM4113 could not be responsible for its effect on food intake, but also add that this conclusion cannot be fully extended to other non-feeding behaviours (Hodge et al., 2008).

Hodge and colleagues’ (2008) conclusions cannot be refuted at this stage, as no similar control yoked experiment has been conducted with nicotine intravenous self-administration. It is possible that the increased grooming and scratching may be playing a role in disrupting the operant responding for nicotine, and not food in our results; however, the degree to which it is impacting it is unknown. Also, grooming and scratching could also just be a side effect unrelated to AM4113’s effectiveness on nicotine intravenous self-administration. Rimonabant also caused increased grooming and scratching at the preclinical level (Jarbe et al., 2002; 2006), and, in addition, rimonabant and taranabant (another CB1 inverse agonist), in clinical trials commonly precipitated itching (pruritus) as a side effect (European Medicines Agency, 2008; Cahill and Ussher, 2011; Kirkham, 2008). It seems possible, that the itching side effect may be a direct result of CB1 blockade (Hodge et al., 2008) versus a property of CB1 inverse agonism as confirmed by our own observations of itching with the neutral CB1 antagonist AM4113. In the human trials however, there would be no response competition between
itching and smoking, and although CB1 inverse agonists displayed itching as a possible side effect, they still retained efficacy in terms of promoting smoking cessation (Cahill and Ussher, 2011). The question now lies in whether the itching side effect will precipitate in humans as well (Hodge et al., 2008) if AM4113 does indeed reach clinical level of study, and if the side effect does appear, how frequent and how tolerable it is (i.e., “cost”) relative to the drug’s efficacy (i.e., “benefit”) will determine whether AM4113 has potential in the market.

Another possible explanation for AM4113’s effectiveness on nicotine self-administration is that it substitutes for nicotine, and produces the rewarding effects, thus the animal no longer needs to press for nicotine. There is evidence to contradict this theory. Rimonabant, the drug AM4113 development was based on, did not substitute for nicotine in nicotine discrimination studies (Cohen et al., 2002), therefore implying that the drugs act by two different pharmacological mechanisms (Jarbe et al., 2011). In a discriminated drinking aversion study involving rimonabant, Jarbe et al. (2011) found that AM4113 was able to substitute for rimonabant, therefore implying similarity between the two compounds. To further support this, in the Kangas et al. (2013) study where the drug discrimination procedure using AM4054 (full CB1 agonist) was conducted, both rimonabant and AM4113 antagonized the discriminative-stimulus effects of the drug. Therefore, since rimonabant did not substitute for nicotine (Cohen et al., 2002), and since AM4113 substituted for rimonabant (Jarbe et al., 2011) and had the same antagonistic effect on AM4054 (Kangas et al., 2013), it is likely that AM4113 does not produce nicotine-like discriminative effects and does not substitute for nicotine in terms of having a rewarding effect in itself. This assumption is also supported by Cohen and colleagues’ (2002) study, where they demonstrated that nicotine-induced dopamine release in the nucleus accumbens shell and the bed nucleus of stria terminalis could be blocked by treatment with rimonabant. However, when rimonabant was administered alone it did not
produce changes in dopamine levels. It is probable AM4113 would act similarly, although this experiment still needs to be done.

Taking into account all the above mentioned points, it seems probable that AM4113 is effective at reducing the reinforcing effects of nicotine. Several further points support this conclusion. First, by demonstrating no effect in the food self-administration experiments acutely and chronically (with 60-min pretreatment time), there is no indication that AM4113 is affecting memory for the previously trained behaviour. Secondly, it implies that it is unlikely that the drug is affecting general operant responding or having non-specific motor effects. Thirdly, since there is no disruption of food self-administration under the progressive ratio of reinforcement it indicates that AM4113 is not having an effect on general motivation. All in all, this leads to the probable conclusion that AM4113, acting on the CB1 receptor as a neutral antagonist, is effective at attenuating the reinforcing effects of nicotine and reducing the motivation to self-administer it.

