Examining the role of the insular cortex in addiction-relevant behaviours

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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University of Toronto

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Abstract

INTRODUCTION: Recently recognized as a critical brain region underlying the neurocircuitry of addiction, the body of evidence supporting the role of the insular cortex across multiple aspects of addiction is growing rapidly. Understanding the complex role of the insular cortex is crucial to moving forward with the development of therapeutics targeting this brain region for substance-related and addictive disorders.

OBJECTIVES: The current series of experiments were conducted to investigate the effects of modulating activity in two insular subregions, through the use of multiple modalities (chemical lesions, pharmacological inactivation or electrical stimulation), on behaviours relevant to addictive disorders, including drug self-administration, reinstatement, and decision-making.

METHODS: In the first series of experiments, we investigated the effects of chemical lesions or transient pharmacological inactivation of two distinct subregions of the insular cortex, the rostral agranular (RAIC) vs. caudal granular (CGIC), on the acquisition or performance of choice behaviour in the Rat Gambling Task (rGT). In the next series of experiments, we examined the effects of pharmacological inactivation of the RAIC on nicotine self-administration under a fixed ratio-5 (FR-5) schedule of reinforcement and the reinstatement of nicotine-seeking induced by
nicotine-associated cues. Finally, we examined the effects of electrical modulation of the insular region on nicotine self-administration under both FR-5 and progressive ratio schedules of reinforcement and the reinstatement of nicotine-seeking by cues and nicotine priming injections.

RESULTS: Lesions of the RAIC, but not CGIC, resulted in higher preference for options which produced lower reward amounts with higher reward consistency while inactivation of the RAIC, but not CGIC, shifted previously acquired preferences towards those same low reward/high consistency options. Inactivation of the RAIC attenuated nicotine self-administration and cue-induced reinstatement. Finally, electrical modulation functionally inactivated the insular region, resulting in attenuated nicotine self-administration, under FR-5 and progressive ratios, and of cue- and priming-induced reinstatement.

CONCLUSIONS: This work demonstrates involvement of the RAIC, but not CGIC, in cognitive aspects of addictive disorders, such as gambling-relevant behaviours. Yet both regions were demonstrated to be implicated in substance-based addictive disorders. Thus, therapeutics using electrical modulation to target the CGIC may be preferable for substance dependence, with limited cognitive side effects.
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<tr>
<td>5CSRTT</td>
<td>5-choice serial reaction time task</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
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<tr>
<td>AID</td>
<td>agranular insular cortex - dorsal part</td>
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<tr>
<td>AIP</td>
<td>posterior agranular insula</td>
</tr>
<tr>
<td>AIV</td>
<td>agranular insular cortex - ventral part</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
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<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BOLD</td>
<td>blood oxygen level dependent</td>
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<tr>
<td>BP</td>
<td>break point</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of the amygdala</td>
</tr>
<tr>
<td>CGIC</td>
<td>caudal granular subregion of the insular cortex</td>
</tr>
<tr>
<td>Cl</td>
<td>Claustrum</td>
</tr>
<tr>
<td>CPA</td>
<td>conditioned place aversion</td>
</tr>
<tr>
<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>dACC</td>
<td>dorsal anterior cingulate cortex</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>dopamine- and cyclic adenosine monophosphate-regulated phospoprotein</td>
</tr>
<tr>
<td>DBS</td>
<td>deep brain stimulation</td>
</tr>
<tr>
<td>DI</td>
<td>dysgranular insula</td>
</tr>
<tr>
<td>DMN</td>
<td>default-mode network</td>
</tr>
<tr>
<td>DSM-5</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition</td>
</tr>
<tr>
<td>ECN</td>
<td>executive control network</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
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<tr>
<td>FR</td>
<td>fixed ratio</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GI</td>
<td>granular insula</td>
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<tr>
<td>HFS</td>
<td>high frequency stimulation</td>
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<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>IGT</td>
<td>Iowa Gambling Task</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Interperitoneal</td>
</tr>
<tr>
<td>IR-DIC</td>
<td>infrared differential interference contrast</td>
</tr>
<tr>
<td>ITI</td>
<td>intertrial interval</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K&lt;sub&gt;Dir&lt;/sub&gt;</td>
<td>delayed inward-rectifying K&lt;sup&gt;+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>LO</td>
<td>lateral orbital cortex</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-methylenedioxy-methamphetamine</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAcc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>nAChRs</td>
<td>nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NIDA</td>
<td>National Institute for Drug Abuse</td>
</tr>
<tr>
<td>NRT</td>
<td>nicotine replacement therapy</td>
</tr>
<tr>
<td>OFC</td>
<td>orbitofrontal cortex</td>
</tr>
<tr>
<td>PCC</td>
<td>posterior cingulate cortex</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PHNO</td>
<td>(+)-4-propyl-9-hydroxynaphthoxazine</td>
</tr>
<tr>
<td>PR</td>
<td>progressive ratio</td>
</tr>
<tr>
<td>RAIC</td>
<td>rostral agranular subregion of the insular cortex</td>
</tr>
<tr>
<td>rCET</td>
<td>rat cognitive effort task</td>
</tr>
<tr>
<td>rGT</td>
<td>rat Gambling Task</td>
</tr>
<tr>
<td>RM</td>
<td>repeated measures</td>
</tr>
<tr>
<td>rsFC</td>
<td>resting-state functional connectivity</td>
</tr>
<tr>
<td>rTMS</td>
<td>repetitive transcranial magnetic stimulation</td>
</tr>
<tr>
<td>S1J</td>
<td>primary somatosensory cortex - jaw region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SA</td>
<td>self-administration</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SN</td>
<td>salience network</td>
</tr>
<tr>
<td>TAS-20</td>
<td>Toronto Alexithymic Scale</td>
</tr>
<tr>
<td>tDCS</td>
<td>transcranial direct current stimulation</td>
</tr>
<tr>
<td>vmPFC</td>
<td>ventromedial prefrontal cortex</td>
</tr>
<tr>
<td>VO</td>
<td>ventral orbital cortex</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>zif268</td>
<td>zinc finger protein 225</td>
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CHAPTER 1. Introduction

1.1 Substance-related and Addictive Disorders

1.1.1 Addiction: A Major Burden for Canada and the World

Substance-related and addictive disorders, commonly referred to as addictions, are often viewed as behavioural deficits limited to the fringes of the population, and as such they are not given the level of funding or attention compared to health issues such as cancer and cardiovascular disease. Yet two of the three leading risk factors for global disease burden are those associated with substance-related and addictive disorders: tobacco smoking (including second hand smoke) and alcohol use (Lim et al., 2012) while nearly 9% of the total global burden of disease can be attributed to alcohol, tobacco and illicit drugs as a group (Rehm et al., 2006). Importantly, Canada does not appear to be immune from this burden with substance use being estimated to have caused 23% of all potential years of life lost in this country in 2002, with over 3.8 million hospital days and 40,000 deaths directly resulting from substance abuse (Rehm et al., 2007).

From these numbers, it is not difficult to begin to understand the obvious health implications to the individual, yet it is important to realize that addiction is a major cause of economic and social burden. Together with mental health, substance use disorders are the leading cause of years lived with disability worldwide (Whiteford et al., 2013) resulting in a tremendous social burden on the families and communities in which these patients reside. In the year 2002, in Canada alone, the direct healthcare costs of tobacco, alcohol, and illegal drugs was estimated to be $8.8B, while the costs of law enforcement for these issues were $5.6B (Rehm et al., 2007). Yet the largest economic tolls were indirect costs or productivity losses which accounted for an estimated of $24.4B. Thus, the major burden that substance-related and addictive disorders impose upon Canadian society, and the world as a whole, cannot be ignored.
1.1.2 Defining Addiction: Diagnostic Criterion Underlying Addictive Disorders

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) describes the diagnostic criteria for Substance-Related and Addictive Disorders, which encompasses 10 separate classes of drugs along with a single non-substance related disorder: gambling disorder (APA, 2013). The inclusion of gambling disorder within this edition reflects the evolving understanding of addiction within the medical and scientific community. Thus, before proceeding to examine the role of the insular cortex within addiction-relevant behaviours, it is necessary to first present a general overview of the fundamental shared features underlying addictive disorders and thus what makes certain behaviours relevant to addiction. These fundamental features are their shared clinical expression, comorbidity, treatment, and neurobiological basis.

Of the nine DSM-5 diagnostic criteria for gambling, eight can easily be understood within the context of substance dependence. Individuals with substance dependence will often display many clinical features similar to those indicative of gambling disorder: (1) restless or irritable when attempting to cut down or stop their addictive behaviour, (2) have made repeated unsuccessful efforts to control, cut back, or stop their addictive behaviour, (3) thoughts which are primarily preoccupied with reliving or planning future addictive behaviour, (4) often engage in addictive behaviour when feeling distressed, (5) lie to conceal the extent of involvement with their addictive behaviour, (6) has jeopardized or lost a significant relationship, job, or educational or career opportunity because of their addictive behaviour, (7) relies on others to provide money to relieve desperate financial situations caused by their addictive behaviour. Finally, (8) the need to gamble with increasing amounts of money in order to generate excitement is parallel to the need to consume increasing amounts of drug in order to achieve the
desired subjective effects. Though the latter case is often presumed to be purely due to pharmacological tolerance, there is substantial literature demonstrating significant neuroplasticity in the brain regions underlying reward, following repeated exposure to drugs of abuse or non-drug rewards, such as gambling (Olsen, 2011). Importantly, not all criteria will be present in every case of substance dependence or gambling disorder; however, the degree of overlapping criteria clearly demonstrates their shared clinical expression.

With such similar clinical expression, it is not surprising to find high prevalence of comorbidity, both co-occurrence and sequential, of substance dependence and gambling disorder (Petry et al., 2005) as well as the existence of polysubstance dependence (Connor et al., 2014). Additionally, both substance dependence (Schuckit, 2006) and gambling disorder (Petry et al., 2005) are noted for having high prevalence of comorbidity with mood, anxiety and personality disorders, though substance abuse is much more prevalent in comorbidity for psychiatric disorders in general. It should be noted that the likelihood of an individual diagnosed with an addictive disorder having a comorbid psychiatric disorder is significantly higher than vice versa, suggesting the possibility that addictive behaviour may induce or trigger concurrent or subsequent psychiatric disorder (Jane-Llopis and Matytsina, 2006). Finally, both substance dependence and gambling disorder are associated with a high degree of impulsiveness (Petry, 2001), though whether it is a cause or consequence remains unclear (Grant and Chamberlain, 2014).

The neurobiological basis of addictive disorders, including gambling disorder will be described throughout the introduction, in the sections following.
1.1.3 Treating Addiction: A Lack of Highly Efficacious Therapies

Currently, there are two well accepted treatment approaches for substance dependence, pharmacological and behavioural therapies, which are generally combined as part of a therapeutic process beginning with detoxification and followed by treatment and relapse prevention (NIDA, 2009). In the case of gambling disorder, there is no particular detoxification phase requiring a formal in-patient process; however, this is often the case for drugs such as tobacco and cannabis as well. Gambling disorder also lacks any Food and Drug Administration (FDA) -approved pharmacological therapies at this time; however, the same is true for stimulant (cocaine, methamphetamine) dependence and cannabis dependence. Tobacco (treated with nicotine replacement therapy [NRT], varenicline, or buproprion), alcohol (treated with naltrexone, acomprosate, and disulfiram) and opiate dependence (treated with methadone, buprenorphine, and naltrexone) all have FDA-approved pharmacological therapies. Naltrexone is currently the only medication FDA-approved for use in both alcohol and opiate dependence, though varenicline has shown promise as a potential treatment for alcohol dependence (Erwin and Slaton, 2014).

Though individual research studies focus on drug targets for a particular substance of abuse or gambling disorder, these targets are often found to be involved in dependence for multiple substances and gambling disorder, as shall be discussed further in subsequent section on the shared neurobiological underpinnings of addictive disorders. As such, it is also understandable that various behavioural treatment approaches, such as motivational interviewing, contingency management, cognitive behavioural therapy and mindfulness-based relapse therapy, are relevant across addictive disorders (NIDA, 2009; Potenza et al., 2013).
Regardless of treatment modality, whether pharmacological and/or behavioural approaches, relapse rates following established treatment regimens for addictive disorders remain high, with conservative estimates of relapse being 40-60% (McLellan et al., 2000). Thus there is a significant need for novel therapeutics for the treatment of addictive disorders. The identification of targets in the search for novel therapeutics is focused on understanding the complex neurobiological underpinnings of addictive disorders.

1.1.4 Identifying Novel Therapies: The Use of Animal Models

There are several approaches used for studying the neural circuitry involved in addictive disorders; however, the study of human subjects is limited in terms of the interventions and approaches approved for use in such research. Animal models provide the opportunity to utilize highly invasive approaches, such as the direct brain lesions utilized in the present thesis research. As such, I will briefly overview the major animal models utilized within this thesis with regards to their particular advantages and limitations for assessing the neurobiology of addictive behaviour.

1.1.4.1 The Operant Self-Administration Paradigm

Of most interest in the context of this thesis, is the animal model of operant self-administration, particularly intravenous drug self-administration under fixed and progressive ratio schedules of reinforcement. The operant self-administration model is considered the gold standard for the study of the voluntary responding for and direct reinforcing capacity of a stimulus, including natural rewards and addictive drugs. This model typically involves an animal being trained to perform a behaviour, usually the pressing of a lever, in order to receive reinforcement in the form of an appetitive stimuli, such as food, water or an addictive substance (van Ree et al., 1978). Thus these stimuli serve as positive reinforcers as they are the direct and immediate
consequences of the behaviour which reliably increase their probability of occurrence (Stewart and Venzina, 1988).

Often, and in the case of the experimental work presented herein, the delivery of the unconditioned appetitive stimulus is paired with the presentation of a neutral discrete cue(s) that develops its own conditioned reinforcing properties (Stewart et al., 1984), and which can often become a required component of the reinforcing capacity of the addictive behaviour (Caggiula et al., 2001). Discrete or contextual cues can be instrumental in reinstating lever responding following the extinction of lever responding (Bossert et al., 2013), as will be discussed further below. Importantly, the drug self-administration model has high face and construct validity, as drugs capable of reinforcing self-administration in animals are those known to have high abuse liability in human subjects (Griffiths et al., 1979), while changes in dose are capable of affecting responding for said drugs (Kalivas et al., 2006).

The self-administration paradigm is most frequently studied using a fixed ratio (FR) schedule of reinforcement, where animals are required to make a fixed number of responses in order to receive each reinforcement, as it is the easiest way to ensure a large majority of animals learn the required behaviour (Schindler et al., 2002). Using the FR schedule of reinforcement, animals self-administer addictive substances in a fairly consistent manner, in an attempt to maintain a relatively consistent level of dopamine in the nucleus accumbens (NAcc) throughout the session (Robbins and Everitt 1992) with characteristic U-shaped dose-response curves having been established for several major drugs of abuse (Corrigall and Coen, 1989). At very low doses there is no significant responding, but as dose is increased responding and number of reinforcements increases before peaking (at approximately 0.03mg/kg/infusion for nicotine) and then declining thereafter. As such, this schedule is difficult to utilize for confirming whether an
experimental treatment potentiates or diminishes the effects of the reinforcer. For example, if a treatment significantly potentiates the dopamine release caused by an intravenous infusion of an addictive substance, the animal will reduce its responding and intake of the addictive substance. Yet if another treatment significantly diminishes dopamine release by the same addictive substance, the animal may either increase their responding to compensate or their responding will begin to extinguish as it is no longer sufficiently reinforced.

Another method of assessing reinforcing efficacy (i.e. the motivational capacity of a stimulus to elicit responding) is the progressive ratio (PR) schedule of reinforcement, where the animal is required to increase the number of responses made to receive each successive reinforcement. The PR schedule used within the experimental work presented in this thesis is exponential in nature, and lever responding becomes relatively stable after an acquisition period of a few sessions (Stafford et al., 1998). Break point, the largest ratio requirement the animal completes to obtain its final reinforcer, is typically used to assess the effect of a treatment on the reinforcing capacity or "potency" of a stimulus (Poling, 2010). Though the PR schedule provides valuable additional information to FR schedule assessment, it must be noted that no single schedule can completely characterize the effect of a treatment on reinforcer efficacy (Arnold and Roberts, 1997). A wide range of parameters utilized under FR and PR schedules can significantly alter responding levels, while differences are often also observed across drugs of abuse (Stafford et al., 1998). To truly characterize reinforcer efficacy, consistent findings across multiple schedules and varying parameters are best compared with non-behavioural measures, such as in vivo microdialysis or electrophysiology (Roane, 2008).
1.1.4.2 The Reinstatement Paradigm

In this section, I will delve into the reinstatement model in rats, as induced by cues/context, drug-priming, and stress. The reinstatement model is based on the operant self-administration model described above, where animals are trained to respond for a reinforcing stimulus. In the reinstatement model, following acquisition of stable responding for the reinforcer, behaviour is then extinguished by removing access to the reinforcer and allowing the animals to respond and learn the reinforcer is no longer availability (Shalev et al., 2000). Critically, the discrete cues previously paired with the delivery of the reinforcer are also withheld during the extinction period, or in the case of context-induced reinstatement the extinction training occurs in an environment different from that in which responding was acquired.

The effects of chronic performance of an addictive behaviour results in adaptations within the neurocircuitry underlying reward and memory, which biases attention towards addiction-related cues and contexts (Shaham et al., 2003). As such, the re-introduction of cues or context, which were removed during extinction training, can reinstate lever pressing behaviour in rats (Shalev et al., 2002). Alternatively, the reinforcer itself can be presented and is capable of reinstating responding behaviour, which is termed priming-induced reinstatement (Schmidt et al., 2005). While for addictive substances, certain forms of stress, including electric footshock and pharmacological stressors such as yohimbine, are also capable of reinstating drug seeking behaviour (Shaham et al., 2000). Importantly, all three methods of inducing relapse in animals are known to induce craving in human addicts and are associated with relapse to addictive behaviour (O'Brien, 2005; Sinha et al., 2006; Sinha and Li, 2007).

Briefly, these three methods of reinstatement do overlap to some degree with respect to neurobiological underpinnings, particularly with regards to the involvement of the mesolimbic
dopaminergic system and the extended amygdala (Shalev et al., 2002). The neurobiology underlying reinstatement induced by an addictive substance is varied to a degree as it is fairly dependent upon the particular substance, though generally they all appear to share common a common pathway of dopaminergic projections from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) which sends glutamatergic projections to the NAcc, and finally the NAcc sending inhibitory projections to the ventral pallidum and driving behaviour (Kalivas and McFarland, 2003; Kalivas and O'Brien, 2008). Cue-induced reinstatement also shares components of the same pathway; however, the process appears to begin in the basolateral amygdala (BLA) before feeding forward to the PFC (Weiss et al., 2001). Finally, the neurobiology underlying stress-induced reinstatement overlaps to a large degree with that described previously in the section on stress systems in withdrawal, specifically the noradrenergic and corticotropin-releasing factor (CRF) systems of the extended amygdala, particularly the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) in rats (Shaham et al., 2000; Shalev et al., 2002).

Interestingly, though it seems logical that the attentional bias towards addiction-related cues and context should begin to diminish as individuals abstain from the addictive behaviour, this expected time course has been demonstrated to be incorrect in animal models of cue-induced reinstatement of addictive substances (Pickens et al., 2011). Specifically, the ability of drug-related cues to induce the reinstatement of drug-seeking behaviour has been demonstrated to increase for a significant period of time following initiation of abstinence from drug exposure, a phenomenon termed "incubation" (Grimm et al., 2001). Recently, this finding has been verified in human methamphetamine addicts, with cue-induced craving being greater at three months, but lower at six months, of abstinence when compared to cue-induced craving measured immediately following the initiation of abstinence (Wang et al., 2013).
1.1.4.3 The Rat Gambling Task

Cognitive deficits are known to be negatively correlated with good treatment outcomes for addictive disorders (Aharonovich et al., 2006) as they underlie a broader impairment in self-regulation of behaviour driven by prefrontal circuitry, which include impulsive and risk taking components. In this regard, several animal models have been developed to examine impulsivity and/or risk taking in decision-making behaviours. The model utilized within the experimental work presented within this thesis is known as the rat Gambling Task (rGT), a rodent version of the Iowa Gambling Task (IGT), a human decision-making paradigm under risk and uncertainty (Zeeb et al., 2009). In this task animals are placed within a chamber where they are trained to associate four illuminated holes with their respective outcomes differing in probability and amount of reward (food pellets) or punishment (time out period). After they have had significant time to learn the hole-outcome pairing, they are allowed to make their own decision of which holes to choose from in daily sessions involving multiple trials.

Certain holes (named P1 and P2) in the rGT are considered behaviourally optimal, in that if they are chosen consistently they provide greater overall number of pellets and least time out duration in a session compared to suboptimal holes (P3 and P4). Importantly, the two optimal holes provide low reward per trial amounts (one and two pellets for P1 and P2, respectively) and low punishment per trial durations (5 and 10 seconds for P1 and P2, respectively) but with high probability of reward per trial (90% and 80% for P1 and P2, respectively). The suboptimal holes (P3 and P4) provide the inverse, with high reward and punishment but low probability of reward. Thus animals are faced with a situation in which they must weigh immediate reward and risk against long term overall reward (ie. obtaining the greatest number of pellets they can in a session).
Chronic exposure to alcohol during acquisition of the rGT has been demonstrated to result in increased choice of the suboptimal options (Spoelder et al., 2015), which is similar to decision-making behaviour observed in abstinent alcohol dependent subjects on the IGT (Bechara et al., 2001; Tomassini et al., 2012; Brevers et al., 2014; Korner et al., 2015). Similar suboptimal decision-making behaviour on the IGT has been observed in nicotine dependent individuals (Briggs et al., 2015), opiate dependent individuals (Lemenager et al., 2011), 3,4-methylenedioxy-methamphetamine (MDMA) dependent individuals (Hanson et al., 2008), cannabis dependent individuals (Whitlow et al., 2004; Verdejo-Garcia et al., 2007; Wesley et al., 2011) and cocaine dependent individuals (Stout et al., 2004; Verdejo-Garcia et al., 2007; Kjome et al., 2010) though there is some evidence to suggest that the acute effects of cocaine can transiently reverse this prefrontal deficit (Garavan et al., 2008; Hulka et al., 2015). Of course, pathological gamblers also consistently display suboptimal decision-making behaviour in the IGT (Achab et al., 2014).