4.3.2. *AM4113’s mechanism of action*

At the current time, to the author’s knowledge, there is no completely confirmed mechanism of action for AM4113, or for that matter, for CB1 inverse agonists (Le Foll et al., 2008; Lupica and Riegel, 2005). More research is needed in this area. However, based on the work on rimonabant and the similarity of AM4113 to rimonabant, it is likely AM4113 works by a mechanism of action similar to that of rimonabant [see Figure 9] (Cohen et al., 2005b; Cheer et al., 2007).
Figure 9. Potential mechanism of action of AM4113/Rimonabant on nicotine’s reinforcing effects. Nicotine-stimulated dopamine neurons release endocannabinoids in addition to dopamine. The endocannabinoids bind to CB1 receptors on glutamate and GABA neurons, and inhibit glutamate and GABA release. The inhibition of GABA release due to endocannabinoid-CB1 receptor interaction is more predominant. Therefore, this ultimately results in less inhibition of dopamine release in the nucleus accumbens, and thus endocannabinoids help to promote the reinforcing effects of nicotine. When AM4113 is administered, it blocks the CB1 receptors on GABA neurons in the ventral tegmental area from endocannabinoids, resulting in the disinhibition of GABA release onto dopamine neurons in the ventral tegmental area, and thus inhibiting dopamine release in the nucleus accumbens. By indirectly inhibiting dopamine release, AM4113 attenuates the reinforcing effects of nicotine. PFC: prefrontal cortex; NAcc: nucleus accumbens; VTA: ventral tegmental area. Figure adapted from Cohen et al., 2005b; Maldonado et al., 2006; and Picciotto, 2003.

As presented in the introduction, nicotine promotes dopamine release through direct and indirect modulation of dopamine neurons (Lupica and Riegel, 2005; Pidoplichko et al., 1997; 2004). Through the direct route, nicotine binds to $\alpha_4\beta_2^*$ nAchR located on dopamine
neurons in the ventral tegmental area and nucleus accumbens to promote dopamine release in the nucleus accumbens (Cohen et al., 2005b; Pidoplichko et al., 1997). For indirect modulation, nicotine binds to the α\textsubscript{4}β\textsubscript{2} \* nAchR on GABA-ergic neurons and the α\textsubscript{7} nAchR on glutamate neurons in the ventral tegmental area. The α\textsubscript{4}β\textsubscript{2} \* nAchR however, de-sensitizes more quickly, leaving the α\textsubscript{7} nAchR active longer and thus, since the glutamate neuron is active longer, the net effect is it stimulates the dopamine neuron longer to promote further dopamine release in the nucleus accumbens (Cohen et al., 2005b; Pidoplichko et al., 2004). In addition to this, nicotine, in particular under chronic administration, increases endocannabinoid levels in the limbic areas of the brain (Gonzales et al., 2002), where CB1 receptors in the nucleus accumbens and ventral tegmental area are found (Cohen et al., 2005b; Herkenham et al., 1991; Le Foll et al., 2008; Maldonado et al., 2006). The endocannabinoids bind to the CB1 receptors on GABA neurons in the ventral tegmental area, and this results in less GABA being released onto dopamine neurons in the ventral tegmental area. This ultimately results in less inhibition of dopamine release in the nucleus accumbens, and thus endocannabinoids help to promote the reinforcing effects of nicotine (de Bruin et al., 2011; Le Foll et al., 2008). Endocannabinoids also bind to CB1 receptors located on glutamate neurons, however the inhibition of GABA release due to endocannabinoid-CB1 receptor interaction is more predominant (Cheer et al., 2007; Lupica and Reigel, 2005; Riegel and Lupica, 2004; Szabo et al., 2002).

Since CB1 receptors are not found on dopamine neurons (Lupica and Riegel, 2005), and since there is no interaction between rimonabant and the nAchR (Cohen et al., 2005b), it is unlikely that rimonabant works through a direct mechanism in preventing nicotine-induced dopamine release. When rimonabant is administered, it binds to the CB1 receptors on GABA neurons in the ventral tegmental area, and thereby blocks endocannabinoids from binding to
the receptor. Therefore, resulting in the disinhibition of GABA release onto dopamine neurons in the ventral tegmental area, and this inhibits dopamine release in the nucleus accumbens (Cohen et al., 2002; 2005; de Bruin et al., 2011). By indirectly inhibiting dopamine release, rimonabant attenuates the reinforcing effects of nicotine. Although rimonabant is an inverse agonist by classification, it resembles AM4113 in that it blocks endocannabinoids from binding to the CB1 receptor. Thus, it is believed AM4113, the neutral CB1 antagonist, will act via the same proposed mechanism.

4.4. RECOMMENDATIONS FOR FUTURE RESEARCH

4.4.1. Investigating AM4113’s side effect profile

Validating the therapeutic potential and side effect profile of AM4113 at the preclinical and clinical level still remains to be completed. The preclinical results presented here do show promise for AM4113 as a possible future smoking cessation aid, but more verification is still required. However, at the present time, examining the side effect profile of AM4113 in animals should be the next phase of testing. As is this case with all drugs, although it may be an effective treatment for a given disorder, if its side effects are too serious, its therapeutic value is irrelevant. The main question with AM4113 is whether it will display the side effects of CB1 inverse agonists, such as anxiety, depression, and thoughts of suicide (European Medicines Agency, 2008). These side effects caused the withdrawal of this class of drug as an anti-obesity agent and potential as a smoking cessation aid.

Some of the crucial work on side effects has already been initiated. Sink and colleagues (2010) have already tested whether acute AM4113 treatment has anxiogenic properties and have found no effect on the elevated plus maze. This is a promising outcome as AM251 (Sink et al., 2010) and rimonabant (Navarro et al., 1997) both demonstrated an anxiogenic effect in
similar tests. However, as mentioned, they only tested acute AM4113 in their paradigm, and this is not how the compound would be taken in the clinical setting. Therefore, the next set of tests should involve the chronic administration of AM4113, followed by subsequent anxiety test measures (e.g., elevated plus maze) to see whether chronic CB1 blockade via AM4113 can induce anxiogenic effects. If no effects are found, this will make the transition from animal to human work more likely and feasible. There is evidence to indicate that this may well be the case. O’Brien et al. (2013) have acutely and chronically administered tetrahydrocannabivarin, a cannabis-derived CB1 neutral antagonist, and, under both administrations, they found no anxiogenic response in the light-dark immersion anxiety model, a model which did show anxiogenic effects of rimonabant. This would suggest that the inverse agonist properties of drugs like rimonabant and AM251 were responsible for their anxiogenic effects. This initial data holds great promise for AM4113, but nevertheless, AM4113 itself needs to be tested to confirm its safety.

The other side effect that contributed to rimonabant’s and other CB1 inverse agonists’ demise as therapeutic agents was the occurrence of depression (European Medicines Agency, 2008). This was later supported by animal work (Beyer et al., 2010), which showed that the chronic administration of rimonabant induced depressive-like behaviours, similar to those expressed by chronic mild stress rats in both the forced swim test and the sucrose-consumption test. It is imperative to determine whether this side effect is attributable to the inverse agonist properties of rimonabant or whether it is a result of CB1 blockade. Jutkiewicz et al. (2010) have already demonstrated AM4113 to have no effect in the forced swim test, but the testing of AM4113 chronically using both the sucrose-preference test and the forced swim test is still required.
4.4.2. Investigating AM4113’s effectiveness on nicotine-seeking behaviour