Finally, much work with both the rGT and IGT has examined the role of particular brain regions in these tasks, using either functional magnetic resonance imaging (fMRI) or individuals with lesions for studies of the IGT and transient pharmacological inactivation or lesions for the rGT. In this regard, there has been consistent demonstration for involvement of the orbitofrontal cortex (OFC) and PFC (Bechara et al., 1994; Bechara et al., 2000; de Visser et al., 2011a; Rivalan et al., 2011) and amygdala (Bechara et al., 1999; Zeeb and Winstanley, 2011). Thus the rGT appears to have good face validity though predictive and construct validity remain to be demonstrated (de Visser et al., 2011b).
1.2 The Insular Cortex in the Neurocircuitry of Addiction

1.2.1 The Cytoarchitecture of the Insular Cortex

First identified by Johann Christian Reil, the insular cortex was so named for being an island hidden in the banks of the Sylvian fissure, thus being termed the "Island of Reil" in the seminal textbook *Gray's Anatomy* (Binder et al., 2007). The insular cortex is composed of three distinct cytoarchitectural subregions ordered from the dorsal to ventral cortex, known as the granular, dysgranular and agranular. (Paxinos and Watson, 1986). The caudal granular region (CGIC; bregma to -3.8mm in rats) receives both general viscerosensory unimodal inputs (Cechetto and Saper, 1987) and nociceptive thalamic inputs (Gauriau and Bernard, 2004) along with somatosensory cortex inputs, while sending projections primarily back to the thalamus and somatosensory cortex along with projections to the caudate-putamen (Shi and Cassell, 1998a).

The CGIC is reciprocally connected to the rostral agranular insula cortex (RAIC) primarily through intermediate relays in the dysgranular insular subregion (Shi and Cassell, 1998b). The entire granular insula, including the CGIC, is the only component of the insula that does not send projections to amygdalar nuclei (Shi and Cassell, 1998b).

It should not be surprising to find that the insular cortex has been identified to receive projections from and send projections to several brain areas which are well known to play roles in addictive behaviour. The RAIC sends projections to the caudate-putamen, NAcc, and extended amygdala (McGeorge and Faull, 1989; Wright and Groenewegen, 1996; Shi and Cassell, 1998b; McDonald et al., 1999), while having reciprocal connections with the BLA and the prelimbic cortex (Gerfen and Clavier, 1979; Saper, 1982; Shi and Cassell, 1998b; Vertes, 2004). The RAIC is considered a high-order multimodal cortical region due to its inputs from the medial subdivision of the mediodorsal thalamic nucleus (Krettek and Price, 1977; Allen et
al., 1991), which in itself is considered a high-order thalamic nucleus (Van der Werf et al., 2002), along with inputs from various medial thalamic nuclei thought to convey motivational/affective components of nociception. Critically, along with its reciprocal connections with the extended amygdala discussed above, the insula also receives a high density of terminals coming from dopaminergic neurons that arise mainly from the VTA and substantia nigra (Ohara et al., 2003). Cortical glutamatergic projections, including those from the RAIC (McGeorge and Faull, 1989; Wright and Groenewegen, 1996; Shi and Cassell, 1998b), are believed to influence dopamine neuronal reactivity and dopamine release in the NAcc, critical for addictive behaviour (Kalivas and Volkow, 2005).

Given the high degree of connectivity between the insular cortex, particularly the RAIC, and the key brain areas in the neurocircuitry of addiction, it is somewhat surprising that the involvement of the insular cortex has remained hidden until quite recently.

1.2.2 The Insular Cortex and Interoception

The insular cortex has been implicated in behaviors such as conscious urges, anxiety, pain, cognition, and mood amongst several others (Hardy, 1985; Suhara et al., 1992; Goldman-Rakic, 1998; Damasio et al., 2000; Craig, 2002; Paulus and Stein, 2006). Though it is involved in a multitude of behaviours, the insular cortex was rarely studied in depth and was relegated to the mere role of "primary taste cortex" for decades (Penfield and Faulk, 1955; Pritchard et al., 1986). This is not to say that the insular cortex does not play a large role in taste, as studies of epileptic seizures in the insula (Isnard et al., 2004), stroke-induced lesions to the insula (Pritchard et al., 1999; Cereda et al., 2002), lesioning studies in rats (DeCoteau et al., 1997; Ragozzino and Kesner, 1999; Fresquet et al., 2004), and direct electrical stimulation studies in primates...
(Verhagen et al., 2004; Kadohisa et al., 2005) have confirmed that the insula is critical for perception, recognition and working memory in taste.

Even quite early on in the direct study of the insular cortex, the importance of such visceral perception in addiction was beginning to be noted, as dopaminergic innervation of the insular cortex was found to play an important role in the conditioning of the aversive aspects of opiate withdrawal (Zito et al., 1988), and thus the active avoidance of withdrawal, which can be observed as a key feature of opiate addiction (American Psychiatric Association, 2013). The learning and memory of gustation, particularly of disgust, plays a critical evolutionary role in mammals and thus it is not surprising that the insular cortex is critical to both the experience of disgust and its recognition in others (Calder et al., 2000; Wicker et al., 2003; Woolley et al., 2015). The importance of recognizing and experiencing disgust along with its resulting immune responses is now beginning to be recognized with relation to addiction (Sinha, 2014).

Yet the role of the insular cortex is much broader than merely as a primary taste cortex. Its critical thalamic connections described above allow it to integrate a wide variety of visceral sensations, including those from the airways, gut and cardiovascular systems (Cechetto and Saper, 1987; Allen et al., 1991), all known to be relevant with regards to bodily responses to the performance of addictive behaviours (Verdejo-Garcia et al., 2012). The realization of the true function of the insular cortex was made by AD Craig who recognized the insula as the critical brain region underlying interoception (Craig, 2002). Interoception is defined as the complete representation of all bodily signals and integration with external stimuli to guide behaviour towards or away from said stimuli for the purposes of maintaining homeostasis (Craig, 2003).

To put it simply, the caudal granular insular cortex keeps a constant account of the current state of the body as it is the converging point for numerous bodily signals relayed
through the thalamus, as described above. This information is then relayed through the
dysgranular insular cortex which appears to integrate salient external stimuli (Craig, 2004).
Finally, in the rostral agranular subregion of the insular cortex, the current state of the body and
environment are compared to prior states and environments in order to motivate behaviours for
the purpose of maintaining homeostasis (Craig, 2009b). This process only reaches conscious
awareness at the final stages and thus one does not need to consciously consider whether they are
cold before they are motivated to rush indoors during a winter snowstorm.

1.2.3 Addiction-Relevant Neuronal Receptor Populations within the
Insular Cortex

Several receptor populations known to play roles in motivation, stress and addiction are present
within the insular cortex, particularly the RAIC. The dopaminergic terminals from the VTA and
substantia nigra release onto a high density of dopamine D1-subtype receptors (Hurd et al.,
2001), with the agranular subregion being noted as an extrastriatal site of unusually high
dopamine utilization (Gaspar et al., 1989). When compared to the medial PFC, there are fewer
dopaminergic terminals innervating the agranular insula, yet the rate of dopamine release is
much high in the agranular insula, suggesting that there may be less autoreceptor-mediated
control of said release when compared to the medial PFC (Jones et al., 1986). Confirming the
role of insular dopamine receptors in addictive behaviour, a recent study demonstrated that a D1,
but not D2, receptor antagonist infusion into the RAIC was capable of attenuating nicotine self-
administration (Kutlu et al., 2013).

The high density of terminals coming from dopaminergic neurons arising from the VTA
and substantia nigra (Ohara et al., 2003) project to the agranular subregion of the insula which
contains large pyramidal neurons in layer 5 possessing gamma-Aminobutyric acid (GABA) B-
subtype receptors on their dendrites (Margeta-Mitrovic et al., 1999). Our own work has
demonstrated the ability of a mixture of GABA agonists, for both A and B subtypes, infused in the CGIC are capable of attenuating nicotine taking and seeking behaviours (Forget et al., 2010a). There are close appositions between dopamine fibers and GABAergic interneurons within the insula, with these GABA B-subtype receptor-bearing glutamatergic neurons projecting principally to the amygdala and NAcc (Ohara et al., 2003). Therefore, the insula is centrally placed to receive and integrate the information about the salience and relative value of environmental stimuli and of drugs effects coming from brain structures that have been implicated in reward and addiction.

The agranular insular cortex is also known to possess a high density of CRF subtype 1 receptors (Sanchez et al., 1999) with CRF being noted above for its role in both distress during withdrawal, stress-induced relapse. CRF is known to play a role in the motivation to consume drugs in human subjects with substance dependence (Contoreggi et al., 2003) and in the cue-induced reinstatement of cocaine-seeking (Cosme et al., 2015). Additionally, several studies have identified a dense innervation of orexin/hypocretion from the lateral hypothalamus into the granular insular cortex (Peyron et al., 1998; Date et al., 1999) with the granular insular cortex neurons recently being confirmed to possess a high density of hypocretin subtype-1 receptors (Hollander et al., 2008) which play a role in nicotine addiction. Finally, muscarinic acetylcholine receptors in the CGIC, specifically subtypes M1 and M4, have recently been identified to play a role in the associative conditioning effects of morphine, as assessed using the conditioned place preference task (Wu et al., 2014).

Given its well described role in pain processing (Berthier et al., 1988; Ostrowsky et al., 2002; Frot and Mauguiere, 2003; Afif et al., 2008; Mazzola et al., 2009; Segerdahl et al., 2015), it is not surprising to find that the insular cortex also has a high level of endogenous opioids
(Gaspar et al., 1989) along with a significant density of µ-subtype opioid receptors (Baumgartner et al., 2006). Thus, opiates likely act directly upon the insular cortex in their ability to mediate pain and potentially also in their addictive properties. Since the insula also expresses a high level of nicotinic acetylcholine receptors (nAChRs) containing the β2 subunit (Rubboli et al., 1994), the major subtype of nAChR implicated in nicotine reward (Maskos et al., 2005; Grabus et al., 2006), it is also highly likely that activation of insula occurs during exposure to nicotine and plays a role in its the addictive properties as well.

A recent study has also established the involvement of mu opioid receptor populations in the insula of smokers (Domino et al., 2015). That study demonstrated that cigarette smoking decreases [11C]carfentanil binding potential in the left insula, suggesting that cigarette smoking is capable of causing endogenous opioid release in the insula. Another study has identified a correlation between lower endogenous opioid levels in the insula at the start of treatment and shorter duration of cocaine abstinence (Ghitza et al., 2010). The novel findings of these studies demonstrates that much work remains to be conducted in exploring the receptor populations of the insula involved in mediating the effects of addictive substances and behaviours.

1.2.4 Damage to the Insular Cortex Disrupts Smoking Addiction

Although many of the neurobiological underpinnings of addiction have been identified painstakingly through methodical study in animal models and human imaging, occasionally targets are identified through unconventional means. One of such targets is the insular cortex which, until quite recently, has been relatively overlooked within the addiction literature. The seminal work of Naqvi and colleagues (2007) in *Science*, was the first to propose that the insula may in fact play a critical role in nicotine addiction (Naqvi et al., 2007). This study took the unique approach of examining a phenomenon observed by neurologists in which stroke patients
occasionally report immediate smoking cessation without having previously stated any active initiative to quit. The authors collected a sample of stroke patients who were smokers at the time of brain injury and assessed whether they demonstrated a stroke-induced disruption of smoking, exemplified by four criteria: ‘quitting smoking easily, immediately, without relapse, and without persistence of the urge to smoke’. It was identified that individuals with stroke-induced lesions to either the right or left insula had a significantly greater likelihood of having a disruption of smoking as compared to individuals with stroke-induced lesions to non-insula areas.

Though several limitations of these findings have been noted (Vorel et al., 2007), along with a study which failed to replicate them (Bienkowski et al., 2010), a recent prospective study has demonstrated that stroke-induced insular damage (vs. stroke damage sparing the insula) is associated with increased odds of three-month continuous smoking abstinence and total cessation from the use of any nicotine product, including NRT (Abdolahi et al., 2015c). The average time to relapse was longer in these insular damaged patients and, among quitters, resulted in greater odds of experiencing a disruption of addiction (see criteria above). The same authors found that smokers with stroke-induced insular damage had lower scores on both the Wisconsin Smoking Withdrawal Scale and Minnesota Nicotine Withdrawal Scale during their hospitalization post-stroke (ie. forced abstinence period) and were less likely to use nicotine replacement therapies compared to non-insular damage stroke-suffering controls (Abdolahi et al., 2015b). Several other studies have also confirmed that insular damage is associated with increased odds of abstinence at 1-year post-stroke follow-up (Suner-Soler et al., 2012; Gaznick et al., 2014). A recent study has expanded this finding of insular ischemic-damage induced disruption of addictive behaviour to opiate dependence, where individuals were more likely to stop using opium, or if they did not stop they were more likely to change route of administration, when compared to individuals with non-insular ischemic damage (Yousefzadeh-Fard et al., 2013).
Recent work has attempted to define whether there is a particular influence of the dominant vs. non-dominant insular areas in this finding of smoking disruption; however, preliminary findings suggest little difference between unilateral dominant insular damage over non-dominant damage in regulating urge, withdrawal, and smoking cessation (Abdolahi et al., 2015a). Another study has observed similar null findings when examining the effect of unilateral right vs. left basal ganglia-insular lesions on incentive motivation, though there was an interesting observation in right, but not left, lesion patients of reduced preference and viewing of stimuli they rated as only slightly positive, and particularly an avoidance of images with sexual content (Vijayaraghavan et al., 2013). This finding of hemispheric differences is consistent with prior studies where patients with right insular lesions demonstrated neglect of tactile, visual and auditory stimuli (Berthier et al., 1987; Manes et al., 1999b) along with more frequent subjective anergia, hypoactivity, and fatigue (Manes et al., 1999a) compared with non-insular or left insular lesion patients, while left insula dysfunction has been observed to more likely result in aphasia (Shuren, 1993; Marshall et al., 1996; Nestor et al., 2003).

Thus, the work of Naqvi and colleagues (2007) brought the insular cortex into the spotlight of critical neurocircuitry underlying addiction. It should be noted here that though several studies have noted increased risks for neurological deficits following insular ischemic stroke, including somatosensory deficits, aphasia, dysarthria (Cereda et al., 2002), and to a limited degree vestibular-like syndrome (Baier et al., 2013) and motor deficits (Lemieux et al., 2012), though the outcome generally leaves the patient with relatively good functionality in daily life.
1.2.5 The Insular Cortex within the Neurocircuitry Underlying Addiction

The process of prolonged, intermittent exposure which results in addiction can be viewed as three phases: (1) performance of the addictive behaviour, followed by (2) a period of withdrawal or abstaining from performing the addictive behaviour, and finally (3) craving/urges prior to the resumption of performance the addictive behaviour (Koob and Volkow, 2010). All three stages are critical for understanding why addiction is a complex learned process involving both associative learning and operant conditioning, in the forms of positive and negative reinforcement and punishment. The focus of this thesis will be in understanding the critical role of the insular cortex in these learning processes and thus addictive disorders.

1.2.5.1 Neurocircuitry of Addictive Behaviour and a Role for the Insular Cortex

A) Evidence from Animal Studies

The earliest studies of the neurocircuitry underlying the positive reinforcement of behaviour were performed in a rodent model known as "brain stimulation reward" or "intracranial self-stimulation" (Olds and Milner, 1954). This model involves implanting electrodes into a particular site within the brains of rodents and allowing them to perform a behaviour (eg. wheel turning or lever pressing) which results in the delivery of electrical current to the electrode and thereby to the brain site in which it is implanted, with the particular parameters of the electrical current being set to cause an increase in neuronal firing (Carlezon and Chartoff, 2007). This early work identified that the medial forebrain bundle (MFB), which critically includes dopaminergic projections from the VTA to the NAcc, was the most sensitive of the several sites in which electrodes were implanted (Olds and Milner, 1954). This was the first evidence that identified VTA dopaminergic projections to the NAcc as being involved in the neurocircuitry underlying the reinforcement of behaviours. Critically, the acute administration of most
addictive substances has been demonstrated to decrease the "brain reward threshold", or the intensity of electrical current delivered to the MFB required to elicit the associated behavioural response (Kornetsky et al., 1979).

The operant self-administration paradigm, described above, has been used to demonstrate that activation of the midbrain dopamine system is responsible for the attribution of salience to the environmental stimuli associated with the addictive behaviour, including contextual cues which are present throughout self-administration sessions or discrete cues paired with reinforcement as described above (Robinson and Berridge, 1993). This activation is also responsible for the promotion of goal-directed behaviours, including the addictive behaviour itself (Salamone and Correa, 2002). However, the time course of dopamine release is a critical component of the addictive potential for a behaviour and/or substance (Schultz, 2006) with short bursts (phasic) of dopamine being critical to reinforcement and the valuation of predicted outcomes, while slow steady (tonic) dopamine release causes more general activation and the priming of behaviour related systems, particularly habitual behaviour.

Dopaminergic receptors also appear to be involved in the role of the insular cortex in drug self-administration behaviours. Di Pietro and colleagues (2008) examined the role of RAIC dopamine D1 receptors in cocaine self-administration behaviour. The D1 receptor antagonist, SCH23390, delivered intracranially directly to the RAIC, was able to significantly decrease lever responding and cocaine intake. Indeed it has been noted that cocaine self-administration, with or without cues, results in modest increases of insular Arc messenger ribonucleic acid (mRNA) expression (Zavala et al., 2008), an immediate early gene associated with activity-dependent plasticity as well as learning and memory (Guzowski, 2002; Tzingounis and Nicoll, 2006), and which requires D1 receptor stimulation (Fosnaugh et al., 1995; Yamagata et al., 2000; Fumagalli
et al., 2006; Wirtshafter, 2007). A more recent study demonstrated that SCH23390, but not the D2 receptor antagonist haloperidol, infusions into the RAIC attenuated nicotine self-administration (Kutlu et al., 2013). However, it should be noted, that D1 receptor blockade in the RAIC also resulted in significant decreases in operant food-maintained responding and overall intake, thus suggesting a non-drug specific mechanism (Di Pietro et al., 2008). Regardless, these studies of dopaminergic receptors in the insular cortex suggest that addictive substances may indirectly affect dopamine release onto these receptor populations and the resulting effects on insular neurons are critical to the reinforcing capacity of drugs and natural rewards.

In our own laboratory, prior to the start of the experimental work presented within the body of this thesis, our first study examining the insular cortex looked at examining its role using the intravenous nicotine self-administration paradigm in rats. This study involved local intracranial infusions of GABA agonists (baclofen and muscimol), in order to temporarily inactivate the CGIC bilaterally for the period of the behavioural testing sessions (Forget et al., 2010a). Essentially, this strategy allowed for a functional replication of the insular lesions observed by Naqvi and colleagues (2007). Utilizing this strategy, we were able to demonstrate that insular inactivation is capable of significantly reducing nicotine taking behaviour as assessed under an FR schedule of reinforcement and motivation for nicotine as assessed under a PR schedule of reinforcement (Forget et al., 2010a). In contrast, insular cortex inactivation did not affect lever presses for food or motivation to get food, or food seeking behaviors, indicating that those results were selective for nicotine-seeking (Forget et al., 2010a). Apart from the D1 receptor involvement described above (Kutlu et al., 2013), the nicotine self-administration model has also identified a role for the hypocretin/orexin system in nicotine addiction, specifically the blockade of hypocretin-1 receptors in the granular insula decreasing nicotine self-administration.
in rats (Hollander et al., 2008). The same study also demonstrated that hypocretin-containing fibers innervate the insula and hypocretin-1 receptors are located on insular neurons. Finally, Seif and colleagues (2013) utilized an optogenetic approach to demonstrate that inactivating glutamatergic neurons within the RAIC projecting to the NAcc was capable of attenuating aversion-resistant alcohol self-administration and intake, but had no effect on undeterred alcohol self-administration (Seif et al., 2013).

Apart from self-administration models, the acute effects of addictive substances has been studied using the conditioned place preference model. Specifically, the conditioned preference of an amphetamine-associated environment stimulated neuronal activity in the insula while the inactivation of the granular region of the insula blocked the expression of amphetamine-induced conditioned place preference (CPP) (Contreras et al., 2007). A follow-up study also demonstrated a loss of amphetamine-induced CPP following a local injection of a protein synthesis inhibitor, anisomycin, into either the CGIC or RAIC shortly after memory retrieval (Contreras et al., 2012). This loss was coupled with a decrease in the expression of zinc finger protein 225 (zif268) in both insular regions, but not the primary somatosensory cortex, after anisomycin was injected into the RAIC. That study also demonstrated that inactivating the CGIC, but not the RAIC, could facilitate the extinction of amphetamine-induced CPP. Another study in rats found involvement of the CGIC, but not RAIC, in the acquisition of morphine induced CPP (Li et al., 2013). A more recent study has supported this finding demonstrating that local CGIC infusions of a nonselective muscarinic acetylcholine receptor antagonist significantly inhibits morphine-induced CPP expression in both contextual cue- and priming-induced CPP reinstatement (Wu et al., 2014). That study also demonstrated opposing roles of the M1 and M4 receptor subtypes, with an agonist of the M1 and antagonist of the M4 both enhancing CPP expression while an antagonist of the M1 and agonist of the M4 both inhibited CPP expression.
This CGIC, but not RAIC, involvement may be specific to morphine-induced CPP as a study in rats demonstrated that zif268, another immediate early gene) expression in both the CGIC and RAIC were significantly greater when CPP was induced by cocaine but not by social interaction (El Rawas et al., 2012). A study in mice has also demonstrated a role for the insula in nicotine-induced CPP (Scott and Hiroi, 2011); however, the lesions utilized in that study were not specific to a subregion of the insula.

The ability of addictive substances to produce activity in particular brain regions can be demonstrated in rodents through the use of immunohistochemistry for the c-Fos protein, an immediate early gene, where an intraperitoneal injection of nicotine or the peripherally-limited nicotine pyrrolidine methiodide are both able to induce c-Fos immunoreactivity in the insula (Dehkordi et al., 2015). Additionally, a recent study has also demonstrated the capability of oral ethanol (20%) stimulation to induce robust c-Fos immunoreactivity in the insula (Brasser et al., 2015), suggesting that the insula may also play a role in the associative conditioning between the central and gustatory aspects of alcohol. Finally, the availability of high-resolution MRI scanners in recent years has allowed for the use of such scanners in imaging the rodent brain. Utilizing such technology, it has recently been demonstrated that acute infusions of intravenous nicotine produced a significant dose-dependent increase of blood oxygen level dependent (BOLD) signal in the insula (Bruijnzeel et al., 2015). Acute subcutaneous administration of mecamylamine alone produced a significant decrease of BOLD signal in the insula and was able to completely block nicotine's ability to increase BOLD signal in the insula.

B) Evidence from Human Studies

In human subjects, brain imaging studies have demonstrated that the speed of dopamine release is critical to the subjective effects experienced, with the fast, large release of dopamine caused by
addictive substances resulting in subjective reports of pleasure and euphoria (Volkow et al., 1997a) that are not reported by therapeutic drugs, such as those used for attention deficit hyperactivity disorder, which cause the slow, steady release of dopamine (Chait, 1994; Grace, 1995; Volkow et al., 2001; Volkow and Swanson, 2003). Thus, it is the particular timing between the performance of a behaviour and its contingent reinforcement are critical for determining whether prolonged, repeated exposure to the behaviour is capable of resulting in an addictive disorder (Nutt et al., 2015). Well understood in this regard, is that the pharmacokinetic properties of addictive drugs (eg. heroin vs. morphine) and the influence of route of administration (eg. oral vs. intravenous) play a critical role in their relative abuse liabilities (Schultz, 2002). Parallel in the case of gambling, if one was to examine the timing of games played within a casino, the times between betting behaviour and the contingent result are all relatively short in duration when compared to similar non-casino games (Clark, 2010).