If the expected safety profile of AM4113 can be confirmed, attention should next be directed to validating its therapeutic potential in the prevention of relapse (i.e., reinstatement of nicotine-seeking behaviour). As alluded to in the introduction, there are three main preclinical models of relapse: cue-, drug- and stress- induced reinstatement. Although in general there is a poor correlation between the findings in these preclinical studies and the findings in human clinical studies (Katz and Higgins, 2003), and the translational validity could be improved (Lerman et al., 2007), it is still reasonable to test AM4113 in these paradigms. Rimonabant was found to be efficacious in reducing cue-induced reinstatement (Cohen et al., 2005a; Forget et al., 2009). It is believed that CB1 inverse agonists, and we hypothesize that CB1 neutral antagonists may be effective as well at attenuating cue-induced reinstatement by acting on the mesocorticolimbic dopaminergic system similar to how it is described to work on nicotine-taking. The findings of Kodas and colleagues (2007) supports this hypothesis as they were able to show that rimonabant produced its attenuating effect on cue-reinstatement by acting on the nucleus accumbens shell, prelimbic cortex and the basolateral amygdala, all important structures of the mesocorticolimbic system (Forget et al., 2009; Le Foll et al., 2008), which would probably be targeted by AM4113 as well. Furthermore, rimonabant also attenuates nicotine priming-induced reinstatement (Forget et al., 2009) and, since rimonabant has demonstrated a good correlation between preclinical and clinical studies, it is therefore likely that the same correlation will be present for AM4113. Also, it is important to remember at this stage that these are the best models available, and gaining a little information from them provides some knowledge versus having none, and proceeding into human testing blindly.
4.4.3. Investigating AM4113’s mechanism of action

The last important aspect of AM4113 that should be addressed at the preclinical level relates to its site and mechanism of action. One approach to this question would be to conduct a microdialysis experiment similar to that of Cohen et al. (2002), to assay the dopamine levels in key mesocorticolimbic structures (e.g., nucleus accumbens). This would determine the effect of AM4113 (intraperitoneally) pretreatment on nicotine (subcutaneously) induced dopamine release. In addition, the effect of direct intracerebroventricular or intra-ventral tegmental area administration of AM4113 on nicotine self-administration behaviour could be studied. This experiment would be similar to how Sink and colleagues (2009b) conducted intracerebroventricular administration of AM4113 on food-taking behaviour. This would be a feasible study to conduct, and this approach would give insight into the hypothesis that AM4113 does indeed work on CB1 receptors located in the ventral tegmental area to help reduce the reinforcing effects of nicotine.

Lastly, it has been shown (Laviolette et al., 2002; Laviolette and van der Kooy, 2003a, b) that there are dopamine dependent and independent reinforcing effects associated with nicotine administration, and that by infusing nicotine into the ventral tegmental area it is possible to differentiate between the reinforcing and aversive motivational effects related to nicotine dependence. Challenging with AM4113 under such a paradigm would be a unique opportunity to possibly see if and how AM4113 affects the reinforcing and aversive motivational effect related to nicotine.

4.4.4. Studying AM4113 at the clinical level

If AM4113 demonstrates effectiveness preclinically against both nicotine-taking and – seeking behaviour, and is free of the side effects that plagued rimonabant’s use, validating the
drug at the clinical level would be the ultimate goal. Since there is a lack of consistent effective smoking cessation aids available today, additional therapeutic agents that could help curb nicotine dependence would be welcomed. However, AM4113 must also demonstrate that it is free of anxiogenic and depressive side effects that can lead to thoughts of suicide. In addition, if AM4113 is indeed found to be effective against nicotine dependence it will also be important to see if itching (pruritus), which has been seen with animals, will present itself, and, if so, to what frequency/extent and whether AM4113’s effectiveness can trump this unpleasant side effect.

Taking all that has been discussed into consideration, and with the preclinical results and knowledge at hand, AM4113 looks to be a promising therapeutic agent for smoking cessation.

4.4.5. Expanding studies with AM4113 to other substances of abuse

The scope of investigating AM4113’s potential should not be limited to its effects on nicotine addiction and obesity. Rather, we should test its efficacy as an aid for treating other substances of abuse. Tobacco dependence is not the sole addiction problem faced by society. In Canada alone, in individuals aged 15 years and older, the rate of abuse or dependence in their lifetime for alcohol is 18.1%, for cannabis 6.8%, and for other drugs of abuse 4.0% (Statistics Canada, 2012). Treatments for dependence to these other substances of abuse is required and, if a viable option is available, than it should be tested. AM4113 would fit the category of a viable option because past studies with rimonabant have shown it to be efficacious at attenuating the effects of other substances of abuse in addition to nicotine.
4.4.6. Rationale for investigating AM4113 for alcohol dependence