It is not surprising that dopamine release has also been significantly correlated with fMRI activation of dopaminergic midbrain nuclei during the anticipation of monetary reward, as is experienced while gambling (Schott et al., 2008). Similarly, the subjective effects and brain response to an addictive drug which is expected is greater than that experienced by drug delivery when it is unexpected (Volkow et al., 2003b). The role of context and expectation suggest that cortical glutamatergic projections, likely including those from the RAIC (McGeorge and Faull, 1989; Wright and Groenewegen, 1996; Shi and Cassell, 1998b), influence dopamine neuronal activity and release in the NAcc (Kalivas and Volkow, 2005). Thus addictive drugs themselves, even if administered in a somewhat similar fashion to that normally experienced by an individual with addiction, do not necessarily elicit the same degree of subjective or brain responses as when they are administered through the individual's routinely experienced addictive behaviour (Nutt et al., 2015). This result lends support to the critical role the insula plays in the associative
conditioning that occurs between the central and peripheral aspects of nicotine, along with the consequent association to discrete and environmental cues, in human smokers (Rose and Levin, 1991; Rose et al., 1993; Westman et al., 1996; Rose et al., 2003; Palmatier et al., 2006).

Though the early studies on the neurobiological basis of addiction began with the acute effects of addictive drugs, there has been a gradual understanding that addictive disorders all involve a rather long course of disease progression. The point at which an individual is diagnosed and/or seeks treatment for an addictive disorder is often after they have performed the addictive behaviour in question for a prolonged period, as can be easily understood by reviewing the DSM-5 diagnostic criteria listed above. Thus, though we have begun with a brief review of the neurobiology of acute reinforcement, it is the repeated and prolonged exposure to the effects of addictive behaviours which result in changes to the underlying neurocircuitry, and thus addiction. However, it should be noted that neuroadaptions in addiction are not mediated solely by the performance of the addictive behaviour but more likely a result of repeated cycling between performance and withdrawal periods (Koob, 1996), or periods during which the individual refrains from or cannot perform the addictive behaviour.

### 1.2.5.2 Neurocircuitry of Withdrawal and a Role for the Insular Cortex

**A) Evidence from Animal Studies**

Though the experimental work contained within this thesis does not directly deal with withdrawal or negative affect experienced during periods of abstaining from addictive behaviours, it is important to briefly discuss this stage of the addictive process in order to fully understand the neurobiological and behavioural aspects of addiction, including the involvement of the insula as discussed below. Most critically, repeated and prolonged exposure to addictive behaviours results in significant neuroadaptations within several brain regions. From the
perspective of behavioural effects, these neuroadaptations can be understood by the DSM-5 diagnostic criterion of being restless or irritable when attempting to cut down or stop their addictive behaviour.

Returning to the concept of brain reward threshold discussed above, acute exposure to addictive substances has been observed to decrease in this threshold; however, chronic prolonged exposure to such substances results in an increased threshold when the animal is finally deprived of drug (Kornetsky et al., 1979). Thus the primary system underlying this acute withdrawal period is of course the midbrain dopamine system where activity is decreased (Rossetti et al., 1992; Weiss et al., 1992) along with decreased serotonergic neurotransmission in the NAcc (Weiss et al., 1996). This decreased dopaminergic tone results in decreased locomotor activity (Pulvirenti and Koob, 1993) along with a decreased motivation to work for natural rewards (Barr and Phillips, 1999; Orsini et al., 2001).

Using a conditioned place aversion (CPA) paradigm in rats, the aversive nature of withdrawal from an addictive substance can be established along with the ability to study underlying neurobiology or therapeutics potentially capable of alleviating this negative state (Stolerman, 1985; Tzschentke, 2007). Inactivating subregions (either granular or agranular) of the insular cortex is capable of preventing the acquisition of CPA established by naloxone-precipitated acute morphine withdrawal (Li et al., 2013). However, this effect was not observed in mice with insular cortex lesions when CPA was established by mecamylamine-precipitated acute nicotine withdrawal (Scott and Hiroi, 2011).

The stress modulation systems appear to up-regulate in order to overcome the neurochemical results of prolonged, repeated performance of the addictive behaviour and maintain brain homeostasis (Koob, 2008). Thus during the acute and protracted withdrawal
phase, these systems remain upregulated resulting in the experience of irritability and distress. Both the hypothalamic–pituitary–adrenal (HPA) axis and the corticotropin-releasing factor (CRF)-based brain stress system cause psychological distress during acute withdrawal through elevated adrenocorticotropic hormone, corticosterone, noradrenaline and extended amygdalar CRF (Koob and Kreek, 2007). For all addictive substances, cessation of consumption following prolonged use is known to result in psychological distress characterized by irritability, emotionality, dysphoria and sleep disturbances (Koob and Volkow, 2010). Importantly, similar symptoms are also reported during withdrawal during periods of cessation from pathological gambling behaviour and to contribute to relapse (Grant et al., 2010; el-Guebaly et al., 2012; Romanczuk-Seiferth et al., 2014). There is also a high concentration of CRF receptors in the insular cortex indicating the potential for involvement of the insula in withdrawal and the infusion of a CRF1 antagonist into the RAIC has already been demonstrated to attenuate cue-induced reinstatement of cocaine seeking (Cosme et al., 2015).

B) Evidence from Human Studies

In human subjects with drug dependence, few studies have been conducted during acute withdrawal periods; however, during protracted withdrawal, decreased activity in the midbrain dopamine system has been observed in the form of decreased dopamine release in the ventral striatum (Volkow et al., 1997b; Martinez et al., 2005; Volkow et al., 2014) and decreased expression of dopamine D2-subtype receptors across all subregions of the striatum, including the NAcc (Volkow et al., 1996; Martinez et al., 2004). Importantly, though this reduced dopaminergic state results in a motivational withdrawal syndrome characterized by dysphoria, irritability, emotional distress, and sleep disturbances (Volkow et al., 2003a), there is a concurrent increased sensitivity to the ability of addiction-associated stimuli or "conditioned
cues" to induce craving that correlates with brain activity in a variety of areas, including the insular cortex (McClernon et al., 2005; McClernon et al., 2009), as discussed further below. It is this concurrent deficit in motivation for natural rewards and enhanced sensitivity to conditioned cues that results in relapse to addictive behaviour (Childress AR, 1993).

Apart from the work in individuals with insular lesions described above (Abdolahi et al., 2015b), there is evidence to support a role for the insula in withdrawal. In individuals with nicotine dependence, overnight nicotine withdrawal results in increased resting state functional connectivity of the amygdala-insula circuit and the insula-default mode network (DMN consisting of posterior cingulate cortex, medial prefrontal cortex, parahippocampus) circuit (Sutherland et al., 2013a). These elevations are blunted by both transdermal nicotine and varenicline, suggesting that insula-related circuits are likely involved in the subjective symptoms of nicotine withdrawal. Similar findings of increased resting state functional connectivity of the amygdala-insula have been found in abstinent heroin dependent individuals and correlated with impulsivity as measured by the Barratt Impulsive Scale (Xie et al., 2011). When comparing individuals who had recently consumed cocaine to those who had negative urine tests, again amygdala-insula resting state functional connectivity was again found to be increased in abstinent individuals suggesting that the resumption of drug intake may renormalize this circuitry (Gu et al., 2010). Recent work has also demonstrated that there is overall increased global brain connectivity of the insular cortex during acute abstinence in smokers, and that smoking a cigarette reduces this enhanced insular connectivity (Wang et al., 2014).

Psychological disturbances during abstinence are observed in all cases of addictive disorders, and are thereby a criterion in their clinical diagnosis. However, for certain drugs of abuse, particularly opiates and alcohol, ceasing drug intake following prolonged use results in
highly distressful physical symptoms which can potentially be fatal and often must be managed medically (Koch-Weser et al., 1976; Gold et al., 1982). These symptoms arise as several processes, in both the brain and body, are up or down-regulated to maintain homeostasis in the presence of the addictive drug and continue to remain so during the acute withdrawal period. Though the specifics of such somatic withdrawal symptoms are not be discussed here, they should be understood to play a major role in the active avoidance of withdrawal in individuals with alcohol or opiate addiction. With the role the insular cortex plays in evaluating the somatic state, it is likely that this brain region plays a role in said active avoidance, though this has yet to be examined in human subjects.

Neuroadaptations are capable of explaining the critical role of withdrawal in the operant conditioning processes of addiction. Firstly, abstinence results in both positive punishment in the form of distress/irritability and negative punishment through loss of the appetitive aspects of natural rewards (ie. the lack of motivation for and decreased reinforcement of natural rewards). Secondly, individuals will engage in active avoidance, a form of negative reinforcement, in which continued addictive behaviour is driven by the desire to prevent experiencing the distress of withdrawal. Lastly, withdrawal also contributes to escape behaviours, another form of negative reinforcement, where individuals resume addictive behaviour in order to relieve the distress of withdrawal.

1.2.5.3 Addiction-Relevant Neuroadaptations of the Insular Cortex
Here I will examine the specific findings with regards to neuronal changes induced within the insular cortex as a result of exposure to addictive substances or behaviours. As the majority of this data has been derived from human-imaging, I will not review animal model data separately. The ability of repeated, prolonged use of addictive drugs or excessive performance of addictive
behaviours to cause profound damage across several brain regions has been well described (Buttner, 2011). The insular cortex appears to be no exception with decreased gray matter density noted in several addicted populations compared to healthy controls, including heroin (Gardini and Venneri, 2012), cannabis (Lopez-Larson et al., 2011; Battistella et al., 2014), cocaine (Franklin et al., 2002; Makris et al., 2008a; Weller et al., 2011), methamphetamine (Schwartz et al., 2010; Mackey and Paulus, 2013), alcohol (Makris et al., 2008b; Durazzo et al., 2011; Senatorov et al., 2015), nicotine (Fritz et al., 2014; Hanlon et al., 2014; Morales et al., 2014), and internet addiction (Zhou et al., 2011b; Weng et al., 2013; Yuan et al., 2013; Zhu et al., 2015). In post mortem examinations of subjects with a history of alcoholism, reduced anterior insular volume has been attributed to loss of von Economo neurons (Senatorov et al., 2015), large bipolar neurons with an atypical spindle- or corkscrew-shaped soma and thick basal and apical dendrites that have been noted for their implication in neuropsychiatric disorders involving emotional regulation and social cognition.

There is some evidence to suggest that though left insula cortical thinning is consistent with substance dependence regardless of sex, there is an interaction of sex in bilateral insula volume and right insula cortical thickness with females with substances dependence having lower volumes/thickness than gender-matched healthy controls while males with substance dependence have greater volumes/thickness (Tanabe et al., 2013). The question of whether these reductions in grey matter volumes are caused by or predispose individuals to additive behaviour remains unclear, as individuals who experience chronic stress during development are noted to have reduced insula volumes and are predisposed to addiction (Ansell et al., 2012).

Interestingly, nicotine delivered chronically through osmotic mini-pumps implanted in rats has been demonstrated to cause novel dendritic branching in the insular cortex (Ehlinger et
al., 2012). This finding in animals suggests the possibility that though overall insular gray matter volume is generally found to be decreased in individuals with substance dependence, they may still have increased addiction-specific neuronal connectivity, or essentially a insular cortex that is focused particularly on addiction relevant visceral and external stimuli, as is suggested by the functional connectivity, craving and relapse studies described below. This hypothesis is supported by a recent finding in adolescents receiving substance use treatment, where increased left insular white, but not gray, matter was linked with drug enhancement motives (eg. reported using drugs in order to "get high") and thereby with frequency of binge drinking while right insula white matter was correlated with obsession/craving for alcohol (Chung and Clark, 2014).

In contradiction to the general loss of gray matter in the insular cortex, one study indicated that smokers have an increased left insular cortex gray matter density compared to non-smokers and that this increased density is correlated with a higher score on the Toronto Alexithymia Scale (TAS-20) (Zhang et al., 2011a). Alexithymia is a personality trait characterized by the inability to identify and describe one's emotional experiences (Taylor and Bagby, 2004; Parker et al., 2008) and is associated with substance use severity for both alcohol (Bruce et al., 2012) and methamphetamine (Saladin et al., 2012). Though originally posited to be a stable trait which may predispose individuals to addiction (Mann et al., 1995), recent findings have suggested that TAS-20 scores vary during disease progression (de Haan et al., 2012). Alexithymia has been shown to mediate negative emotionality and emotional arousal to addictive substances (Bonnet et al., 2013), and insular activation is greater in response to emotional stimuli in alexithymic individuals compared to healthy controls (Karlsson et al., 2008). Another contrary finding in young adult amphetamine-related stimulant abusers observed a positive correlation between amphetamine use history and grey matter volume in the left mid-
insula (Mackey et al., 2014). This last finding may represent the possibility that higher left insula volumes predispose individuals to drug taking behaviours.

The gross structural changes which can be observed in individuals with prolonged exposure to addictive substances and behaviour, are merely the "tip of the iceberg" in regards to the overall level of significant change which occurs at all levels of the brain. Of great recent interest in the study of addiction has been the use of blood BOLD signal fluctuations of fMRI to detail changes in functional connectivity during the resting-state (ie. task-free settings), termed "resting-state networks" or "intrinsic connectivity networks" (Biswal et al., 1995; Greicius et al., 2003; Beckmann et al., 2005; Fox et al., 2005). It should be noted that the anterior insula, along with the dorsal anterior cingulate cortex (dACC) comprises the "salience network" (SN) which has been implicated in focusing attention towards the currently most prominent homeostatic events (Seeley et al., 2007). Though often co-active during cognitive tasks, the SN appears to be distinct from that underlying executive processing, known as the "executive control network" (ECN), which is comprised of dorsolateral frontal and parietal neocortices. The co-activation of these two networks, SN and ECN, is termed the "task-activation ensemble" which is known to be inversely correlated with that of a third distinct network, known as the "default mode network" (DMN), which is comprised of several brain areas focused around the posterior cingulate cortex (PCC) as the major node (Greicius et al., 2003; Buckner et al., 2008).

Increased resting state functional connectivity (rsFC) has been observed between the right insula and several DMN regions, including the dorsomedial and dorsolateral PFC, in cocaine dependent individuals (Cisler et al., 2013), though it should be noted here that this study did not account for frequency or recency of drug use, thereby it cannot be determined whether their findings were due to current intoxication or withdrawal effects. Cocaine users with higher
alexithymia demonstrated reduced rsFC of SN and DMN regions, including that of the bilateral insula and DMN, which interestingly correlated with current levels of cocaine use (Liang et al., 2015).

Acutely abstinent smokers demonstrate increased rsFC of the insula-DMN when compared to both non-smokers (Huang et al., 2014) and satiated smokers (Wang et al., 2014), though satiated smokers still have higher insula-DMN rsFC than non-smokers. Smoking cues, when compared to food cues, result in greater left insula functional connectivity with the right insula, orbitofrontal cortex, and striatum with that greater connectivity being positively correlated with nicotine dependence, as judged by scores on the Fagerstrom Test of Nicotine Dependence (Claus et al., 2013). Smoking cue-elicited activity in the insula has been found to be negatively correlated with rsFC of the insula-dorsomedial PFC in smokers (Zhang et al., 2011b). rsFC between the dACC, insula and striatum also negatively correlates with smoking severity (Moran et al., 2012). Reduced rsFC of the insula-NAcc-amygdala has been observed in patients with prescription opiate dependence (Upadhyay et al., 2010). In combination, these results suggest that the differential functional connectivity of the insula observed during addiction-relevant tasks and abstinent states vs. non-relevant tasks and non-abstinent states may be a potential biomarker of addiction severity.

Interestingly, smokers with higher alexithymia, appear to have lower levels of increased rsFC between the right anterior insula and the ventromedial PFC, a core node of the DMN, and that this mediates craving following overnight withdrawal, though this mediation is disrupted by varenicline or NRT (Sutherland et al., 2013b). This same group reported that varenicline or NRT reduces rsFC between the amygdala-insula and insula-DMN regions following overnight withdrawal and that this effect also correlates with reduced withdrawal severity (Sutherland et
al., 2013a). This group has recently concluded, through an activation likelihood estimation meta-analysis, that nicotinic acetylcholine receptor agonists reduce left mid-insula activations in both smokers and healthy controls, while the agonists also cause reduced activation of the right anterior insula in smokers (Sutherland et al., 2015).

Individuals with internet gaming disorder (Petry et al., 2014) have also been observed to have a large range of insula centered increases in rsFC, including amygdala-insula (Ko et al., 2015), right anterior insula-ACC, anterior insula-DMN, left anterior insula-right putamen, and posterior insula-somatosensory/sensorimotor cortices (Zhang et al., 2015). Increased rsFC of the right anterior insula-ACC was correlated with the duration of internet gaming disorder, while increased rsFC of the anterior insula-DMN and posterior insula-somatosensory/sensorimotor cortex was correlated with the severity of internet gaming disorder. Finally, resting state cerebral blood flow has been observed to be increased in the bilateral insula (Feng et al., 2013) along with increased glucose metabolism in the right insula (Park et al., 2010) of adolescents with internet gaming disorder compared to control subjects. These findings suggest that the insula may play similar roles between substance dependence and internet gaming disorder.

Recent approaches using graph theoretical analysis have allowed for the examination of vast functional interactions within the entire brain, termed the functional connectome (Wang et al., 2011). Through this approach it has recently been observed that the brains of addicted individuals have a hyperconnected and altered resting brain network, which interestingly includes the insula, that may enable quick, subconscious execution of behaviors directed toward drug-related goals (Wang et al., 2015). The same study demonstrated these individuals have a loss of normal inter-regional communications which may underlie their loss of cognitive control and inhibition.
1.2.5.4 Neurocircuitry of Craving and Relapse and a Role for the Insular Cortex

A) Evidence from Animal Studies

As described earlier, the prior work of our own laboratory examined the effect of pharmacologically inactivating the CGIC using GABA agonist infusions (mixture of baclofen/muscimol) on nicotine-taking behaviour under FR5 and PR schedules of reinforcement (Forget et al., 2010a). Following acquisition of nicotine self-administration, lever pressing was then extinguished as described in the section above. Again, pharmacological inactivation of the CGIC prior to reinstatement by either nicotine-associated cue light or priming injections of nicotine decreased nicotine-seeking behaviour. Supporting the role of the insular cortex in the reinstatement of drug-seeking behaviour, incubation of cue-induced reinstatement of nicotine seeking behaviour in rats has also been correlated with enhanced protein kinase A signaling in the insular cortex via phosphorylation of the dopamine- and cyclic adenosine monophosphate-regulated phosphoprotein (DARPP-32) at threonine-34 (Abdolahi et al., 2010). Supporting the clinical relevance of these findings in rodents is the observation of increased insula activation by smoking cues after extended abstinence compared to a pre-quit scan session (Janes et al., 2009). Contrarily, a cocaine challenge following a 21, but not 1-day, withdrawal period produced a decrease in zif268 expression in rats previously exposed to cocaine, suggesting that the role of the insula in cue incubation differs from its role in re-exposure to drug following short or extended withdrawal (Unal et al., 2009).

In contrast to our own findings, RAIC, but not CGIC, inactivation attenuated the cue-induced, but not priming, reinstatement of cocaine seeking behaviour (Cosme et al., 2015). This finding of RAIC inactivation on cue-induced reinstatement is similar to that of a prior study using a light-odor (discrete+contextual) cue (Di Pietro et al., 2006). The work of Cosme and
colleagues (2015) also demonstrated no significant effects of RAIC inactivation on food seeking behaviour induced by cues or priming. Additionally, the infusion of a CRF1 antagonist into the RAIC was also able to attenuate cue-induced reinstatement of cocaine seeking. The level of cue-induced reinstatement of cocaine seeking behaviour has also been correlated to Fos protein expression, a marker of neuronal activity, in the RAIC (Kufahl et al., 2009). Cocaine-associated contextual odor cues have also been demonstrated to cause a greater fMRI BOLD response in the insula, particularly the agranular subregion, when compared to neutral odor in rats trained to self-administer cocaine (Johnson et al., 2013). Contrary to these findings, lesions of the anterior insula, including the RAIC, increased cocaine-seeking in rats reintroduced to the cocaine-seeking context following forced abstinence (Pelloux et al., 2013).

B) Evidence from Human Studies

The stage of relapse to addictive behaviour is likely most critical with regards to the development of therapeutic approaches for the clinical situation yet, until recent years, it has been the least studied in comparison to the stages of performance and withdrawal from addictive behaviour (O’Brien, 2003). Though relapse to addictive behaviour is now well understood to be the most critical aspect for the treatment of all addictive disorders, there are several questions as to the most reliable predictors of relapse. Aspects related to the prolonged exposure to the addictive behaviour (ie. attentional and self-regulatory deficits) along with more acute aspects immediately preceding relapse (ie. exposure to related cues/contexts and stressors) appear to have varying degrees of involvement in relapse for various addictive disorders (Donovan, 1996; Li, 2000; Sinha, 2011). With regards to these latter aspects, the reinstatement paradigm described above has been used, along with neuroimaging studies in human subjects, to establish the neurocircuitry underlying the acute triggers for relapse.
The findings of Naqvi and colleagues (2007) described above were the first to suggest a crucial role for the insula in nicotine addiction yet the area was already known to have some involvement in addiction, particularly with regards to drug urges in human imaging studies (Naqvi and Bechara, 2009; Naqvi and Bechara, 2010). However; such studies, due to their broad observational nature, did not specifically focus on the insular cortex. Naqvi and Bechara, following their initial publication described above, conducted a review of such functional imaging studies in which the insula was observed to be activated during drug urges (Naqvi and Bechara, 2009).

Importantly, insular activity has been correlated with cue or context induced urges to consume cannabis (Filbey et al., 2009), heroin (Sell et al., 1999; Lou et al., 2012), alcohol (Myrick et al., 2004; Tapert et al., 2004), cocaine (Wang et al., 1999; Garavan et al., 2000; Kilts et al., 2001; Wexler et al., 2001; Bonson et al., 2002; Kilts et al., 2004), nicotine (Brody et al., 2002; Lee et al., 2005; McClernon et al., 2005; McBride et al., 2006; Franklin et al., 2007; Wang et al., 2007), gambling (Goudriaan et al., 2010) and high calorie foods in obese subjects (Killgore et al., 2003; Geliebter et al., 2006; Grill et al., 2007; Rothemund et al., 2007; Stoeckel et al., 2008; Brooks et al., 2013; Goldman et al., 2013). One study correlated insula reactivity to alcohol cues to the severity of the patient's alcohol use disorder (Claus et al., 2011a) while others have correlated insula reactivity to smoking cues to the severity of the patient's nicotine dependence (Franklin et al., 2009b; Franklin et al., 2011a). A more recent meta-analysis of fMRI studies on smoking cue reactivity also concluded that smoking cues, as compared to neutral cues, reliably evoke larger fMRI responses in the insula (Engelmann et al., 2012). Finally, one study demonstrated that the left insula was the only brain region with greater activation to smoking images over all other images, including highly activating erotic images, in smokers (Versace et al., 2011).
In contrast, a recent study has found greater left posterior insula activation for neutral images than for drug images, though the authors suggest that the reduced activation of this subregion may be due to diminished aversion to the drug images (Gray et al., 2014). A similar finding was seen in a study cited above, where posterior insula activity was decreased for drug memory recall when compared to neutral memory recall, yet anterior insula activity was increased for drug memory recall over anger memory recall (Kilts et al., 2001).

A very interesting study has parsed smoking cues based on their temporal relation to smoking behaviour, examining the effect of smoking initiation-related cues (eg. lighter) vs. smoking conclusion-related cues (eg. cigarette butt in ashtray) on smokers who were either content with their smoking behaviour or discontent with their behaviour (Stippekohl et al., 2012). Discontent smokers actually showed greater insular activation in response to initiation-related cues than content smokers and the authors speculated that insula activation may counteract the influence of reflective reasoning about the danger of smoking, and make it difficult for dissonant smokers to quit smoking. This finding is supported by the fact that anterior insula activation is observed during intrinsic, but not extrinsic, motivation (Lee et al., 2012; Lee and Reeve, 2013). A recent study where smokers attempting to quit were asked to use either an intrinsic (eg. I want to quit smoking for my own health) or extrinsic (ie. My family wants me to quit smoking for my health) coping strategy to resist when presented with a smoking opportunity, demonstrated insular activation only in participants using the intrinsic coping strategy (Wilson et al., 2013).

With regards to insular connectivity during cue presentation, the intensity of craving evoked by smoking cue exposure during withdrawal has been observed to correlate with insula-precuneus connectivity, suggesting that the greater the conscious awareness of the current bodily state (ie. lack of drug), the greater the craving induced by cues (Moran-Santa Maria et al., 2015).
Additionally, the inferior frontal cortex, a region identified to play a role in self-regulation across motor, affective and craving (Berkman et al., 2009; Tabibnia et al., 2011), has shown negative functional connectivity with the insula during a craving self-control task in abstinent smokers (Tabibnia et al., 2014), suggesting that the inferior frontal cortex inhibition of the insula may be critical to maintaining self-control during craving. Mindful attention has been shown to reduce self-reported cue-induced craving and reduce functional connectivity between the ACC and bilateral insula in smokers (Westbrook et al., 2013). Interestingly, the cue reactivity of the dorsal striatum has been associated with the rsFC of the medial PFC-left insula in smokers, suggesting the possibility that this rsFC may itself cause the heightened dorsal striatum cue reactivity (Janes et al., 2012). Finally, there appears to be some generalization of this enhanced cue response as both food and cocaine cues, vs. neutral cues, cause greater activation of overlapping brain regions, including the insula, in cocaine abusers (Tomasi et al., 2015).

Apart from these extensive findings with cue-induced craving, left insula activation has been correlated with stress-induced cocaine craving and subjective distress (Sinha et al., 2005). It should be noted, in the same study, right insula activation was inversely correlated with subjective distress suggesting differential roles between hemispheres. Interestingly, cocaine-dependent women appear to have greater stress-induced activation of the insula as compared to cocaine-dependent males (Potenza et al., 2012).

With regards to neuroimaging biomarkers of relapse, a positive correlation was found between smoking cue-induced insula activation and lapse during a 8-week smoking cessation in a clinical trial utilizing nicotine replacement therapy (Janes et al., 2010a). These individuals also had decreased functional connectivity between the insula and inhibitory control regions such as the dorsal anterior cingulate cortex and the dorsal lateral prefrontal cortex. Insula reactivity to
high-calorie food pictures has also been demonstrated to be predictive of short and long term outcomes in a weight-loss program (Murdaugh et al., 2012). Stress-induced left posterior insula activation in treatment-engaged, abstinent cocaine-dependent individuals has been positively correlated with greater number of days of cocaine use reported at 90-day follow-up (Sinha and Li, 2007). Contradictory to these findings, a study of abstinent subjects with alcohol dependence correlated hypoactivity of the insula during stressful memory recall with alcohol use severity after relapse (Seo et al., 2013).

Further in conflict with the simple conclusion that increased insular activity correlates to relapse, a recent prospective study demonstrated greater anterior insula activation during reward contingency learning predicted abstinence of cocaine-dependent individuals at 1 year follow-up (Stewart et al., 2014). A study examining the stop signal task in cocaine-dependent individuals under fMRI found that in male participants, decreased stop error-related activations of the left insula predicted relapse and earlier time to relapse (Luo et al., 2013). Similar results have been obtained in treatment-engaged, methamphetamine-dependent individuals where a negative correlation was observed between right insular activation during a simple decision making task performed during early recovery and the likelihood of relapse at 1 year follow-up (Paulus et al., 2005). Those authors had similar findings using a risky decision making task, where abstinent methamphetamine-dependent patients had lower insula activation during safe decisions than risky decisions while the subjects who relapsed showed similar insula activation during safe and risky decisions (Gowin et al., 2014). A combination of cocaine and methamphetamine dependent patients also showed a negative correlation between right insular activation during a selective attention task and relapse (Clark et al., 2014b).
Additionally, insula activation during the cognitive-control Stroop task has been associated with better treatment outcome - lower cotinine levels - in young adult smokers (Krishnan-Sarin et al., 2013) which has recently been demonstrated to be enhanced by treatment with the noradrenergic α2a agonist guanfacine (3mg/day) (McKee et al., 2015). It should be noted however that contradictory results were found in a recent 12-week open label smoking cessation trial with varenicline, where non-completers of the study had significantly greater insular activation during Stroop task performance (Wheelock et al., 2014).

With regards to non-task related fMRI studies, recent work examining the effects of either acute (Franklin et al., 2012) or chronically (Franklin et al., 2011b) administered baclofen, the GABA-B agonist being investigated as a therapeutic for smoking cessation (Franklin et al., 2009a) and alcohol dependence (Garbutt et al., 2010), is capable of reducing cerebral blood flow to the anterior ventral insula, potentially contributing to its therapeutic effects. Weaker rsFC of the posterior insula and primary sensorimotor cortices is also observed in relapsed smokers when compared to non-relapsed smokers (Addicott et al., 2015). Lower rsFC of the insula with the NAcc, dorsolateral PFC, ACC, and inferior parietal lobe during early abstinence from alcohol has been demonstrated to significantly predict relapse and is correlated with subsequent alcohol use (Camchong et al., 2013). Contradictory to these findings, another study in cocaine subjects demonstrated that relapse to cocaine correlated with greater intrinsic connectivity of the insula (Mitchell et al., 2013), where intrinsic connectivity reflects tissue functional connectivity using a whole-brain approach without restricting analyses to a priori regions of interest (Scheinost et al., 2012).

Finally, recent evidence has demonstrated the capability of FDA graphic image warning labels on cigarette packs to effectively reduce craving, with a stronger effect in adolescent
smokers; however, these labels elicit a blunted response in the insula and dorsolateral PFC when compared to non-smokers (Do and Galvan, 2015). The authors' also identified a parametric relationship between insula activation and craving reduction which brought them to conclude that the insular activation is important for allowing the visceral reaction to the graphic warning labels to subsequently reduce self-reported craving.

Of importance, the majority of these studies correlating increased insular activation with reduced craving/relapse did not involve addiction-related cues (apart from the indirectly associated FDA graphic image warning labels) or stress, suggesting that individuals prone to relapse may have their insular activity focused on addiction relevant stimuli and thus learning for non-addiction stimuli may be compromised. This interpretation is supported by a study in which heavy drinkers showed significantly greater activity in the insula relative to light drinkers during NoGo trials when the NoGo signal was represented by images of beer bottles (Ames et al., 2014). The authors of that study suggested that the heavy drinkers may have had to exert increased working memory demand and control efforts to withhold a response due to poorer inhibitory control from enhanced salience of alcohol cues on the beer NoGo trials. In contrast, importantly in a study in which no addiction-relevant stimuli were used, there was an association between right insula activation with inhibition success and increased abstinence duration (Bell et al., 2014).

1.2.5.5 Neurocircuitry of Addiction-Relevant Cognition & Decision-Making and a Role for the Insular Cortex

A) Evidence from Animal Studies

Relapse due to the urges induced by re-exposure, cues/context and/or stress, in individuals who state that they wish to abstain from addictive behaviour, is the crux of all addictive disorders.
The inability to prevent oneself from relapsing to the addictive behaviour can be considered a
deficit of self-regulation. In this regard, there have been a number of animal models used to
identify deficits in cognitive performance and brain changes following exposure to addictive
substances, particularly stimulants.

Involuntary exposure to cocaine has been demonstrated to impair reversal learning
(Schoenbaum et al., 2004) while cocaine self-administration results in long-lasting deficits
following withdrawal associated with abnormal OFC functioning (Calu et al., 2007). Extended
access (vs. 1hr limited access) to cocaine has been demonstrated to result in significant
impairments to the PFC and hippocampus as observed through working memory (George et al.,
2008), object recognition (Briand et al., 2008b) and sustained attention (Briand et al., 2008a)
tasks. In the latter study, cognitive deficits were associated with decreased dopamine D2
receptor mRNA in the PFC and OFC. Finally, quinolinic acid lesions of the RAIC result in a
failure to acquire increased anticipatory discriminability, suggesting a direct role of this
subregion in the anticipation of previously experienced rewards, such as would be seen clinically
in substance dependence and gambling disorder (Kesner and Gilbert, 2007).

To my knowledge, only three studies have examined the insular cortex in a rodent model
of decision-making under risk (St Onge and Floresco, 2010; Ishii et al., 2012; Ishii et al., 2015)
though a recent study has demonstrated that a dopamine D1 antagonist infused into the rostral
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studies examining decision-making under risk inactivated the RAIC, with one study finding no
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recordings in the RAIC with increased activity changes during risk-taking choice, but not sure
choice (Kaizu, 2015). Their recent work has demonstrated differential roles for dopamine and serotonin receptors populations where blocking D2 receptors in the RAIC increased risk preference, particularly after winning gambles, while blocking 5-HT1A receptors in the RAIC increased risk preference, particularly after losing gambles (Ishii et al., 2015).

B) Evidence from Human Studies

Recent studies have continued to observe insula activation in a variety of addiction-relevant tasks. Left insular activation has also been correlated with cigarette craving during a smoking-related attentional bias task (Janes et al., 2010b; Luijten et al., 2011) while right insula hyperactivity is observed in smokers during inhibition of an immediately rewarding stimulus in order to obtain a larger, delayed reward (Luijten et al., 2013). Another study found similar activation of the right anterior insula between alcohol dependent individuals and healthy controls on the same task (Amlung et al., 2014). Smokers undergoing acute withdrawal have been shown to have slower reaction times and increased insular activation when undertaking a probabilistic decision-making task (Addicott et al., 2012). Smokers, with or without nicotine replacement therapy, demonstrate bilateral activation of the anterior insula during a working memory task (Sutherland et al., 2011).

Right posterior insular activation has been observed during smoking or alcohol cue presentation has been correlated with lower capability for behavioural control in individuals with episodic drinking and regular smoking (Liu et al., 2014), which suggests that the insula may play a general overarching role in behavioural control. In other study, smoking dependence (assessed with the Fagerström questionnaire) correlated with the magnitude of BOLD change in the right insula during a high-attention emotion recognition task (Artiges et al., 2009). Successful memory retrieval of smoking-related, but not neutral, images presented during a delayed-match-
to-sample task was correlated with greater activation of the left insula (Janes et al., 2015). The authors suggest that the storage and retrieval of smoking-related images is aided by association to the interoceptive cues of smoking stored in the insular cortex, which is consistent with the finding that awareness of somatic states leads to better recognition of previously viewed affective images (Pollatos et al., 2007).

Contrary to those findings in addiction-relevant tasks, methamphetamine dependent individuals show hypoactivation of the right insula after making an error on the cognitive-control Stroop task when compared to healthy controls (Nestor et al., 2011). Again, this suggests that insular cortex activity is changed by exposure to addictive substances and behaviour to become biased towards addiction-relevant stimuli. Errors on the Stroop task are a situation in which an individual's behaviour conflicts with their intention (i.e. they intend to say red when seeing text in the colour red, but accidentally say blue because the text of the word reads BLUE). The fact that individuals with methamphetamine dependence show hypoactivation of the insula suggests that they may have less of a bodily reaction to this conflict of behaviour-intention, something that would support their high degree of relapse in the face of a stated intention to abstain. Supporting this interpretation is the finding that individuals acting based on intrinsic motivations (i.e. spontaneous self-satisfaction) had greater insular activity compared to when they acted based on extrinsic motivations (i.e. socially-acquired stored values) which corresponds to posterior cingulate activation (Lee et al., 2012). Interestingly, a stimulant medication prescribed for narcolepsy, modafinil, is capable of enhancing brain activation of the bilateral insula and eliminating the deficits in learning observed in methamphetamine dependent individuals (Ghahremani et al., 2011).
Menon and Uddin (2010) have proposed that the insular cortex, together with the anterior cingulate, constitute a salience network responsible for allocating resources between the DMN and the ECN (consisting of the dorsolateral PFC and posterior parietal cortex) and that the insular cortex is responsible for initiating switching between these two networks (Sridharan et al., 2008; Menon and Uddin, 2010). With this in mind, the studies demonstrating increased functional connectivity of the insula-DMN would suggest that the ability of the insula to allocate resources is compromised, and thus may contribute to the deficits in executive functioning observed in individuals during withdrawal and relapse to drug dependence (Goldstein and Volkow, 2011). Further work in patients with stroke-induced lesions are required to examine how DMN and ECN functioning differs in those patients compared to healthy controls and individuals with drug dependence.

Addiction is a disorder that compels individuals to perform behaviours that conflict with social norms, as can be easily observed by examining the criterion stated in the DSM-5. It is thus important to understand the role of the insular cortex in the representation of these social norms, detecting whether one's behaviour deviates from social norms, and the ability to correct behaviour which has deviated from social norms (Sanfey et al., 2003; Xiang et al., 2013). Individuals with insular lesions playing a fairness or "ultimatum" game detect norm violations with greater sensitivity than controls, yet show abnormally slow adaptation speed to norm violations (Gu et al., 2015). The authors posited that the insula patients in their could not correctly characterize the bodily feelings coupled with deviations from norm expectations and thus, stayed with their original representation of the norms and not to adapt. The same group of authors also observed that individuals with anterior insular lesions have deficits in the explicit and implicit perception of pain in other people (Gu et al., 2012). Interestingly, individuals with methamphetamine dependence have a reduced emotional response to threatening scenes,
empathy for another's pain, and fearful or angry face, all of which correlate with hypoactivation of the insula (Kim et al., 2011). A similar effect of anterior insular activity attenuation has been observed during emotional face-processing after the consumption of alcohol in healthy volunteers (Padula et al., 2011).

The anterior insular cortex is well known to play a role in the anticipation and valuation of rewards, with anticipation of gain/loss posited to be composed of an ‘alerting’ signal from the thalamus that converges with anterior insular interoceptive representations to influence action selection in the NAcc (Cho et al., 2013). An imaging study in human subjects with alcohol dependence demonstrated that the severity of dependence correlated with greater impulsivity in a delayed reward discounting task and both of these correlated with greater activation of the posterior insula (Claus et al., 2011b) though interestingly, when choosing the delayed reward and resisting the impulsive choice these individuals showed greater left anterior insula activation. Thus, there may be a differential role of the posterior vs. anterior insula in decision-making behaviour involving delay discounting, though the authors posit that the increased anterior insula activation represents the negative feelings experienced by the individual when delaying gratification. Importantly, this finding of the anterior insula was not observed in healthy controls, suggesting a disease specific deficit.

Abstinent individuals with gambling disorder have been observed to have a negative correlation between their insular activation during reward anticipation and the duration of their disorder (Tsurumi et al., 2014). The activation of the insula during reward anticipation has been observed in healthy individuals (Samanez-Larkin et al., 2007; Liu et al., 2011). Thus, the finding of this negative correlation with the duration of illness appears similar in concept to the increasing tolerance developed with chronic substance abuse.
Also with respect to decision-making behaviour, preference for risk can be observed in individuals with substance dependence and those with gambling disorder, as can easily be recognized from the DSM-5 criteria described above, and the involvement of the insular cortex appears to play a prominent role in this preference. Functional imaging in humans has demonstrated increased insular activity preceding risk-averse decisions (Kuhnen and Knutson, 2005), correlated with risk/loss avoidance (Paulus et al., 2003; Fukunaga et al., 2012), anticipation of risk (Preuschoff et al., 2006; Rudorf et al., 2012), monetary uncertainty (Critchley et al., 2001) and the interaction between urgency and expected values (Jones et al., 2011).

Hypoactivity of the anterior insula has been observed in individuals with substance dependence and this was correlated with poor performance on the IGT, particularly when they were given explicit knowledge of certain decks being worse in the long run (Fukunaga et al., 2013). Contrary to this, increased regional cerebral blood flow in the insula has been observed in chronic marijuana users performing the IGT and correlated with earlier age of first use (Vaidya et al., 2012). Yet others have observed hypoactivity of the left insula in response to loss and loss avoidance-related stimuli (Nestor et al., 2010) and hypoactivity of the right insula correlated with error-awareness rates in chronic marijuana users (Hester et al., 2009). Stimulant users show increased left insular activity during low error rates yet decreased activity during high error rates as compared to healthy controls in a decision-making task (Paulus et al., 2008). Regardless, such data have supported a broader role for the insula in signalling aversive consequences via interoceptive signals (Paulus and Stein, 2006) and thus, it has been suggested that insular activity is required primarily for preventing disadvantageous risk (Clark et al., 2008).

Yet another study has noted increased insular activity prior to a decision to take a risk and immediately after taking a risk (especially if risk resulted in a win), and decreased activity prior to refusing a gamble (Xue et al., 2010). As well, individuals with insular lesions have a lower
propensity for risk in the rewarding “Gains” trials of the Cups Task (though no difference in propensity for risk in the punishing “Loss” trials) compared to healthy controls (Weller et al., 2009). These studies together suggest that insular activity does not solely signal aversive consequences but more likely relays an overall interoceptive representation of the options available, along with the influence of contextual conditions, and plays a significant role in decision-making under risk. A study in rats has demonstrated that pharmacological inactivation of the RAIC reduces the risk-taking behaviour on a two-choice (risky vs. sure) task (Ishii et al., 2012) with the same group producing preliminary data using single neuron recordings in the RAIC with increased activity changes during risk-taking choice, but not sure choice (Kaizu, 2015). Their recent work has demonstrated differential roles for dopamine and serotonin receptors populations where blocking D2 receptors in the RAIC increased risk preference, particularly after winning gambles, while blocking 5-HT1A receptors in the RAIC increased risk preference, particularly after losing gambles (Ishii et al., 2015).

Additional support for the conclusion of a broader role for the insula in decision-making is the finding that individuals with insular cortex lesions do not demonstrate the “near-miss” effect (Clark et al., 2014a), which is a greater motivation to play a slot-machine task after a loss that is close to a win (e.g. a loss with two out of three stars); an effect that is reliably observed in healthy controls (Clark et al., 2009; Billieux et al., 2012; Clark et al., 2012). In individuals with gambling disorder, ventral striatal connectivity with the bilateral insula during the experience of near-misses is correlated with gambling severity (van Holst et al., 2014). The involvement of both the anterior (Mizuhiki et al., 2012) and posterior (Asahi et al., 2006) insula in reward expectation has been established using single neuron recording in non-human primates. Preliminary data has also utilized single neuron recordings in rats who received operant conditioning with alcohol as a reinforcer to demonstrate that insular neurons encode reward
expectancy for alcohol (Vicencio, 2015). There is some data to demonstrate that insular activity during reward anticipation correlates with impulsivity and that this may be associated with genetic polymorphisms of the GABA α2 receptor subunit in families with histories of alcoholism (Villafuerte et al., 2012). Specifically examining anticipation for a nicotine infusion, smokers during withdrawal were shown to also have hyperactivity of the insula following verbal cues of nicotine delivery (Gloria et al., 2009).

Individuals with insular lesions have also been observed not to demonstrate “the gambler’s fallacy” (Clark et al., 2014a), which is the erroneous perception that recent consecutive outcomes are somehow less likely to occur even though events are known to be independent (e.g. belief that ‘red is due to win now’ on a roulette spin because multiple consecutive prior spins landed on black); also reliably observed in healthy controls (Ayton and Fischer, 2004). Individuals with insular lesions actually demonstrate a positive recency bias on the roulette task (i.e. their choice of a colour increases in likelihood as a function of the preceding run of that color), again suggesting an increased reliance on reward frequency (Clark et al., 2014a).

Overall, the vast literature described above illustrates the critical role of the insular cortex in decision-making behaviour in addictive disorders, but also in general healthy subjects. Understanding the neuroadaptations of the insular cortex following prolonged drug use or gambling behaviour, is likely to greatly aid in our understanding of the aberrant decision-making behaviour observed in addictive disorders.

1.3 Experimental Rationales

The work presented here was conducted to investigate the effects of modulating activity in two insular subregions, the CGIC and RAIC, through the use of multiple modalities (chemical
lesions, pharmacological inactivation or electrical stimulation), across a variety of behaviours relevant to addictive disorders, including drug self-administration, reinstatement, and decision-making. A greater understanding of the addiction-specific role of the lower-order, sensory-focused CGIC in comparison to the downstream, cognition and affect-focused RAIC, will allow us to determine how best to target these insular cortex subregions, individually or together, for various behavioural aspects of addictive disorders.

1.3.1 Rationale for Investigating the Differential Involvement of the Agranular vs Granular Insular Cortex in the Acquisition and Performance of Choice Behavior in a Rodent Gambling Task

With a long term aim of examining the potential of modulating insular activity as a therapeutic approach for addictive disorders, understanding the role of the insular cortex in decision-making under risk will allow us to determine whether such an approach can be expected to result in adverse effects (eg. increase risky decision-making), additional positive impacts (eg. decreased risky decision-making), or no significant clinically-relevant impact on behaviour. In addition, our particular focus on the individual subregions, CGIC vs. RAIC, was to determine whether the lower-order sensory-focused CGIC would also be involved in addiction-relevant behaviour not previously reinforced by addictive substances, or whether only the higher-order cognitive and affect-focused RAIC would mediate said behaviours.

As described earlier, to our knowledge, only three studies have examined the insular cortex in a rodent model of decision-making under risk (St Onge and Floresco, 2010; Ishii et al., 2012; Ishii et al., 2015) though a recent study has demonstrated that a dopamine D1 antagonist infused into the RAIC promotes impulsive decision-making (Pattij et al., 2014). Both studies examining decision-making under risk inactivated the RAIC, with one study finding no effect (St Onge and Floresco, 2010) and the other study finding a reduction in risk taking behaviour (Ishii et al., 2012). The latter group produced preliminary data using single neuron recordings in the
RAIC with increased activity changes during risk-taking choice, but not sure choice (Kaizu, 2015). Their recent work has demonstrated differential roles for dopamine and serotonin receptors populations where blocking D2 receptors in the RAIC increased risk preference, particularly after winning gambles, while blocking 5-HT\textsubscript{1A} receptors in the RAIC increased risk preference, particularly after losing gambles (Ishii et al., 2015).