Studies on the effects of rimonabant on alcohol motivated behaviour are second only to nicotine in the category of substance abuse studies. Based on these studies, the endocannabinoid system seems to be involved in the reinforcing and motivational properties of alcohol in both alcohol-taking and seeking through modulation of the mesocorticolimbic dopamine pathway (Justinova et al., 2009; Maccioni et al., 2010). Economidou and colleagues (2006) have demonstrated that rimonabant administration significantly decreases responding for alcohol under both fixed ratio-1 and progressive ratio schedules of reinforcement in regular Male Wistar rats. Cippitelli et al. (2005), using the same rats have also observed the attenuating effect of rimonabant on alcohol self-administration. Furthermore, these researchers also conducted rimonabant challenges to operant self-administration of alcohol in Marchigian Sardinian alcohol-preferring rats, and found the CB1 inverse agonist to be effective and even more potent in these genetically selected alcohol preferring rats compared to the Wistar rats (Cippitelli et al., 2005). In a different strain of alcohol-preferring rats, Alko, Alcohol (AA), Malinen and Hyytia (2008) have also demonstrated that rimonabant attenuates responding for ethanol. These studies strongly support rimonabant’s efficacy with respect to alcohol-taking behaviour, and its effectiveness in different strains of animals with different baseline alcohol preferences.

In regards to the modulation of alcohol-seeking behaviour, rimonabant has been found to significantly reduce cue-induced reinstatement in both Wistar (Cippitelli et al., 2005; Economidou et al., 2006) and in Marchigian Sardinian alcohol-preferring rats (Cippitelli et al., 2005). However under the foot-shock stress-induced reinstatement paradigm, rimonabant was not able to reduce alcohol-seeking behaviour (Economidou et al., 2006). Therefore, from these
preclinical results it currently seems like CB1 blockade would be effective for the prevention of cue-induced relapse, but not stress-related relapse.

The success of rimonabant on alcohol self-administration at the preclinical level has led to studying and validating these findings at the clinical level. In a 12-week phase IIa (proof-of-concept), double-blind, placebo-controlled trial, rimonabant’s efficacy with respect to alcohol relapse prevention in alcohol-dependent subjects has been evaluated. Although the primary measure was not statistically significant, there was a minor difference in relapse rate with 47.7% from the placebo treatment group and 41.5% of the rimonabant treatment group relapsing (Maccioni et al., 2010; Soyka et al., 2008). In another double-blind, placebo-controlled trial of 3-weeks (2-week treatment schedule) duration in non-treatment-seeking heavy alcohol drinkers, the investigators did not find rimonabant to have an effect on alcohol consumption during either the outpatient reporting or the laboratory measurement sessions (George et al., 2010; Maccioni et al., 2010). The lack of correlation between the preclinical and clinical results is disappointing. In the former clinical study, the investigators cite the high response rate of the placebo treatment group as a possible factor contributing to the lack of significant effects in their study (Soyka et al., 2008). In the latter study the short treatment duration (i.e., 2 weeks), and the baseline bias of the subjects being that they are not looking for treatment, could explain the lack of significant effects in this study.

The lack of significant effects with rimonabant at the clinical level should not be the sole determining factor in discouraging the testing of AM4113 preclinically and clinically on alcohol’s reinforcing effects. The repetitive success of rimonabant at the preclinical level on alcohol-taking and alcohol-seeking should provide reason enough to at least validate AM4113 in a similar battery of tests. If AM4113 is found to have efficacy like rimonabant at the preclinical stage, AM4113 testing should be translated to clinical studies even if only
rimonabant did show a non-significant benefit over placebo-treatment. There is potential for AM4113 to be effective in the treatment of alcohol dependence/abuse and in the prevention of alcohol relapse.