Thus, in addition to our examination of the effects of inactivating the CGIC, we decided to also examine the effect of RAIC inactivation on behavioural performance of the rGT, a rodent model of the IGT described above. Further, due to the amount of data on the effects of insular lesions on decision-making in human patients (Bar-On et al., 2003; Weller et al., 2009; Clark et al., 2014a), we decided to utilize a neurotoxin lesion approach to examine the role of the CGIC and RAIC during the acquisition of behaviour in the rGT. The neurotoxin lesion approach was better able to parallel the findings with insular lesions in human patients, not only due to the similar permanent destruction of tissue in both cases, but also because the human patients examined in those studies had not previously been trained on the tasks they were tested with prior to their injury. Thus the effect of their insular lesions could have been due either to disruption of performance of the tasks and/or disruption of the ability to learn the task.

Due to the reciprocal connectivity of the RAIC, but not the CGIC, with areas such as the basolateral amygdala and orbitofrontal cortex, previously determined to be involved in choice behaviour on the rGT (Zeeb and Winstanley, 2011), we hypothesized that only the RAIC would be involved in both the acquisition and performance of the rGT.
1.3.2 Rationale for Examining the Involvement of the Rostral Agranular Insular Cortex in Nicotine Self-Administration in Rats

Having clearly established differential roles of the CGIC vs. RAIC in decision-making behaviour, we next sought to determine whether such differences were also present in behaviours reinforced by addictive substances. Already having established the critical role of the CGIC across nicotine-taking and seeking behaviours, here we repeated the same experimental protocol to determine whether pharmacologically inhibiting the RAIC would produce similar or differential effects as that of the CGIC.

Others have examined the RAIC in the self-administration and reinstatement of various addictive substances. Seif and colleagues (2013) utilized an optogenetic approach to demonstrate that inactivating glutamatergic neurons within the RAIC projecting to the NAcc was capable of attenuating aversion-resistant alcohol self-administration and intake, but had no effect on undeterred alcohol self-administration (Seif et al., 2013). Another recent study examined the effect of local infusions of either D1 or D2 receptor antagonists into the RAIC on nicotine self-administration and demonstrated that only D1 receptor antagonists were capable of attenuating nicotine taking (Kutlu et al., 2013). This finding is consistent with an older study which demonstrated that D1 receptor antagonists delivered into the RAIC were capable of attenuating cocaine self-administration (Di Pietro et al., 2008). Finally, the infusion of a CRF1 antagonist into the RAIC has also been demonstrated to attenuate cue-induced reinstatement of cocaine seeking (Cosme et al., 2015).

Pharmacological inactivation of the RAIC has also been demonstrated to attenuate cue-induced reinstatement of cocaine-seeking, without having any effects on the reinstatement of food seeking behaviour (Di Pietro et al., 2006; Cosme et al., 2015). The level of cue-induced reinstatement of cocaine seeking behaviour has also been correlated to Fos protein expression, a
marker or neuronal activity, in the RAIC (Kufahl et al., 2009). The incubation of nicotine-seeking is also correlated with increased levels of DARPP-32, a phosphoprotein enriched in dopamine neurons containing D1 receptors, specifically in the RAIC (Abdolahi et al., 2010). Interestingly, though either inactivation of the RAIC or CGIC were demonstrated to block conditioned place aversion established by naloxone-precipitated morphine withdrawal, only CGIC inactivation was demonstrated to block conditioned place preference established by morphine (Li et al., 2013).

With strong evidence for the involvement of the RAIC in drug taking and seeking behaviour, we hypothesized that inactivation of the RAIC would attenuate nicotine self-administration and reinstatement behaviour, without affecting food self-administration behaviour.

1.3.3 Rationale for Examining the Effect of Electrically Modulating Insular Cortex Activity on Nicotine Taking and Seeking Behaviour

Our work with both the CGIC and RAIC inactivations on nicotine self-administration and reinstatement (Forget et al., 2010a; Pushparaj et al., 2015a), confirmed the critical involvement of both regions of the insular cortex in an animal model of addiction following the findings of Naqvi and colleagues (2007) in patients with stroke-damage to the insular cortex (Naqvi et al., 2007). Thus, the next obvious thoughts were to explore the potential of inhibiting insular cortex activity as a therapeutic approach for addictive disorders; however, the direct brain cannulation and pharmacological inactivation conducted in our animal models is clearly not a viable option.

Currently, there exist a number of methods for modulating activity within specific brain regions. The oldest and most studied approach is DBS which involves the surgical implantation of an electrode(s) into a particular brain region, and the passing of electrical current through that
electrode as coordinated by an external neurostimulator which can be programmed by a clinician to run continuously. DBS was first approved by the FDA for the treatment of essential tremor and is now also approved for the treatment of Parkinson's Disease, dystonia and most recently for Obsessive Compulsive Disorder. DBS is also being studied for use as a treatment for chronic pain and major depression, along with a number of other psychiatric disorders (Fitzgerald and Segrave, 2015).

To my best knowledge, apart from the work presented here, there have been no DBS studies targeting the insular cortex in humans or animals. Thus, in order to explore the potential of targeting the insular cortex as a therapeutic for smoking cessation with DBS or other modalities for modulating electrical activity within the brain, we examined the effect of high frequency stimulation of the insular region on nicotine self-administration and reinstatement behaviour. In order to determine the specific functional result of high frequency stimulation on insular neurons, we utilized an in vitro electrophysiological approach. To ensure that high frequency stimulation within the insular region did not disrupt natural reward or operant behaviour in general, we examined the effect of said stimulation on food self-administration under both fixed and progressive ratio schedules of reinforcement.

We hypothesized that high frequency stimulation of insular neurons would result in a functional inactivation in the in vitro electrophysiology study, and thus it would also result in an attenuation of nicotine taking and seeking behaviour in the in vivo setting. With the belief that the amplitude of the stimulation was sufficiently limited to prevent the functional effects from extending far beyond the insular cortex, we hypothesized that there would be no significant effects on food self-administration behaviour. Finally, it should be noted that the term insular region is utilized here, and not insular cortex or a particular subregion of the insular cortex, as
the electrical stimulation could not be specifically demonstrated to be constrained to a particular area.

CHAPTER 2. Differential Involvement of the Agranular vs Granular Insular Cortex in the Acquisition and Performance of Choice Behavior in a Rodent Gambling Task


Based on Neuropsychopharmacology, 2015

Pushparaj A designed all experiments with guidance from Winstanley CA and Le Foll B. Kim AS and Musiol M were responsible for animal training and feeding as well as the collection and preparation of data for subsequent analysis. Pushparaj A was responsible for all surgical and cortical inactivation procedures. Pushparaj A performed all statistical analysis and wrote the manuscript. All authors were involved in responding to reviewer comments and making necessary revisions to the manuscript.
2.1 Abstract

Substance-related and addictive disorders, particularly gambling disorder, are known to be associated with risky decision-making behavior. Several neuroimaging studies have identified the involvement of the insular cortex in decision making under risk. However, the extent of this involvement remains unclear and the specific contributions of two distinct insular subregions, the rostral agranular (RAIC) and the caudal granular (CGIC), have yet to be examined. Rats were trained to perform a rodent gambling task (rGT), in which subjects chose between four options that differed in the magnitude and probability of rewards and penalties. In order to address the roles of the RAIC and CGIC in established choice behavior, pharmacological inactivations of these two subregions via local infusions of GABA receptor agonists, were performed following 30 rGT training sessions. The contribution made by the RAIC or CGIC to the acquisition of choice behavior was also determined by lesioning these areas prior to behavioral training. Inactivation of the RAIC, but not of the CGIC, shifted rats’ preference towards options with greater reward frequency and lower punishment. Prior to rGT acquisition, lesions of the RAIC, but not the CGIC, likewise resulted in a higher preference for options with greater reward frequency and lower punishment, and this persisted throughout the 30 training sessions. Our results provide confirmation of the involvement of the RAIC in rGT choice behavior and suggest that the RAIC may mediate detrimental risky decision-making behavior, such as that associated with addiction and gambling disorder.
2.2 Introduction

Gambling disorder is classified as an addictive disorder in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) due to findings establishing its similarity to substance use disorders in terms of clinical expression, brain origin, comorbidity, physiology and treatment (APA, 2013). The insular cortex, or insula, is a brain area involved in interoception and thus numerous behaviors including conscious urges, anxiety, pain, cognition, mood and substance abuse (Hardy, 1985; Watanabe et al., 1997; Goldman-Rakic, 1998; Damasio et al., 2000; Craig, 2002; Paulus and Stein, 2006; Craig, 2009b). As such, it is not surprising that insular involvement has been identified in decision-making under risk and/or uncertainty (Clark, 2010). Importantly, the insula is composed of three distinct cytoarchitectural subregions ordered from the dorsal to ventral cortex, known as the granular, dysgranular and agranular. (Paxinos and Watson, 1986) and research has focused on the role of either the caudal granular (CGIC) or rostral agranular (RAIC) regions. Thus the present study was undertaken to explore the differing roles of these two subregions in a rodent decision making task.

Following the work of Naqvi and colleagues in human smokers (Naqvi et al., 2007), Our prior work identified the involvement of the CGIC in nicotine taking and seeking behavior using a rodent model (Forget et al., 2010a; Pushparaj et al., 2013). The CGIC (bregma to -3.8mm in rats) receives both general viscero sensation unimodal inputs (Cechetto and Saper, 1987) and nociceptive thalamic inputs (Gauriau and Bernard, 2004) along with somatosensory cortex inputs, while sending projections primarily back to the thalamus and somatosensory cortex along with projections to the caudate-putamen (Shi and Cassell, 1998a). The CGIC is reciprocally
connected to the RAIC primarily through intermediate relays in the dysgranular insular subregion (Shi and Cassell, 1998b). An important distinction between these two regions is that the entire granular insula, including the CGIC, is the only component of the insula that does not send projections to amygdalar nuclei (Shi and Cassell, 1998b). The RAIC sends projections to the ventral caudate-putamen and the lateral nucleus accumbens (McGeorge and Faull, 1989; Shi and Cassell, 1998b), while having reciprocal connections with the basolateral amygdala and the prelimbic cortex (Shi and Cassell, 1998b; Vertes, 2004). The RAIC is considered a high-order multimodal cortical region due to its inputs from the medial subdivision of the mediodorsal thalamic nucleus (Krettek and Price, 1977; Allen et al., 1991), which in itself is considered a high-order thalamic nucleus (Van der Werf et al., 2002), along with inputs from various medial thalamic nuclei thought to convey motivational/affective components of nociception. Others have confirmed the involvement of the CGIC (Contreras et al., 2007; Hollander et al., 2008; Scott and Hiroi, 2011), along with that of the RAIC, using various rodent models of addiction (Di Pietro et al., 2008; Abdolahi et al., 2010; Contreras et al., 2012; Seif et al., 2013).

Models of decision-making under risk are particularly useful for studying gambling disorder. Models of decision-making under expected uncertainty, or risk, involve overt presentation of specific probabilities and quantities for reward/loss while models of unexpected uncertainty, or ambiguity, involve a clear understanding of the existence of risk, but not the specific probabilities/quantities. To our knowledge, only two studies have examined the insular cortex in a rodent model of decision-making under risk (St Onge and Floresco, 2010; Ishii et al., 2012) though a recent study has demonstrated that a dopamine D1 antagonist infused into the RAIC promotes impulsive decision-making (Pattij et al., 2014). Both studies examining decision-making under risk inactivated the RAIC, with one study finding no effect (St Onge and Floresco, 2010) and the other study finding a reduction in risk taking (Ishii et al., 2012).
The current study examines the involvement of two insular subregions, the RAIC and CGIC, in a rodent model of decision-making under risk, the rat Gambling Task (rGT), which is similar to the Iowa Gambling Task (IGT), a human model of decision-making under risk (Zeeb et al., 2009). The rGT shares a similar design and contingency structure with the IGT but differs as rodents are given a "forced-choice" training period to learn the contingencies for each of the four options (i.e. reward amounts and punishment durations, along with probabilities). Human subjects in the IGT are presented with 4 decks of cards to choose from but are never informed of the contingencies associated with each and must learn these through choosing from each deck. Thus the rGT is a model of decision-making under risk, but not ambiguity. To differentiate between involvement in performance and acquisition of the task, we either inactivated the insula subregions, using local infusions of GABA receptor agonists, after 30 sessions of rGT training had established stable choice preference or lesioned them prior to any behavioural training. Due to the reciprocal connectivity of the RAIC, but not the CGIC, with areas such as the basolateral amygdala and orbitofrontal cortex (Shi and Cassell, 1998b; Vertes, 2004), previously determined to be involved in choice behaviour on the rGT (Zeeb and Winstanley, 2011), we hypothesized that only the RAIC would be involved in both the acquisition and performance of the rGT.

2.3 Materials and Methods

Subjects

Male Long-Evans rats (Charles River, Lachine, QC) weighing 300-325 g at the start of experiments were maintained on ~20 g of rat chow daily and ad libitum water while in their home cages. Animals were single-housed in a temperature-controlled room on a 12h reverse light cycle with all behavioral testing occurring during the dark phase.

Experimental Design
Experiments were conducted to examine the role of insular subregions (CGIC vs. RAIC) at different rGT performance periods (acquisition of rGT choice behavior vs. prior established rGT choice behavior). Acquisition experiments involved bilateral lesions of the RAIC or CGIC given prior to any behavioral training. Experiments examining established rGT performance involved bilateral cannulation of the RAIC or CGIC following acquisition of choice behavior (ie. animals experienced 30 days of daily rGT sessions).

Surgery

Animals were anaesthetised in an isoflurane (5%) induction chamber before being positioned in a stereotaxic apparatus (Kopf, Model 900) after which anaesthesia was maintained using isoflurane (1-2%) delivered via nose cone. The animals’ heads were shaved and local anesthetic (0.1ml bupivicaine, 0.125%) was injected at the incision site before Betadine was applied to clean the area. An incision was made along the midline and the skull exposed. Location of bregma and lambda were determined and the skull was leveled. Sites of interest were determined relative to bregma as follows, CGIC: anteroposterior -0.4 mm, lateral ± 4.8 mm; RAIC: anteroposterior +2.8 mm, lateral ± 4.0 mm. Small holes were drilled at the respective sites for each animal. For animals receiving bilateral cannulation, 22G stainless-steel guide cannulae (Plastics One, Roanoke, VA) were lowered relative to the dorsoventral coordinate taken from the cranial surface at the site of interest which was as follows, CGIC: +5 mm; RAIC: +6.5 mm, with CGIC cannulae being implanted at a 10º divergent from the vertical. Guide cannulae were then fixed to the skull with screws and dental cement and sealed with a stainless-steel occluder. For animals receiving bilateral lesions, a 28G stainless-steel injector, coupled by polyethylene tubing to a 10µl Hamilton syringe in a microinfusion pump (Harvard Apparatus, Model 22, South Natick, MA), was slowly lowered relative to the dorsoventral coordinate taken from the cranial
surface at the site of interest which was as follows, CGIC: +6 mm; RAIC: +7.5 mm, with the CGIC injector again at a 10º divergent from the vertical. Over the course of 2 min, ibotenic acid (0.2 ul/side, 20 mg/ml in phosphate buffered saline) or vehicle was delivered to the site of interest and the injector was left in place for 5 min to allow for diffusion and finally the incision was closed with wound clips. All animals were given prophylactic antibiotics (Derapen; 30,000 U, IM) and analgesic (Ketoprofen; 0.1 mg/kg, SC) and given a minimum of 1 week recovery in their home cages.

**Behavioral Equipment**

Behavioral testing occurred in traditional 5-choice serial reaction time task (5CSRTT) chambers (Med Associates, Roanoke, VA) controlled by software written in MED-PC running on an IBM compatible computer (see Zeeb et al., 2009 for further details). The chambers had five holes on one wall of the chamber, with a houselight above the holes, and a food receptacle in the middle of the opposing wall to which pellets (45 mg Dustless Precision Pellets, Bio-Serv, Flemington, NJ) could be delivered by an external dispenser. All five holes and the receptacle could be illuminated by a light contained within and nose-poke responses could be detected by infrared sensor. Only the outer 4 of the 5 holes were utilized in all experimental procedures (ie. the middle hole, #3 of 5, was unused).

**Pre-rGT Training**

Animals were trained in 30 min daily sessions on a task similar to the 5CSRTT with the exception of the middle hole (hole 3 of 5) being unused. Animals were trained to make a nose-poke response into the illuminated hole within 10 seconds which resulted in a sucrose pellet being delivered to the food receptacle. The illuminated hole changed at each trial with equal number of presentations of each possible hole over the course of the session. Once all animals
demonstrated two consecutive sessions where 100 trials were completed with 80% of trials being correct (ie. nosepoke made into illuminated hole and not an incorrect nosepoke into one of the other holes) and less than 20% were omitted (ie. no response made within 10 seconds), the group experienced 12 daily sessions (30 min each) of forced-choice version of the rGT where only one of the holes was illuminated at each time. This ensured that all animals had equal experience with the specific number of rewards (ie. 45 mg pellets) and specific duration of punishment (ie. time-out period), and respective probabilities of each, for the four different holes. Animals were counterbalanced on the forced-choice rGT with version A or version B, corresponding to that which they would undergo in the subsequent rGT acquisition. Version A and B differed only based on the assignment of each contingency (P1-P4; see Figure 1 for contingencies) to its respective hole (Version A: hole 1 = P1, hole 2 = P4, hole 3 = P2, hole 4 = P3; Version B: P4, P1, P3, P2).
Figure 1. Trial structure of the rGT. Number of pellets rewarded and duration of punishing timeout are stated for each option above their respective probabilities of occurrence. Assuming exclusive choice of a specific option throughout the session, its maximum number of pellets obtainable are stated at the bottom of the diagram, thus indicating optimal choice order of P2>P1>P3>P4. (Schematic taken with permission from Zeeb et al, 2009).
The rGT has been previously described (Zeeb et al., 2009) and is outlined in Figure 1. Briefly, animals were given 30 daily sessions (30 min each) where they were required to make a nose-poke response into the illuminated food receptacle to start a trial. Once the response was made, the chamber was darkened for a 5 second intertrial interval (ITI) during which responses made were recorded as being premature responses and resulted in a 5 second houselight illumination and time-out period (during which responding had no effect) followed by an illumination of the food receptacle which allowed the animal to begin another trial. If no premature response was made during the ITI, the four response holes were illuminated and the animals had 10 seconds to nosepoke into any of the four holes or else these lights were turned off and the food receptacle light was turned back on with the trial being counted as an omission. If the animal responded into one of the four holes before 10 seconds, the four lights were turned off and the trial was either rewarded (food receptacle illuminated and pellet(s) delivered according to contingency for chosen hole – see figure for contingencies; collection of reward resulted in initiation of new trial) or punished (stimulus light of chosen hole flashed at 0.5 Hz for duration of time-out period, according to contingency of chosen hole – see figure for contingencies, following which food receptacle was illuminated allowing for new trial to be initiated).

Cortical Inactivation

For experiments examining insular subregion involvement in established rGT choice behavior, animals were implanted with guide cannulae, as described above, after completing a total of 30 rGT sessions. Following surgical recovery, animals were given an additional 10 rGT sessions for
reacquisition before testing began. Stability of choice, trials and premature responding were statistically confirmed across the last three sessions (p>0.05, choice: two-way repeated-measures ANOVA for both choice X session and session; trials and premature responding: one-way repeated measures ANOVAs for session). On testing days, injection cannulae were coupled to a 10 µL Hamilton syringe by a polyethylene tubing (inner diameter .58 mm; Plastics One, Roanoke, Virginia) filled with a GABA agonist mixture (muscimol, .03 nmol/side and baclofen, .3 nmol/side; Sigma-Aldrich, St. Louis, MO) or the vehicle (sterile saline) and inserted into the guide cannula after removing the occluder. Rats were first habituated to the procedure of inserting the injection cannulae one day before testing. Over the course of 1 min, 0.5 µL of the GABA agonist mix (or saline) was injected into each side (driven by a microinfusion pump, Harvard Apparatus, Model 22, South Natick, MA), 5–10 min before the session. After the infusion, 1 min was allowed for diffusion, the injectors were removed, and occluders were replaced. Inactivation vs. vehicle testing was counterbalanced and animals were given 5 rGT sessions between testing with stability being statistically confirmed for each respective group (ie. animals receiving inactivation first vs. vehicle first).

Data Analysis

All statistical analyses were conducted using SPSS for Windows. The main variables analyzed were percentage choice for each option (P1-4) and percentage of optimal (P1+P2) choice. All choice data was arcsine transformed to control for the effect of a ceiling. Data from the experiments involving lesions were analyzed using repeated measures ANOVAs with choice (four levels, P1-4) and session as within-subject factors and group (lesion vs. sham) as the between-subjects factor. Sham groups for the lesions were pooled after no statistically significant difference was found from an ANOVA conducted with choice, session and sham
region as factors. Data from the experiments involving cortical inactivation were analyzed using a two-way repeated measure ANOVA with choice (four levels, P1-4) and condition (inactivation vs. vehicle) as within-subject factors with group (optimal vs. suboptimal) as a between subject factor. Animals were defined as optimal if their responding for P1+P2 was greater than 50% for the 2 sessions prior to each inactivation/vehicle session (average of 4 sessions total). If the outcome of the main ANOVA yielded significant effects (p<0.05), further post hoc tests were performed.

Histology

Following completion of behavioral testing, animals were given an overdose of sodium pentobarbital before brains were extracted and flash-frozen in liquid isopentane (kept at approximately -50ºC). Brains were stored at -80ºC before being sliced into coronal serial 30 µm sections throughout the respective subregion of interest and stained with cresyl violet. The extents of the lesions or the injector tips were mapped onto standardized sections of the rat brain (Paxinos and Watson, 1986).