4.4.7. *Rationale for investigating AM4113 for marijuana and other illicit drug dependence*

The effects of CB1 blockade on the reinforcing effects of Δ⁹-tetrahydrocannabinol (THC; partial agonist for CB1 receptor), the main psychoactive ingredient in marijuana (Justinova et al., 2003, 2008; Tanda et al., 2000), and other illicit drugs of abuse, such as cocaine, have also been studied using rimonabant. To model human marijuana abuse, currently the best available animal model is THC self-administration in squirrel monkeys, as THC self-administration in rats and mice has not been successful to date (Justinova et al., 2009). In fact at first, establishing THC self-administration even in squirrel monkeys had its challenges. The first successful acquisition of THC self-administration in monkeys occurred only after they were previously trained to self-administer cocaine (Tanda et al., 2000). The confounding factor in this early study is that the previous exposure to cocaine might have promoted a predisposition for these monkeys to then self-administer THC (Justinova et al., 2003). However, Justinova and colleagues (2003) have since established self-administration of clinically relevant THC doses in drug naïve squirrel monkeys, permitting the use of this animal model for the study of marijuana abuse. With respect to the effects of CB1 blockade on THC taking behaviour, Tanda and colleagues (2000) showed rimonabant pretreatment to reduce the number of THC infusions earned compared to vehicle pretreatment levels. Furthermore, rimonabant was also effective at attenuating THC-seeking in cue-induced and THC priming reinstatement tests (Justinova et al., 2008).

Initial studies of rimonabant on cocaine self-administration showed no effect in squirrel monkeys (Tanda et al., 2000) and rats (De Vries et al., 2001) under fixed ratio schedules of
reinforcement, suggesting that CB1 receptors might not be involved in cocaine’s reinforcing effects (Justinova et al., 2009). Contrary to this, more recent studies utilizing a progressive ratio schedule of reinforcement have found rimonabant to be efficacious at reducing the breaking point in both mice (Soria et al., 2005) and rats (Orio et al., 2009). These findings suggest that rimonabant might be effective at attenuating the reinforcing and motivational properties of cocaine. It is possible, that the effects of CB1 blockade on cocaine self-administration may only be seen under a more demanding schedule of reinforcement where more motivation is required to achieve the next infusion due to the progressive increase in responding required to achieve this next cocaine infusion. With respect to CB1 blockade efficacy on cocaine-seeking behaviour, rimonabant reduced cue- and cocaine-induced reinstatement, but it demonstrated no such effect on reinstatement tests that were stress-induced (De Vries et al., 2001).

Therefore, there is enough preclinical evidence with rimonabant to support the notion that CB1 blockade utilizing AM4113 might also be efficacious in attenuating THC and cocaine’s reinforcing effects. Furthermore, it seems highly plausible that AM4113 would also be efficacious in reducing cue- and drug-induced seeking behaviour for both drugs. Hence, if validated, AM4113 might also serve as a very useful treatment for marijuana and cocaine dependence and in the prevention of relapse to both drugs of abuse.

4.5. OVERALL CONCLUSION

The neutral CB1 antagonist, AM4113, is effective at attenuating nicotine-taking behaviours. AM4113 (60-min pretreatment time) is able to attenuate the reinforcing effects of nicotine acutely, while having no non-specific effects in food control experiments. Furthermore, chronic administration of AM4113 was demonstrated to be equally effective at
reducing nicotine’s reinforcing effects with no tolerance developing during the course of the
treatment, and it showed little effect in the similar food control self-administration
experiments.

Before further research is conducted on further validating AM4113’s therapeutic value,
attention should focus on testing its side effect profile. The next phase of AM4113 testing
should involve identifying whether or not its effects can produce depressive- and anxiety- like
behaviours at the preclinical level.

The next priority for research on AM4113 should centre on whether it would be
efficacious for relapse prevention to smoking by conducting nicotine-seeking studies involving
cue-, nicotine- and stress- induced reinstatement models. In concert with continuing to validate
AM4113’s therapeutic value, efforts should also be made to determine how AM4113 works on
the neurocircuitry of addiction and to uncover its mechanism of action.

Pending promising results at the preclinical level for both therapeutic potential and
limited negative side effects, the investigation into AM4113 should be expanded to test its
preclinical effects on other substances of abuse and it should ultimately be translated to clinical
studies. Currently, with the known positive effects of rimonabant, and the preliminary positive
data presented in this study of AM4113 on nicotine’s reinforcing effects, AM4113 has great
promise as the next smoking cessation aid and a possible treatment for other drugs of abuse.
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