2.4 Results

Cannulae placement and lesion analysis

Illustrations of lesion location and extent along with those of cannulae placement are presented in Figures 2 (RAIC) and 3 (CGIC). Animals in the rGT acquisition experiments were excluded due to unilateral or overextended lesions; however, due to the difficulty of targeting specifically the granular or agranular regions without overlap, lesions were only considered incomplete if they did not cover <40% of the targeted area in the 15 brain slices anterior and posterior to the infusion site. Thus a limitation is the sparing of the inferior layers of the target regions,
particularly for the CGIC. Animals in the established rGT performance experiments were
excluded due to incorrect cannulae placement or extensive damage surrounding the infusion site.
It should be noted here, as a limitation, that all animals with CGIC lesions had some degree of
damage extending into the dysgranular insula and secondary somatosensory cortex. Final
numbers included in each experiment were as follows: lesions on acquisition of rGT: sham = 32;
RAIC = 16; CGIC = 14; inactivations on performance of established rGT: RAIC = 14, CGIC =
13.
Figure 2. Location and extent of excitotoxic lesions or placements of injector tips in the rostral agranular insular cortex. (Left Image) An illustration outlining the boundaries of the largest (gray) and smallest (black) area lesioned in any one section are shown for the RAIC lesion groups. (Right Image) Histological reconstruction of the injection sites in the RAIC with black dots indicating locations of injector tips from animals included in statistical analysis. The number beside each reconstructed image indicates the distance (in millimeters) from bregma. Schematic figure was published in The Rat Brain in Stereotaxic Coordinates, 4th ed., by Paxinos and Watson, Copyright Elsevier (1997).
FIGURE 3

Figure 3. Location and extent of excitotoxic lesions or placements of injector tips in the caudal granular insular cortex. (Left Image) An illustration outlining the boundaries of the largest (gray) and smallest (black) area lesioned in any one section are shown for the CGIC lesion groups. (Right Image) Histological reconstruction of the injection sites in the CGIC with black dots indicating locations of injector tips from animals included in statistical analysis. The number beside each reconstructed image indicates the distance (in millimeters) from bregma. Schematic figure was published in The Rat Brain in Stereotaxic Coordinates, 4th ed., by Paxinos and Watson, Copyright Elsevier (1997).
**Effects of CGIC or RAIC lesions on pre-rGT training**

Animals were trained on a modified version of the 5CRTT which only utilized 4 holes in order to correspond to the rGT. Lesioned animals, as compared to sham controls, appeared slower to acquire the behavior with a significantly lower number of trials initiated (Figure 4A; session X lesion, RAIC vs. sham: $F_{11,506} = 2.083$, $p < 0.05$; CGIC vs. sham: $F_{11,484} = 2.196$, $p < 0.05$) and correct responses (Figure 4B; session X lesion, RAIC vs. sham: $F_{11,506} = 2.313$, $p < 0.01$; CGIC vs. sham: $F_{11,484} = 3.014$, $p < 0.001$) and a significantly greater number of incorrect responses (Figure 4C; session X lesion, RAIC vs. sham: $F_{11,506} = 2.994$, $p < 0.001$; CGIC vs. sham: $F_{11,484} = 3.346$, $p < 0.001$). Additionally, CGIC, but not RAIC, lesioned animals showed a significantly greater number of omissions compared to sham controls (Figure 4D; session X lesion, RAIC vs. sham: $F_{11,506} = 1.819$, $p > 0.05$; CGIC vs. sham: session X lesion: $F_{11,484} = 3.590$, $p < 0.001$).

However, by the last three days of this pre-rGT training phase, lesioned animals no longer differed significantly in responding from sham controls (for all variables: lesion, session X lesion, all $Fs < 2.29$).
Figure 4. Effect of insular lesions on pre-rGT training. Animals with insular lesions showed a slower acquisition of response behavior on a modified version of the five choice serial reaction time task, as evidenced by an initial significantly lower number of trials initiated (A), significantly lower percentage of correct responses (B), significantly higher percentage of incorrect responses (C) and in CGIC, but not RAIC, lesioned animals a significantly higher percentage of omissions (D).
Effects of CGIC or RAIC lesions on acquisition of the rGT

rGT Decision Making. The acquisition of choice behavior by RAIC, but not CGIC, lesioned animals significantly differed from that of sham controls (Figure 5 A-C; session X choice X lesion, RAIC vs sham: F_{87, 4002} = 1.387, p < 0.05; CGIC vs sham: F_{87, 3828} = 1.211, p > 0.05).

RAIC, but not CGIC, lesioned rats showed a significantly greater preference for the optimal choices compared to sham controls (Figure 5D; lesion, RAIC vs. sham: F_{1, 46} = 5.495, p = 0.02; CGIC vs. sham: F_{1, 44} = 2.666, p > 0.05). Interestingly, there was no significant effect of session (session: F_{29, 1711} = 0.1914, p > 0.05), suggesting overall optimal choice remained constant for the duration of training. This finding of consistent optimal choice (P1+P2) appears to contradict the observed change in choice pattern; however, this can be understood if a decrease in P1 choice over time is fully compensated for by a corresponding increase in P2 choice, while non-optimal choice remains fairly constant. Indeed this is the case here with significant effects of session observed for P1 (F_{29, 1711} = 1.743, p < 0.01) and P2 choice (F_{29, 1711} = 1.631, p < 0.05) though not for P3 (F_{29, 1711} = 0.233, p > 0.05) or P4 (F_{29, 1711} = 0.346, p > 0.05) choice. The average percentage of optimal animals for each session (made >50% optimal choice) in each group was compared for the entire acquisition period (Figure 5E). A significant effect of lesion was observed for percentage of optimal choice responders (F_{2, 87} = 124.2, p < 0.001) with the RAIC lesioned group having significantly greater optimal responders (87%) compared to CGIC lesioned (71%) and sham groups (69%; p < 0.001).
Figure 5. Effect of insular lesions on acquisition of the rGT. RAIC (A), but not CGIC (B), lesioned animals demonstrated significantly different acquisition of choice behavior as compared to sham controls (C). RAIC, but not CGIC, lesioned animals demonstrated a significantly greater preference for optimal choices (P1+P2) compared to sham controls (D). Finally, RAIC, but not CGIC, lesioned animals had a significantly greater percentage of optimal responders each session on average compared to sham controls (E).
Both RAIC and CGIC lesioned rats showed a significantly greater preference for P1 choice compared to sham controls (lesion, RAIC vs. sham: $F_{1,46} = 6.354, p < 0.05$; CGIC vs. sham: $F_{1,44} = 4.481, p < 0.05$). However, only RAIC lesioned rats showed a significantly greater preference for P2 choice compared to sham controls (lesion, RAIC vs. sham: $F_{1,46} = 4.479, p < 0.05$; CGIC vs. sham: $F_{1,44} = 1.257, p > 0.05$). RAIC, but not CGIC, lesioned rats showed a significantly lower preference for P3 (lesion, RAIC vs. sham: $F_{1,46} = 5.011, p < 0.05$; CGIC vs. sham: $F_{1,44} = 2.245, p > 0.05$) and P4 choice compared to sham controls (lesion, RAIC vs. sham: $F_{1,46} = 3.441, p > 0.05$; CGIC vs. sham: $F_{1,44} = 1.545, p > 0.05$; session X lesion, RAIC vs. sham: $F_{29,1334} = 1.529, p < 0.05$; CGIC vs. sham: $F_{29,1276} = 0.969, p > 0.05$).

**Other Behavioral Measures (Table 1).** Animals with lesions did not differ from sham controls in the level of premature responding, omissions or perseverative responding observed during acquisition of the rGT (for all variables: lesion, session X lesion, all $F$s $< 2.95$). However, lesions of the CGIC, but not the RAIC, resulted in significantly increased latency to choose (lesion, RAIC vs. sham: $F_{1,46} = 3.854, p > 0.05$; CGIC vs. sham: $F_{1,44} = 5.047, p < 0.05$) and collect reward (session X lesion, RAIC vs. sham: $F_{29,1334} = 0.889, p > 0.05$; CGIC vs. sham: $F_{29,1276} = 1.517, p < 0.05$) compared to sham controls along with a decreased number of trials initiated (lesion, RAIC vs. sham: $F_{1,46} = 3.002, p > 0.05$; CGIC vs. sham: $F_{1,44} = 5.883, p < 0.05$).
<table>
<thead>
<tr>
<th></th>
<th>Sham lesion</th>
<th>RAIC lesion</th>
<th>CGIC lesion</th>
</tr>
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<tbody>
<tr>
<td>Trials initiated</td>
<td>112.92 ± 9.67</td>
<td>120.25 ± 12.32</td>
<td>87.67 ± 8.49</td>
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<tr>
<td>Omissions</td>
<td>1.94 ± 0.67</td>
<td>1.41 ± 0.44</td>
<td>2.27 ± 0.72</td>
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<td>Choice latency</td>
<td>1.24 ± 0.22</td>
<td>1.16 ± 0.17</td>
<td>1.93 ± 0.29</td>
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<tr>
<td>Collection latency</td>
<td>0.94 ± 0.07</td>
<td>1.02 ± 0.09</td>
<td>1.33 ± 0.14</td>
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<tr>
<td>Premature responses</td>
<td>13.86 ± 3.41</td>
<td>12.06 ± 2.77</td>
<td>15.24 ± 3.38</td>
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<tr>
<td>Perseverative responses</td>
<td>114.57 ± 22.47</td>
<td>126.34 ± 19.46</td>
<td>130.79 ± 24.55</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM for the last 5 sessions of rGT training. Omissions and premature responses are represented as a percentage. Latency values are in seconds.
Effects of CGIC or RAIC inactivation on established rGT performance

rGT Decision Making. Inactivation of the RAIC, but not the CGIC, significantly altered choice behavior (Figure 6A, B, respectively; inactivation X choice, RAIC: $F_{3,39} = 3.683$, $p < 0.05$; CGIC: $F_{3,36} = 2.167$, $p < 0.05$). Specifically, RAIC inactivation resulted in a significant increase in P1 choice ($t_{13} = 2.283$, $p < 0.05$) and a significant decrease in P3 choice ($t_{13} = 1.912$, $p < 0.05$) with a decrease in P2 choice that did not reach significance ($t_{13} = 1.583$, $p > 0.05$). Animals were defined as optimal if their responding for P1+P2 was greater than 50% for the 2 sessions prior to each inactivation/vehicle session (average of 4 sessions total). When animals were split into optimal vs. suboptimal groups, only RAIC inactivated animals showed a significant interaction effect (group X inactivation X choice, RAIC: $F_{3,39} = 4.323$, $p < 0.05$; CGIC: $F_{3,36} = 1.954$, $p > 0.05$). For suboptimal animals (36% RAIC of group; Figure 6C) RAIC inactivation resulted in a significant decrease in P3 choice ($t_{5} = 3.368$, $p < 0.05$) and a significant increase in P1 choice ($t_{5} = 2.813$, $p < 0.05$) and P2 choice ($t_{5} = 2.199$, $p < 0.05$). Interestingly, for optimal animals (64% of RAIC group, Figure 6D) RAIC inactivation resulted in a significant decrease in P2 choice ($t_{7} = 3.006$, $p < 0.05$) and a significant increase in P1 choice ($t_{7} = 4.259$, $p < 0.01$). Overall, RAIC inactivation resulted in the percentage of animals in the optimal group increasing from 64% to 79%. There were no suboptimal responders who became optimal responders following CGIC inactivation (optimal group remained at 62%).
Figure 6. Effect of insular inactivations on established rGT performance. Inactivation of the RAIC (A) resulted in a significant increase in P1 choice and a significant decrease in P3 choice. Inactivation of the CGIC had no significant effect (B). As only the RAIC inactivation showed a significant interaction effect with choice and optimal (>50% choice of P1+P2 during vehicle session) vs. suboptimal group, these two subgroups were examined separately. For rats in the suboptimal group (C), RAIC inactivation resulted in a significant decrease in P3 choice and significant increases in P1 and P2 choice. While for rats in the optimal group (D), RAIC inactivation resulted in a significant decrease in P2 choice and a significant increase in P1 choice. Data are expressed as the mean ± SEM with asterisks (*) indicating a significant difference (p<0.05) determined by paired t-tests.
Other Behavioral Measures (Table 2). RAIC inactivation did not result in any significant changes in trials initiated, omissions, choice latency, collection latency, premature or perseverative responses (all t’s < 1.22, p > 0.05). CGIC inactivation resulted in a significant increase in choice latency ($t_{12} = 1.914$, $p < 0.05$) and a trend towards an increase in omissions ($t_{12} = 1.581$, $p > 0.05$) while all other measures were not significantly affected (all t’s < 1.28, p > 0.05).
<table>
<thead>
<tr>
<th></th>
<th>RAIC Veh</th>
<th>RAIC Inact</th>
<th>CGIC Veh</th>
<th>CGIC Inact</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trials initiated</strong></td>
<td>99.08 ± 8.97</td>
<td>105.24 ± 9.71</td>
<td>103.84 ± 10.59</td>
<td>95.28 ± 11.41</td>
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<tr>
<td><strong>Omissions</strong></td>
<td>1.31 ± 0.70</td>
<td>1.69 ± 0.63</td>
<td>1.29 ± 0.59</td>
<td>2.34 ± 0.81</td>
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<tr>
<td><strong>Choice latency</strong></td>
<td>1.38 ± 0.21</td>
<td>1.54 ± 0.17</td>
<td>1.43 ± 0.19</td>
<td>2.06 ± 0.29</td>
</tr>
<tr>
<td><strong>Collection latency</strong></td>
<td>1.06 ± 0.08</td>
<td>0.98 ± 0.10</td>
<td>1.11 ± 0.11</td>
<td>1.21 ± 0.14</td>
</tr>
<tr>
<td><strong>Premature responses</strong></td>
<td>15.24 ± 3.16</td>
<td>17.21 ± 2.74</td>
<td>14.52 ± 3.74</td>
<td>18.48 ± 3.49</td>
</tr>
<tr>
<td><strong>Perseverative responses</strong></td>
<td>134.15 ± 19.48</td>
<td>126.57 ± 21.46</td>
<td>127.84 ± 22.64</td>
<td>137.42 ± 18.77</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM. Omissions and premature responses are represented as a percentage. Latency values are in seconds.
2.5 Discussion

These results demonstrate insular involvement in decision-making under risk, as assessed by a rodent task with a contingency structure similar to the IGT. Subregion-specific involvement was demonstrated with RAIC disruption affecting both acquisition and performance of choice behavior while CGIC disruption minimally affected acquisition. However, both RAIC and, to a greater extent, CGIC lesions affected the acquisition of appropriate responding on the pre-rGT serial reaction time task. Thus, it should be clearly noted that this suggests the insula is involved in the learning of a serial reaction time task, apart from its involvement in decision-making in the rGT. RAIC lesions resulted in acquisition of a consistently greater preference for “optimal” choices (options P1 and P2) which, when consistently chosen, produce the greater overall reward potential (295 and 411, respectively vs. 135 and 99 for P3 and P4, respectively). In rodents with established choice behavior, RAIC inactivation increased P1 choice and decreased P3 choice. Examining animals by subgroup of optimal (>50% choice of P1+P2) vs. suboptimal, RAIC inactivation increased P1 choice in both groups and decreased P3 choice in the suboptimal and optimal group. Interestingly, P2 choice decreased in the optimal group but increased in the suboptimal group following inactivation of the RAIC.

A limitation of the negative findings for the CGIC is the observation of increased latencies and decreased trials, particularly following lesions, suggesting potential motor impairments and/or decreased motivation to engage in the task. Although inactivations resulted in a slightly increased (10%) collect latency, our previous findings have demonstrated no effect of CGIC inactivations on pellets obtained or lever presses made for food self-administration under a progressive ratio schedule (Forget et al, 2010). However, the increased choice latency observed from both CGIC inactivations and lesions could be attributed to an increase in decision-
making time. Interestingly, increased choice latency has also been observed following basolateral amygdala (BLA) inactivations in the rat cognitive effort task (rCET; (Hosking et al., 2014) but not for BLA lesions on either the rGT (Zeeb and Winstanley, 2011), loss-chasing or betting tasks (Tremblay et al., 2014). Future work should examine whether this effect of increased choice latency following CGIC lesion/inactivation is also observed in other decision-making models.

To our knowledge, there are only two studies examining RAIC inactivation in rodent models of decision-making under risk with both examining performance not acquisition. St. Onge and Floresco (2010) demonstrated no effects of RAIC inactivation on a risk-discounting task whereas the work of Ishii and colleagues (2012) demonstrated that RAIC inactivation decreased risky (vs. sure) choice and concluded that RAIC activity promotes risk-taking behavior. Due to the nature of the rGT, our study cannot confirm this straightforward conclusion. Importantly, those prior two studies utilized models with only two options (risky vs. sure). The optimal choice in the rGT is P2, which yields an average of 411 pellets per session when chosen exclusively. The other choices, in order of descending expected pellet yield if chosen exclusively, are P1 (295 pellets), P3 (135), and P4 (99). Importantly, this distribution of expected values results in overall choice percentage being high for P2, though individual animals may not prefer this option. Our results suggest that RAIC inactivation does not simply increase choice for the optimal risk-reward option. Both optimal and suboptimal animals appear to have an RAIC inactivation “shift” in choice behavior towards an increase in preference for options with higher probabilities of reward over punishment and lower number of pellets rewarded, but also lower durations of punishment. Unfortunately, due to the design of contingencies in our model (for a single trial, options with greater pellets rewarded = greater punishment duration = greater probability of punishment), we cannot distinguish whether this shift following RAIC
inactivation is due to an increased preference for reward frequency and/or an increased avoidance of larger durations of punishment.

Though historically the insula was considered merely a primary gustatory cortex, more recent evidence has established it as an integrator and processor of somatosensory (Sterzer and Kleinschmidt, 2010), autonomic (Craig, 2002) and cognitive-affective information (Craig, 2009b; Craig, 2010b). According to the somatic-marker hypothesis, such an integral role in emotional feeling establishes this brain area as critical for guiding behavior (Damasio, 1996; Craig, 2004), particularly in situations involving risk and ambiguity (Craig, 2002; Singer et al., 2009; Naqvi et al., 2014). However, only the RAIC, not the CGIC, has been identified to have a large variety of bidirectional connections with critical subcortical and frontal cortical regions, such as the basolateral amygdala and orbitofrontal cortex (Shi et al., 1998b; Vertes, 2004), known to play a role in decision-making behaviour (Zeeb and Winstanley, 2011). Thus it is our belief, based on literature to be presented below, that RAIC activity represents an overall representation/valuation of the options available, along with the influence of contextual conditions (i.e. said representation can be modulated by factors such as urgency, uncertainty, etc.), and that this representation plays a significant role in decision-making under risk.

Functional imaging in humans has demonstrated increased insular activity preceding risk-averse decisions (Kuhnen and Knutson, 2005), correlated with risk avoidance (Paulus et al., 2003), anticipation of risk (Preuschoff et al., 2006; Rudorf et al., 2012), monetary uncertainty (Critchley et al., 2001) and the interaction between urgency and expected values (Jones et al., 2011). Such data have supported a broader role for the insula in signalling aversive consequences via interoceptive signals (Paulus and Stein, 2006) and thus, it has been suggested that insular activity is required primarily for preventing disadvantageous risk (Clark et al., 2008).
Yet another study has noted increased insular activity prior to a decision to take a risk and immediately after taking a risk (especially if risk resulted in a win), and decreased activity prior to refusing a gamble (Xue et al., 2010). As well, individuals with insular lesions have a lower propensity for risk in the rewarding “Gains” trials of the Cups Task (though no difference in propensity for risk in the punishing “Loss” trials) compared to healthy controls (Weller et al., 2009). Together, these results suggest that insular activity relays a contextualized interoceptive representation of available options during decision making under risk, rather than merely identifying averse consequences of potential options. Additional support for this conclusion is the finding that individuals with insular cortex lesions do not demonstrate the “near-miss” effect (Clark et al., 2014a), which is a greater motivation to play a slot-machine task after a loss that is close to a win (e.g. a loss with two out of three stars); an effect that is reliably observed in healthy controls (Clark et al., 2009; Billieux et al., 2012; Clark et al., 2012). The same study demonstrated that individuals with insular lesions do not demonstrate “the gambler’s fallacy”, which is the erroneous perception that recent consecutive outcomes are somehow less likely to occur even though events are known to be independent (e.g. belief that ‘red is due to win now’ on a roulette spin because multiple consecutive prior spins landed on black), also reliably observed in healthy controls (Ayton and Fischer, 2004). Individuals with insular lesions actually demonstrate a positive recency bias on the roulette task (i.e. their choice of a colour increases in likelihood as a function of the preceding run of that color), again suggesting an increased reliance on reward frequency (Clark et al., 2014a).

Individuals with stroke-induced insular lesions have consistently demonstrated impaired decision-making behavior. This has been observed across multiple paradigms, including the IGT (Bar-On et al., 2003), the Cambridge Gambling Task (Clark et al., 2008) and the Cups Task (Weller et al., 2009). The latter two tasks involve decision-making under certain risk with
individuals choosing the value of a bet under differing risk in the Cambridge Gambling Task or choosing between a sure and a risky option in the Cups Task. On the other hand, the IGT involves decision-making under risk and uncertainty with choice between four options. The results of those studies are consistent in their demonstration that individuals with insular lesions lack risk-adjustment, or the ability to adjust behavior with changing expected values. Our seemingly contradictory findings can be explained by the parametrical differences between previous studies and the rGT, and because unlike others, our model featured constant expected values. It should also be noted that any preference for reward frequency was likely not observed for individuals with insular lesions undertaking the IGT (Bar-On et al., 2003) because the original IGT used in that study had “stacked decks” with a greater probability of rewards occurring earlier. As such, overall impaired behavior of individuals with insular lesions on the IGT may have been due to an inability to adjust their choices towards the advantageous decks after having had initial experiences of large, consistent rewards in the disadvantageous decks. As well, greater reliance on reward frequency can explain the lower propensity of individuals with insular lesions to take risks in the rewarding “Gains” trials while having no effect on their propensity for risk in the punishing “Loss” trials of the Cups Task (Weller et al., 2009). Overall, our results cannot confirm whether the RAIC and/or CGiC is the probable site of damage in individuals with stroke-induced insular lesions resulting in impaired risk-adjustment, as our model utilized certain and stable probabilistic contingencies.

Based on the literature described above, one would conclude that both the valuations of reward (# of pellets) and punishment (time out duration) should be encompassed within an interoceptive representation held within the RAIC. Thus assuming a reduction in RAIC function resulted in this interoceptive representation no longer affecting decision-making, one would assume that the relative valuation of reward/punishment amounts for each option no longer
guided decision-making. This leaves the possibility that RAIC manipulated animals instead increased their reliance on outcome probability (regardless of reward/punishment values) in their decision-making behavior. This may be a result of an increased reliance on the striatum in decision-making, as the ventral striatum is suggested to underlie learning associations between stimuli and responses via feedback while the dorsal striatum mediates enacting decisions once those associations are established (Hiebert et al., 2014). Further work should be conducted to confirm whether RAIC manipulations specifically increase reliance on outcome probability, and whether this is mediated by the striatum. Importantly, this reliance on outcome probability does not suggest that lesions/inactivations of the RAIC improve overall performance in all cases, as rats in the optimal subgroup actually decreased choice of the best option, P2, with RAIC inactivations. As well, further work should be conducted under risk-free conditions to verify that the results observed here are specific to decision-making under risk.

In conclusion, our results demonstrate that the RAIC, but not CGIC, is involved in decision-making behavior under risk in the rGT model. Taken along with other findings of insular involvement in the decision-making literature, we suggest that insular activity likely represents an overall interoceptive representation of the available options and thus increased insular activity can precede decisions of both risk avoidance and risk preference. In the case of gambling disorder, where such interoceptive representations likely contribute to detrimental risky decision-making behaviour, manipulations of the RAIC may be of potential therapeutic benefit. Future work should examine the effects of manipulating insular activity, such as repetitive transcranial magnetic stimulation, on decision-making tasks and gambling behaviour in human laboratory models.
CHAPTER 3. Involvement of the Rostral Agranular Insular Cortex in Nicotine Self-Administration in Rats

Pushparaj A, Kim AS, Musiol M, Trigo JM, Le Foll B

Based on Behavioural Brain Research, 2015

Pushparaj A designed all experiments with guidance from Le Foll B. Kim AS and Musiol M were responsible for animal training and feeding for food self-administration experiments and food training prior to nicotine self-administration. Pushparaj A was responsible for animal training and feeding for nicotine self-administration experiments as well as all intracranial surgeries and cortical inactivation procedures. Trigo JM was responsible for all jugular vein catheterization surgeries. Pushparaj A performed all statistical analysis and wrote the manuscript. All authors were involved in responding to reviewer comments and making necessary revisions to the manuscript.

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3.1 Abstract

Our prior work demonstrated the involvement of the caudal granular subregion of the insular cortex in a rat model of nicotine self-administration. Recent studies in various animal models of addiction for nicotine and other drugs have identified a role for the rostral agranular subregion (RAIC). The current research was undertaken to examine the involvement of the RAIC in a rat model of nicotine self-administration. We investigated the inactivating effects of local infusions of a γ-aminobutyric acid agonist mixture (baclofen/muscimol) into the RAIC on nicotine self-administration under a fixed-ratio 5 (FR5) schedule and on reinstatement of nicotine seeking induced by nicotine-associated cues in rats. We also evaluated the effects of RAIC inactivation on food self-administration under an FR5 schedule as a control. Inactivation of the RAIC decreased nicotine, but not food, self-administration. RAIC inactivation also prevented the reinstatement, after extinction, of nicotine seeking induced by nicotine-associated cues. Our study indicates that the RAIC is involved in nicotine-taking and nicotine-seeking in rats. Modulating insular cortex function appears to be a promising approach for nicotine dependence treatment.
3.2 Introduction

Over 5 million deaths per year worldwide result from tobacco-related diseases and this number is expected to double within the next decade (Proctor, 2004), making tobacco smoking a major population health issue. Though multiple medications have been approved for the indication of smoking cessation, the relapse rate of smokers attempting to quit remains quite high (Rigotti, 2002; Le Foll and George, 2007). Considering that nicotine is the primary psychoactive component of tobacco, there is a need to understand the neurocircuitry underlying its role in both smoking behavior and relapse.

Recent focus in this regard has examined the insular cortex, or insula, a brain area involved in interoception and thus in numerous behaviors including conscious urges, anxiety, pain, cognition, mood and substance abuse (Hardy, 1985; Watanabe et al., 1997; Goldman-Rakic, 1998; Damasio et al., 2000; Craig, 2002; Paulus and Stein, 2006; Craig, 2009b). The insula has become more accepted as a critical substrate underlying addiction (Koob and Volkow, 2010), particularly since the 2007 findings of Naqvi and colleagues which observed stroke-induced damage to the insular cortex being correlated to immediate smoking cessation easily achieved without craving or relapse. Though many human imaging studies followed (Naqvi et al., 2014), animal models have been critical to examine the effects of manipulating insular cortex function in various aspects of nicotine addiction (Hollander et al., 2008; Forget et al., 2010a; Scott and Hiroi, 2011; Kutlu et al., 2013; Pushparaj et al., 2013).

The granular, dysgranular, and agranular corticies are the three structurally and functionally distinct subregions that comprise the insula (Paxinos and Watson, 1986). Our previous studies (Forget et al., 2010a; Pushparaj et al., 2013), along with the work of others (Hollander et al., 2008), have demonstrated that the caudal granular insular cortex (CGIC) plays
an important role in rodent models of nicotine addiction. The CGIC integrates nociceptive (Cechetto and Saper, 1987) and unimodal viscerosensory (Gauriau and Bernard, 2004) afferents from the thalamus along with those from the somatosensory cortex. It also primarily sends efferent projections back to those areas as well as to the caudate-putamen (Shi and Cassell, 1998a). Through the dysgranular insular subregion, information initially integrated in the CGIC is relayed and further integrated before reaching the rostral agranular insular cortex (RAIC) (Shi and Cassell, 1998b). Importantly, the granular insular cortex is the only insular subregion lacking projections to nuclei within the amygdala (Shi and Cassell, 1998b).

The RAIC send efferents to the ventral striatum (Groenewegen et al., 1991; Groenewegen et al., 1997) and the lateral nucleus accumbens (McGeorge and Faull, 1989; Wright and Groenewegen, 1996; Shi and Cassell, 1998b), while having reciprocal connections with the basolateral amygdala and the prelimbic cortex (Shi and Cassell, 1998b; Vertes, 2004). As it receives afferents from the high-order (Van der Werf et al., 2002) medial subdivision of the mediodorsal thalamic nucleus (Krettek and Price, 1977; Allen et al., 1991), along with those from multiple medial thalamic nuclei conveying motivational/affective aspects of peripheral stimuli, the RAIC is considered a high-order multimodal cortical region. The involvement of the RAIC has also been observed in certain models of nicotine addiction (Abdolahi et al., 2010; Kutlu et al., 2013) and neural responses to nicotine exposure (Mychasiuk et al., 2013; Dehkordi et al., 2015) in rodents.

The current study examined whether the RAIC was involved in nicotine self-administration and seeking behavior, as we have previously observed with the CGIC. Thus the same intracranial manipulations were used in the current study as used previously with the
CGIC: bilateral infusions of γ-aminobutyric acid (GABA) receptor agonists, baclofen and muscimol, into the RAIC. Food self-administration was also evaluated as a control.

3.3 Materials and Methods

Subjects

Naive male Long-Evans rats (Charles River, Lachine, QC) weighing 300-325g at the start of experiments were maintained on ~20g of rat chow daily and ad libitum water while in their home cages. Animals were single-housed in a temperature-controlled room on a 12h reverse light cycle with all behavioral testing occurring during the dark phase. All experimental procedures described in this report were carried out in compliance with the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the institutional Animal Care Committee.

Drugs

(−)Nicotine hydrogen tartrate (Sigma–Aldrich, St. Louis, MO) was dissolved in saline, and the pH of the resulting solution was adjusted to 7.0 ± 0.2 before being filtered through a 0.22-mm syringe filter (Fisher Scientific, Pittsburgh, PA).

A mixture of baclofen and muscimol (0.3 and 0.03 nmol/side, respectively; Sigma-Aldrich) was dissolved in saline for intracranial infusions.

Initial Operant Training

Procedures were similar to those previously reported (Forget et al., 2010a; Pushparaj et al., 2013). All operant procedures were conducted in experimental chambers with two retractable levers located on a chamber wall (Med Associates, St. Albans, VT). The start of each daily
session was indicated by the extension of these two levers and the concurrent illumination of a house light on the opposing chamber wall. Over the course of 5 daily sessions, all rats were trained to press one of the two levers, the active lever, on a continuous reinforcement schedule in order to receive a 45mg food pellet (Bio-Serv, Flemington, NJ) delivered to a receptacle positioned between the two levers. Responding on the opposite lever, the inactive lever, was recorded but had no consequences. Sessions concluded after rats had obtained 100 pellets or 60 min had passed, whichever occurred first.

**Jugular Vein Catheterization**

Following the initial operant training period (section 2.3), each rat was implanted with an intravenous catheter in the jugular vein exiting between the scapulae. Penicillin G (30,000 Units, SC) was given within 24 hrs before surgical procedures. Anesthesia was induced by a mixture of xylazine and ketamine hydrochloride (10 and 75mg/kg, respectively, IP). Postsurgical analgesia was achieved using buprenorphine (.01mg/kg, SC). Animals recovered for a minimum of 7 days in their home cages.

**Acquisition of Nicotine Self-administration**

Operant chambers were similar to those described in section 2.3 with three major exceptions: (1) no food receptacle was present between the two levers, (2) the catheters described in section 2.4.1 were connected to a spring descending from the top of the chamber which was connected to a pump (Med Associates - Model PHM-104) allowing for reinforcement by rapid delivery (1 s infusion duration) of IV nicotine (0.03 mg/kg/infusion; free base concentration), and (3) a cue light was present above the active lever and would illuminate for the duration of the 60 s timeout period following each reinforcement during which time the house light was turned off and
responding on either lever was recorded but had no consequences. Rats underwent 5 sessions of self-administration under a fixed-ratio 1 (FR-1) schedule of reinforcement, followed by 3 sessions under an FR-2 schedule and then 7 sessions under an FR-5 schedule (ie. five active lever presses required for each reinforcement).

**Intracranial Cannulation**

Following the acquisition of self-administration, rats underwent surgical implantation of bilateral microcannulae guide into the agranular insular cortex as follows. Rats were anaesthetised in an isoflurane (5%) induction chamber before being positioned in a stereotaxic apparatus (Kopf, Model 900) after which anaesthesia was maintained using isoflurane (1-2%) delivered via nose cone. The rats' heads were shaved and local anesthetic (0.1ml bupivicaine, 0.125%) was injected at the incision site before betadine was applied to clean the area. An incision was made along the midline and the skull exposed. Location of bregma and lambda were determined and the skull was levelled. Site of interest were determined relative to bregma as follows, RAIC: anteroposterior +2.8mm, lateral ± 4.0mm. Small holes were drilled at the respective sites for each rat. Guide microcannulae (22G; Plastics One, Roanoke, VA) were lowered 6.5mm relative to the dorsoventral coordinate taken from the cranial surface at the site of interest. Guide cannulae were then fixed to the skull with screws and dental cement (Jet Set 4; Lang Dental, Wheeling, IL) and sealed with a stainless-steel occluder (Plastics One). Postsurgical analgesia was achieved using buprenorphine (.01mg/kg, SC). Rats were again given a minimum of 7 days recovery time in their home cages.

*Cortical Inactivation Procedure*
On testing days, injection cannulae were coupled to a 10µL Hamilton syringe by a polyethylene tubing (inner diameter .58 mm; Plastics One) filled with GABA agonist mixture (muscimol, .03 nmol/side and baclofen, .3 nmol/side) or vehicle (sterile saline) and inserted into the guide cannula after removing the occluder. Rats were first habituated to the procedure of inserting the injection cannulae one day before testing. Over the course of 1 min, 0.5 µL of the GABA agonist mixture (or saline) was injected into each side (driven by a microinfusion pump, Harvard Apparatus, Model 22, South Natick, MA), 5 min before the session. After the infusion, 1 min was allowed for diffusion, the injectors were removed, and occluders were replaced. Inactivation vs. vehicle testing was counterbalanced.

Experiment 1: Inactivating the RAIC on Nicotine Self-Administration

Following intracranial cannulation, some rats were given daily sessions of nicotine self-administration for a minimum of 5 days, until behavior stabilized. Rats were considered to have achieved stable behavior when they had less than 20% variation in the number of reinforcements earned for two consecutive sessions and their number of active lever presses was at least twice the number of inactive lever presses. Once stable, rats were given either GABA agonist or vehicle infusions into the bilateral RAIC, as described in section 2.4.6, before undergoing their usual nicotine self-administration session. Following this first testing, rats underwent a minimum of two self-administration sessions to re-establish stable behavior before undergoing testing again. Rats also underwent a testing session in which nicotine was substituted with saline. All three testing conditions (GABA agonists, vehicle or saline substitution) were counterbalanced using a within-subject design.

Experiment 2: Inactivating the RAIC on Reinstatement of Nicotine-Seeking
Following intracranial cannulation, some rats were given daily sessions of nicotine self-administration for 7 days before beginning extinction sessions. Extinction sessions were conducted under the same conditions as nicotine self-administration with the following exceptions, (1) no nicotine was delivered during the session, (2) the house light remained on for the entire 60 min session and no cue light presentations were made, (3) responding on both active and inactive levers were recorded but had no consequences. Once lever responding had been extinguished (<20 active lever presses/session for 2 consecutive sessions), rats were given either GABA agonist or vehicle infusions into the bilateral RAIC, as described in section 2.4.6, before undergoing a cue reinstatement testing session. Cue reinstatement testing sessions were the same as FR-5 nicotine self-administration sessions described above with the following exceptions: (1) a single cue presentation lasting 60 s was given at the start of the session, (2) though animals could press the active lever under an FR-5 schedule to receive light cue presentations, no nicotine infusions were delivered. Following this first testing, rats underwent a minimum of two extinction sessions to re-establish stable behavior before undergoing testing again with the counterbalanced condition.

*Food Self-Administration Experiments*

Rats for food self-administration experiments did not receive jugular vein catheterization but underwent similar self-administration procedures as described above with the following exceptions: (1) instead of a spring descending from the top of the chamber, a food receptacle was present between the two levers, and (2) instead of nicotine, 45 mg food pellets were delivered as reinforcements. Once food self-administration behavior was acquired, rats underwent bilateral cannulation of the RAIC as described in the section. Following recovery and re-established stable self-administration behavior, rats underwent testing to examine the effect of RAIC
inactivation on food self-administration under an FR-5 schedule, as described for nicotine in the sections above.

Data Analysis

Only rats with bilateral placement of the cannulae in the RAIC were included for data analysis. Nicotine self-administration and reinstatement, as well as food self-administration, data were subjected to repeated measures analysis of variance (ANOVA), followed by post hoc tests for multiple comparisons (dependent variables: reinforcements, active or inactive lever presses).

Histology

Following completion of behavioral testing, animals were given an overdose of sodium pentobarbital before brains were extracted and flash-frozen in liquid isopentane (kept at approximately -50°C). Brains were stored at -80°C before being sliced into coronal serial 30 µm sections throughout the respective subregion of interest and stained with cresyl violet. The location of injector tips were mapped onto standardized sections of the rat brain (Paxinos and Watson, 1986) and can be seen in Figure 7.
Figure 7. Histological reconstruction of the injection sites in the rostral agranular insular cortex (RAIC) in animals trained under either nicotine (A) or food (B) self-administration. Black dots indicate locations of injector tips from the rats that were included in statistical analysis. The number beside each reconstructed image indicates the distance (in millimeters) from bregma. Schematic figure was published in The Rat Brain in Stereotaxic Coordinates, 4th ed., by Paxinos and Watson, Copyright Elsevier (1997). S1J, primary somatosensory cortex – jaw region; GI, granular insula; AID, agranular insular cortex – dorsal part; AIV, agranular insular cortex – ventral part; LO, lateral orbital cortex; VO, ventral orbital cortex; Cl, claustrum.
3.4 Results

Effect of RAIC Inactivation on Nicotine Self-Administration

The ANOVA performed on the number of active lever presses which rats (n = 11) made during the nicotine self-administration testing sessions (Figure 8A; top panel) showed a main effect of treatment [F(3,3024) = 7.26, p < .001]. Pairwise comparisons indicated there was no significant difference between the number of active lever presses made during baseline self-administration or during vehicle infusions into the RAIC. RAIC inactivation before the session (p < .01) or substitution of nicotine by saline (p < .001) significantly reduced the number of active lever presses made compared with vehicle administration. There was no significant difference between the number of active lever presses following RAIC inactivation or during the saline substitution testing session.

The ANOVA performed on the number of inactive lever presses that rats made during the nicotine self-administration testing sessions (Figure 8A; bottom panel) showed no significant effect of treatment [F(3,30) = 2.67, p > .05].

The ANOVA performed on the number of reinforcements that rats earned during the nicotine self-administration testing sessions (Figure 8B) showed a main effect of treatment [F(3,3024) = 6.94, p < .001]. Pairwise comparisons indicated there was no significant difference between the number of reinforcements earned during baseline self-administration or during vehicle infusions into the RAIC. RAIC inactivation before the session (p < .01) or substitution of nicotine by saline (p < .001) significantly reduced the number of reinforcements earned compared with vehicle administration. There was no significant difference between the number of reinforcements earned following RAIC inactivation or during the saline substitution testing session.
Figure 8. Effects of rostral agranular insula cortex inactivation on nicotine self-administration under a fixed-ratio 5 (FR-5) schedule of reinforcement ($n = 11$). Data are expressed as means (± SEM) of the number of lever presses (A) or nicotine reinforcements earned (B): after either an infusion of baclofen/muscimol (INACT) or saline (VEH) into the insula; the average of three normal baseline self-administration sessions the days prior to each condition; or when saline infusions were earned in place of nicotine (SAL SUB). **$p < .01$, ***$p < .001$ vs. BSL, one-way repeated measures ANOVA followed by Bonferroni post-hoc testing.
Effect of RAIC Inactivation on Reinstatement of Nicotine-Seeking by Cues

The ANOVA performed on the active lever presses that rats (n = 10) made during the extinction and reinstatement testing sessions (Figure 9; top panel) indicated a main effect of treatment [F(2,16) = 9.38, p < .01]. The post hoc analysis showed that the cue presentation induced a significant reinstatement of the presses on the active lever (p < .01, vehicle vs. extinction) and that RAIC inactivation significantly decreased this cue-induced reinstatement (p < .05, inactivation vs. vehicle). In addition, there was no difference between responding under extinction and the cue-induced reinstatement with RAIC inactivation.

The ANOVA performed on the number of inactive lever presses that rats made during the extinction and reinstatement testing sessions (Figure 9; bottom panel) showed no significant effect of treatment [F(2,16) = 1.74, p > .05].
Figure 9. Effects of rostral agranular insula cortex inactivation on nicotine-associated light cue-reinstatement ($n = 10$). Data are expressed as means ($\pm$ SEM) of the number of lever presses made after either an infusion of baclofen/muscimol (INACT) or saline (VEH) into the insula followed by a cue-induced reinstatement session; or the average of the two extinction sessions the days prior to each reinstatement session (EXT). **$p < .01$ vs. VEH, one-way repeated measures ANOVA followed by Bonferroni post-hoc testing.
Effect of RAIC Inactivation on Food Self-Administration under FR-5 Schedule of Reinforcement

The ANOVA performed on the number of active lever presses which rats (n = 12) made during the food self-administration testing sessions (Figure 10A; top panel) showed no effect of treatment [F(2,22) = 1.03, p > .05].

The ANOVA performed on the number of inactive lever presses that rats made during the nicotine self-administration testing sessions (Figure 10A; bottom panel) showed no significant effect of treatment [F(2,22) = 1.84, p > .05].

The ANOVA performed on the number of pellets that rats earned during the food self-administration testing sessions (Figure 10B) showed no effect of treatment [F(2,22) = 2.58, p > .05].
Figure 10. Effects of rostral agranular insula cortex inactivation on food self-administration sessions under a fixed-ratio 5 (FR-5) schedule of reinforcement ($n = 12$). Data are expressed as means (± SEM) of the number of lever presses (A) or food pellets earned (B) after either an infusion of baclofen/muscimol (INACT) or saline (VEH) into the insula; or during the average of the two normal baseline self-administration sessions on the days prior to each testing session (BSL).
3.5 Discussion

The results presented here demonstrate that inactivation of the RAIC is capable of attenuating operant responding for nicotine and thus the amount of nicotine obtained under a FR-5 schedule of reinforcement. RAIC inactivation is also capable of attenuating nicotine seeking behavior reinstated by a nicotine-associated light cue. Yet RAIC inactivation demonstrated no effect on operant responding for food pellets, even when under a FR-5 schedule of reinforcement. Overall, these results are similar to our prior findings with CGIC inactivation (Forget et al., 2010a) and suggest that the RAIC may play a similar role to the CGIC in this rodent model of nicotine self-administration.

The involvement of the RAIC in nicotine self-administration and reinstatement behaviors is not surprising considering its projections to the ventral striatum (Groenewegen et al., 1991; Groenewegen et al., 1997) and lateral nucleus accumbens (McGeorge and Faull, 1989; Wright and Groenewegen, 1996; Shi and Cassell, 1998b), along with its reciprocal connections with the basolateral amygdala and the prelimbic cortex (Shi and Cassell, 1998b; Vertes, 2004), all areas well-known within the addiction literature, including that of nicotine addiction. A single acute injection of either nicotine or its peripherally-limited analog, nicotine pyrrolidine methiodide, have been observed to induce neural activity, confirmed through c-Fos immunoreactivity, in the RAIC but not the CGIC of mice (Dehkordi et al., 2015). Yet interestingly, the α7 subtype of the nicotinic acetylcholine receptor has been found within the CGIC but not the RAIC of rats (Fuchs, 1989). When examining the incubation enhanced cue-induced reinstatement of nicotine seeking, Abdolahi and colleagues (2010) observed an associated increase in the dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), a phosphoprotein enriched in neurons containing dopamine D1-subtype receptors, specifically within the RAIC but not the CGIC of
rats (Abdolahi et al., 2010). Confirming the ability of direct RAIC manipulation to affect nicotine self-administration, Kutlu and colleagues (2013), found that bilateral infusions of D1, but not D2, antagonists into the RAIC were capable of decreasing nicotine self-administration in rats (Kutlu et al., 2013) similar to the effect found with GABA agonist-induced inactivation in the present study.

It is important to note that the involvement of the insular cortex is not limited to nicotine addiction, but appears to play a much broader role within addictive behaviors as a whole (Naqvi and Bechara, 2009; Clark, 2010; Naqvi and Bechara, 2010; van Holst et al., 2010; Noel et al., 2013; Potenza, 2013; Naqvi et al., 2014). Within animal studies involving insular cortex manipulations, studies have demonstrated both a role for the CGIC (Contreras et al., 2007; Hollander et al., 2008; Forget et al., 2010a; Pushparaj et al., 2013; Wu et al., 2014) or RAIC (Di Pietro et al., 2006; Di Pietro et al., 2008; Ishii et al., 2012; Kutlu et al., 2013; Pelloux et al., 2013; Seif et al., 2013) or both (Scott and Hiroi, 2011; Contreras et al., 2012) in addiction relevant behaviors. To our knowledge, only two studies thus far have compared manipulations of the CGIC vs. RAIC and found differential involvement in addiction-relevant behaviors. Cosme and colleagues (2015) have demonstrated that inactivations of the RAIC, but not inactivations of the CGIC, attenuated cue-induced reinstatement of cocaine seeking behavior in rats (Cosme et al., 2015). Our own unpublished observations have demonstrated a role of the RAIC, but not the CGIC, in a rodent model of decision-making behavior similar to the Iowa Gambling Task (Pushparaj et al., 2015b). Future work should be conducted to directly compare the relative involvements and the specific connections of the RAIC vs. the CGIC in addictive behavior.

The insular cortex, starting from the most caudal granular subregion and moving rostral to culminate in the RAIC, appears to integrate peripheral sensory inputs relevant to
motivation/affect with inputs from other related brain areas (Shi and Cassell, 1998a; Shi and
Cassell, 1998b) in order to form a complete picture of the current homeostatic state, a function
termed interoception by AD Craig (Craig, 2002; Craig, 2003; Craig, 2004; Craig, 2010b).
Importantly, the RAIC appears to be critically involved in comparing the current homeostatic
state to that of prior states in order to motivate and guide behavior (Craig, 2009b; Craig, 2009a;
Craig, 2011). Thus, it is not surprising to find that both the CGIC and RAIC appear to be
concurrently involved in many behaviors, including several of those underlying nicotine
addiction. In the case of the present study, it could be posited that both the integrating capacity
of the CGIC and the comparison capacity of the RAIC are critical to nicotine self-administration
and seeking behaviors, but interestingly not food self-administration behavior.

Of importance with regards to the aspect of food self-administration, it should be noted
that the rats utilized in both the present study and our previous work (Forget et al., 2010a;
Pushparaj et al., 2013) were maintained with food restrictions (~20 g/day) and that the insular
cortex has been demonstrated to be involved in food-related behaviors which are motivated by
aspects apart from hunger (Balleine and Dickinson, 2000; Parkes and Balleine, 2013; Kusumoto-
Yoshida et al., 2015). Neither our present study nor our prior work has demonstrated effects on
any aspects of food self-administration behaviour with either RAIC or CGIC inactivations.
Importantly, under the same food restriction conditions (~20 g/day), Cosme and colleagues
(2015) also demonstrated no effect of RAIC inactivation, also using baclofen/muscimol
infusions, on cue-induced, pellet priming-induced or cue + pellet priming-induced reinstatement
of food seeking behavior. Thus, we do not believe that the RAIC is involved in food-related
behaviour under these conditions of food restriction.
Finally, two important limitations must be stated with regards to the specificity and generalizability of the current findings. Firstly, the possibility exists that the baclofen/muscimol mixture significantly diffused away from the sites of interest though the 0.5 μl infusion volume was chosen in order to limit this possibility. However, given the similarity in findings between the current study and our previous work (Forget et al., 2010a), and without a direct method of demonstrating the extent of diffusion, we cannot rule out the possibility that the actual region of the insula critical to the behaviors examined lies in between the RAIC and CGIC.

Secondly, whether these insular subregion specific findings in rats are translatable to humans is questionable. AD Craig has noted that some of the subregion specific functionality present in the human and primate insula do not exist in lower species (Craig, 2011), particularly with regards to hemispheric differences. That being said, there is some evidence to suggest that such hemispheric differences exist with regards to the critical nature of the insula in smoking behaviour (Naqvi and Bechara, 2010). To our knowledge, no such hemispheric differences have been identified in the rat insula, nor have we found any study explicitly examining this possibility. Overall, it is yet unclear the degree to which differences observed in RAIC vs. CGIC functionality in the rat are meaningful with regards to human research, and vice versa.

The current study demonstrates that inactivation of the RAIC results in an attenuation of nicotine self-administration and the reinstatement of nicotine-seeking behavior but not food self-administration behavior. Our prior work demonstrated similar findings with inactivation of the CGIC. Approaches to modulating the function of brain regions have arisen in recent years, including deep brain stimulation (DBS), transcranial direct current stimulation (tDCS) and repetitive transcranial magnetic stimulation (rTMS). Critically, a prospective, randomized control trial has recently established deep brain rTMS of both the insular and prefrontal cortices
as a promising potential treatment strategy for smoking cessation (Dinur-Klein et al., 2014); however, further studies should be performed using novel TMS coil designs capable of more selectively targeting the insular cortex alone. Thus the present findings support further research utilizing animal models, human laboratory models and clinical trials to evaluate the smoking cessation potential of therapeutics capable of modulating insular cortex function in smokers.
CHAPTER 4. Electrical Stimulation of the Insular Region Attenuates Nicotine-Taking and Nicotine-Seeking Behaviors


Based on Neuropsychopharmacology, 2013

Pushparaj A refined methodological aspects of the experiments with guidance from Hamani C, Nobrega JN and Le Foll B who originally conceived of the experiments. Pushparaj A was responsible for animal training and feeding as well as all surgical procedures with guidance on intracranial electrode implantation from Hamani C. Yu W and Shin DS were responsible for the conduct and analysis of the electrophysiological work. Pushparaj A performed all other statistical analysis and wrote the manuscript, apart from the respective sections on electrophysiology. All authors were involved in responding to reviewer comments and making necessary revisions to the manuscript.

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4.1 Abstract

Pharmacological inactivation of the granular insular cortex is able to block nicotine taking and seeking behaviors in rats. In the present study, we explored the potential of modulating activity in insular region using electrical stimulation. Animals were trained to self-administer nicotine (0.03 mg/kg/infusion) under a fixed ratio-5 (FR-5) schedule of reinforcement followed by a progressive ratio (PR) schedule. Evaluation of the effect of stimulation in the insular region was performed on nicotine self-administration under FR-5 and PR schedules, as well on reinstatement of nicotine-seeking behavior induced by nicotine-associated cues or nicotine priming injections. The effect of stimulation was also examined in brain slices containing insular neurons. Stimulation significantly attenuated nicotine taking, under both schedules of reinforcement, as well as nicotine seeking behavior induced by cues and priming. These effects appear to be specific to nicotine-associated behaviors, as stimulation did not have any effect on food taking behavior. They appear to be anatomically specific, as stimulation surrounding the insular region had no effect on behavior. Stimulation of brain slices containing the insular region was found to inactivate insular neurons. Our results suggest that deep brain stimulation to modulate insular activity should be further explored.
4.2 Introduction

Tobacco-related diseases are a major population health issue resulting in 5 million deaths per year worldwide with this number expected to grow to 10 million by 2025 (Proctor, 2004). Though several pharmacotherapies are currently available for smoking cessation, there is still a relatively high rate of relapse among individuals motivated to quit (Rigotti, 2002; Le Foll and George, 2007). This relapse rate stresses the need for the discovery of novel treatments targeting both a reduction in smoking behavior and the relapse rate.

The insula has been the object of considerable recent interest both in the general sense of its overall role in the brain (Craig, 2009b; Craig, 2010a) as well as its specific role in the neurocircuitry of addiction (Koob and Volkow, 2010). Recent findings demonstrated a correlation between stroke-induced insular damage and a disruption of tobacco addiction, characterized by the ease with which immediate smoking cessation was achieved without craving or relapse (Naqvi et al., 2007). While this effect of insular damage was not observed in another publication (Bienkowski et al., 2010), others have gone as far as to suggest that unintentional abrupt smoking cessation may be a unique lesion localizer (Hefzy et al., 2011). Subsequent work utilizing animal models have shown insular involvement in different aspects of addictive behavior for various addictive substances (Contreras et al., 2007; Hollander et al., 2008; Forget et al., 2010a; Scott and Hiroi, 2011; Contreras et al., 2012). However, even prior to the findings by Naqvi and colleagues, numerous functional imaging studies reported insular activation during subjective drug urges (Naqvi and Bechara, 2009).

Previous work by our group demonstrated that reversibly inactivating the dorsal granular region of the insular cortex in rats, by local infusion of a baclofen/muscimol mixture, decreased nicotine self-administration under both fixed ratio (FR) and progressive ratio (PR) schedules of
reinforcement and also decreased the reinstatement of nicotine seeking behavior induced by a nicotine-associated cue or a priming injection of nicotine (Forget et al., 2010a). Importantly, this inactivation had no effect on food taking behavior assessed as a control.

Deep brain stimulation (DBS) is currently being examined for the treatment of various psychiatric disorders (Krack et al., 2010; Ward et al., 2010; Holtzheimer and Mayberg, 2011; Goodman and Alterman, 2012), including addiction (Halpern et al., 2011; Luigjes et al., 2012). Several reports have established the potential of DBS in the region of the nucleus accumbens (NAcc) in human subjects for the treatment of heroin (Zhou et al., 2011a), alcohol (Kuhn et al., 2007; Muller et al., 2009; Kuhn et al., 2011), and nicotine (Kuhn et al., 2009; Mantione et al., 2010). Animal studies have corroborated these findings with DBS of the NAcc shell attenuating alcohol taking (Knapp et al., 2009; Henderson et al., 2010) and cocaine seeking (Vassoler et al., 2008), while DBS of NAcc core has been shown to attenuate alcohol taking (Knapp et al., 2009) and morphine-induced conditioned place preference (Liu et al., 2008a). Animal studies have also demonstrated an attenuation of cocaine taking with DBS applied to the subthalamic nucleus (Rouaud et al., 2010), the medial prefrontal cortex (Levy et al., 2007) and the lateral habenula (Friedman et al., 2010), and an attenuation of cocaine seeking with DBS applied to the lateral hypothalamus (Levy et al., 2007; Hamani and Temel, 2012). To our knowledge, the effects of DBS in an animal model of nicotine abuse have not yet been investigated.

Here, we evaluated the effects of electrical stimulation in the insular region on nicotine self-administration behavior, under both FR and PR schedules, as well as nicotine seeking behavior reinstated by nicotine-associated cue presentation or nicotine priming injection. We also evaluated the effects of electrical stimulation on food self-administration behavior, as a
control. Finally, we examined the effect of stimulation on brain slices containing insular neurons.

4.3 Materials and Methods

Naïve male Long Evans rats initially weighing 250–275 g were used for all nicotine self-administration work. Rats were individually housed in a temperature-controlled environment on a 12-hour reverse light/dark cycle (lights off from 9:00 am to 9:00 pm) and received 20 g of food pellets with unlimited water access. Naïve male Sprague Dawley rat pups (P23-24) were used for the electrophysiology work. All experimental procedures described were carried out in compliance with the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the local Animal Care Committees.

Initial training procedures and surgical techniques for nicotine self-administration were similar to those previously reported (Forget et al., 2010a; Forget et al., 2010b; Le Foll et al., 2011; Gamaleddin et al., 2012; Yan et al., 2012). Animals were initially trained on a schedule in which each lever press resulted in the delivery of a 45-mg food pellet (continuous reinforcement, no cues associated with food delivery). Once trained, each animal was surgically prepared with a chronic IV catheter implanted in the jugular vein; the catheters exited between the scapulae. Surgery was performed under anesthesia induced by xylazine (10 mg/kg IP) and ketamine hydrochloride (75 mg/kg, IP). Incision sites were infiltrated with the local anesthetic bupivacaine (.125%). Buprenorphine was given for postoperative analgesia (.01 mg/kg SC), and a single dose of penicillin (30,000 U, IM) was administered before surgical procedures. Animals were allowed to recover for a 1-week period before drug self-administration (SA) began.

Drugs
Nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, Missouri) was dissolved in saline, the pH was adjusted to 7.0 (± .2), and the solution was filtered through a .22-mm syringe filter (Fisher Scientific, Pittsburgh, Pennsylvania) for sterilization purposes. All nicotine doses are reported as free base concentrations. Nicotine was administered IV in a volume of 100 μL/kg/injection or SC in a volume of 1 mL/kg.

Acquisition of the Nicotine or Food SA

The SA sessions were carried out in experimental chambers equipped with two levers (Med Associates, St. Albans, Vermont). The start of the session was signaled by illumination of a house-light; switching off this light indicated the time-out period during which time lever responding was recorded but had no consequences. Rapid delivery of the self-administered drug (1-sec delivery time) was achieved with Med Associates Model PHM-104 pumps. Unit doses were 100 μL/kg; volume adjustments were used to accommodate inter-animal or between-session differences in body weight. Responding on one of the levers (active) resulted in drug delivery when schedule requirements were met, whereas responding on the other lever was recorded but did not produce any change of lights or drug infusion (active levers were counterbalanced). The SA sessions occurred 7 days/week.

In this study, rats acquired nicotine SA under an FR schedule of reinforcement, and the unit dose was 30 μg/kg/infusion of nicotine, expressed as base. Session duration was 60 min, and the time-out period (switching off the house light and illumination of a cue light above the active lever) after each infusion was 1 min. During the first 5 days of acquisition, each lever press during the time-in period resulted in the delivery of an infusion (FR-1); the response requirement was then increased to FR-2 for 3 days and finally to FR-5 (i.e., animals were required to make five lever presses for each drug infusion) for 7 days.
The apparatus, the stimuli associated with food delivery, and the schedule of the acquisition for the food SA experiments were exactly the same as described above, except that the rats received a food pellet (45-mg precision pellets; Bioserv, Laurel, Maryland) instead of a nicotine injection.

Electrode implantation

After the acquisition phase of the nicotine SA, stereotaxic surgeries to implant electrodes were done under the same regimen of anesthetics, analgesic, and antibiotic described above. Insulated stainless steel electrodes (125 μm diameter with 0.5 mm exposed surface; Plastics One, Roanoke, VA) were bilaterally implanted, at a 10° divergent angle from the vertical, with the exposed surface within the histological boundary of the granular insular cortex (surgical coordinates: anteroposterior −.40 mm, lateral ± 4.8 mm, and dorsoventral +6 mm; (Paxinos and Watson, 1986). Similar electrodes attached to small screws threaded partially into the skull were used as anodes. Rats were allowed 1 week to recover before the reacquisition of the nicotine SA under an FR-5 schedule of reinforcement.

Electrical Stimulation

Stimulation was conducted with a portable device (St Jude Medical model 3510, Plano, TX), connected to the animals through extension cables to a multi-channel commutator (Plastics One, Roanoke, VA). Animals were stimulated at 100 μA, 90 μs of pulse width, and 130 Hz. These settings are similar to previous publications both from our laboratory (Hamani et al., 2010; Hamani et al., 2012) and others (see introduction). The Sham condition consisted of the animals being connected to the stimulation equipment but not receiving any stimulation. The testing of stimulation and sham conditions were counterbalanced in all experiments. In all testing, animals
were stimulated 5 min before and throughout the duration of the test session (i.e. usually 60 min, except for PR sessions which lasted 4 hrs).

**Testing Under FR**

One week after electrode implantation, the nicotine or food SA under the FR-5 schedule of reinforcement was re-established for all rats until stabilization. Rats were considered to have acquired stable SA when they pressed the active lever more than twice the number of times they pressed the inactive lever and received a minimum of 10 reinforcements during 1-hour session with <20% variation in the number of reinforcements earned per session during two consecutive sessions.

Two groups of rats (n=12 for nicotine SA, and n=9 for food SA) were tested under the FR-5 schedule of reinforcement with stimulation of the insular region or sham in a counterbalanced fashion. Animals were required to achieve stable SA criteria (described above) between testing sessions. Animals were also tested in a session where nicotine was substituted with saline (saline substitution condition) as a positive control measure.

**Testing Under PR**

After the testing under an FR-5 schedule of reinforcement the nicotine group (n=11) was switched to a PR schedule wherein the response requirement increased with each successive reinforcement. A separate group was trained and tested under a PR schedule for food SA (n=9). The response requirement progression was based on the formula $5e^{0.25*(\text{inj. number} + 3)} - 5$, with the first two values replaced by 5 and 10 (modified from (Roberts, 1992). Thus, the response requirements for successive reinforcements were 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, and so forth. The break point (BP) was defined as the highest ratio completed before the first 30-
min period without a response on the active lever. The PR sessions lasted a maximum of 4 hours. The animals were allowed 10 days of SA on the PR schedule before testing began. Animals were once again tested with stimulation of the insular region or sham in a counterbalanced fashion, plus a saline substitution condition for the nicotine SA group.

**Extinction**

After completion of testing on nicotine SA under FR and PR schedules, rats continued on their nicotine SA sessions under a FR schedule for a minimum of five additional sessions to re-establish a stable baseline level of responding for three consecutive sessions. This was followed by an extinction phase that was conducted by withholding nicotine and its associated cues (the house light remained on during the whole session and there was no presentation of the nicotine-associated cues). Responses on the active or inactive lever were recorded but had no consequences. The criterion for extinction was <20 active lever presses per 1-hour session over two consecutive sessions.

**Cue-Induced Reinstatement of Nicotine Seeking**

Animals were then tested for the effect of stimulation of the insular region or sham stimulation on cue-induced reinstatement in a counterbalanced, within-subject design (n=10). Testing days were separated by at least three extinction sessions with a stable extinction responding (under the criteria for extinction) over two consecutive sessions. Reinstatement tests were conducted under conditions identical to that of SA, except that (1) a single presentation of the visual cue (light above the active lever on and house-light off for 60 sec) was delivered response-independently immediately at the start of the session, and (2) responses on the active lever (under an FR-5 schedule) resulted in contingent presentation of the cues (light above the active lever on and
house-light off for 60 sec) without nicotine availability (no infusions). Responses on the inactive lever were recorded but were without consequence. The testing sessions lasted 1 hour.

Nicotine-Induced Reinstatement of Nicotine Seeking

After the cue-induced testing, responding was again extinguished according to the same criteria and subsequently animals were also tested for the effect of stimulation of the insular region or sham stimulation on nicotine-induced reinstatement in a counterbalanced, within-subject design (n=8).

Testing days were separated by at least three extinction sessions with a stable extinction responding over two consecutive sessions. Nicotine priming consisted of a 0.15 mg/kg SC injection of nicotine 10 min before the test session.

Histological Procedures

After completion of behavioral testing, rats were overdosed with pentobarbital (approximately 350 mg/kg, IP). Brains were removed, frozen in methylbutane, and coronal serial sections (15-μm-thick) were stained with cresyl violet for determination of electrode placements. Acceptable histology required that the tip of the electrode lie within the insular region (i.e. granular and agranular subregions) on both sides of the brain. It should also be noted that we utilize the term insular region as we cannot be sure of the exact areas influenced by stimulation, not because we are unaware of the location of electrode tips.

Electrophysiology

The procedure for obtaining brains slices and conducting electrophysiology are described elsewhere (Shin et al., 2007; Hamani et al. 2011). In brief, male Sprague Dawley rat pups (P23-
24) were anesthetized with isoflurane and decapitated. Brains were sliced 300 µm thick in the coronal orientation using a vibratome (VT 1200S, Leica, IL, USA) in chilled dissecting solution containing (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 24 NaHCO₃, 25 glucose and 75 sucrose (oxygenated with 95% O₂/5% CO₂).

Prior to recording, slices were incubated at room temperature for 1 hour in artificial cerebrospinal fluid (aCSF) which contained (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 1 CaCl₂, 25 NaHCO₃, and 10 glucose (oxygenated as above) at a pH of 7.4. To obtain whole-cell patch-clamp recordings, the slice was placed in a RC-26 open bath recording chamber (Warner Instruments, CT, USA) and perfused with aCSF at 2-3 ml/min, aerated and pH balanced with 95%O₂/5%CO₂ at 32.5 to 34°C. Insular neurons were visualized under infrared differential interference contrast (IR-DIC) optics at 40x objective magnification with an Olympus BX51WI upright microscope (Olympus Optical, NY, USA). Whole-cell patch-clamp electrodes were pulled from borosilicate capillaries (World Precision Instruments, FL, USA) and filled with intracellular solution containing (in mM): 110 K-gluconate, 8 NaCl, 20 KCl, 1 MgCl₂, 0.0001 CaCl₂, 10 Na-HEPES, 2 Na-ATP, 0.3 Na-GTP, pH of 7.4. Whole-cell patch-clamp recordings were obtained using an Axopatch 200B (Molecular Devices, CA, USA) in the I-clamp mode, low-pass filtered at 5 kHz and digitized at 10 kHz with a Digidata1440A (Molecular Devices, CA, USA). Signals were then analyzed offline with Clampfit 10 (Molecular Devices, CA, USA).

Insular neurons were stimulated using a 125 µm diameter biconcentric electrode (FHC, ME, USA) that was positioned ≤150 µm from the recorded neuron. Stimulation was monophasic and applied for 30-120 seconds and set to 130 Hz, 60-90 µsec in pulse width and at 100-300 µA
of current with a Grass S48 stimulator (Grass Instruments, MA, USA) that was coupled to a
PSIU6 current isolation unit (Grass Instruments, MA, USA).

Data Analysis

Only rats with correct bilateral placement of the electrodes in the insular region were included
for data analysis (n=12; Figure 11 - left panel) while those with incorrect placements were
separately analyzed as anatomical controls (n=7; Figure 11 - right panel). For all testing,
relevant data (infusions, pellets, or lever responding) were analyzed using one-way repeated
measures (RM) analysis of variance (ANOVA) with Bonferroni comparisons as a post hoc test.
Figure 11. Histological reconstruction of electrode placements in the insula. Black dots indicate locations of electrode tips from the animals with correct bilateral placement (left panel) and those with incorrect placement utilized as anatomical controls (right panel). The numbers in between each reconstructed image indicates the distance (in millimeters) from bregma. Schematic figure was published in *The Rat Brain in Stereotaxic Coordinates*, (Paxinos and Watson, 1986). GI, granular insula; DI, dysgranular insula; AIP, posterior agranular insula.
4.4 Results

*Effect of Stimulation on Nicotine and Food SA under a FR5 schedule*

The one-way RM ANOVA performed on the number of infusions obtained during the sessions (Fig. 12A) showed a significant effect of treatment \([F(3,11)=12.35, p <.0001]\), and Bonferroni comparisons indicated that stimulation of the insular region during the session or substitution of nicotine by saline both significantly reduced the number of infusions compared with sham stimulation \((p < .05 \text{ stim vs. sham}; p <.001 \text{ saline vs. sham})\). Comparisons also revealed that infusions during sham sessions were not significantly different from baseline \((p >.05, \text{ sham vs. baseline})\) and that saline substitution resulted in significantly fewer infusions compared to stimulation \((p <.01, \text{ stim vs. saline})\).

The one-way RM ANOVA performed on the number of pellets obtained during the food SA sessions (Fig. 12B) showed no significant effect of treatment \([F(2,8)=3.28, p >.05]\).
Figure 12. Effects of stimulation of the insular region on nicotine (A) or food (B) self-administration under a fixed ratio-5 schedule of reinforcement (n=12 and 9, respectively). Data are expressed as means (± SEM) of the number of injections or food pellet delivery during regular self-administration (Baseline), animals being connected to the stimulator but not stimulated (Sham), stimulation of the insular region (Stim), or the substitution of nicotine infusions with saline (Saline). The Sham and Stim treatments were counterbalanced in both experiments. *p < .05; ***p < .001 vs. Sham; Bonferroni multiple comparisons following repeated-measures one-way ANOVA.
The one-way RM ANOVA performed on the number of infusions (Fig. 13A) obtained during the sessions by animal with misplaced electrodes (Fig. 13B) showed a significant effect of treatment \([F_{(3,6)}=15.78, p < .0001]\). However, Bonferroni comparisons indicated that stimulation in the insular region during the session had no significant effect while substitution of nicotine by saline significantly reduced the number of infusions compared with sham (p > .05 stim vs. sham; p < .001 vs. saline vs. sham).

Stimulation of the insular region significantly decreased nicotine self-administration but not food self-administration, while stimulation outside this region had no effect.
FIGURE 13

Figure 13. Effects of stimulation of areas surrounding the insular region on nicotine self-administration under a fixed ratio-5 schedule of reinforcement (n=7). Data are expressed as means (± SEM) of the number of infusions during regular self-administration (Baseline), animals being connected to the stimulator but not stimulated (Sham), stimulation of the insular region (Stim), or the substitution of nicotine infusions with saline (Saline). The Sham and Stim treatments were testing in a counter-balanced manner. *p < .05; ***p < .001 vs. Sham; Bonferroni multiple comparisons following repeated-measures one-way ANOVA.
Effect of Stimulation on Nicotine and Food SA under a PR schedule

The ANOVA performed on the number of nicotine injections that the rats received before 30 min of inactivity (BP; Fig. 14A) showed a main effect of treatment \( [F_{(3,10)}=14.79, p < .0001] \) and group comparisons indicated that stimulation of the insular region or substitution of nicotine by saline significantly reduced the BP compared with sham \( (p < .01 \text{ stim vs. sham}; p < .001 \text{ saline vs. sham}) \). BPs during sham sessions were not significantly different from baseline \( (p > .05 \text{ sham vs. baseline}) \) but saline substitution resulted in significantly lower BPs compared to stimulation \( (p < .05 \text{ stim vs. saline}) \).

The one-way RM ANOVA performed on the number of pellets obtained during the food SA sessions (Fig. 14B) showed no significant effect of treatment \( [F_{(2,8)}=3.93, p > 0.05] \).
Figure 14. Effects of stimulation of the insular region on nicotine (A) or food (B) self-administration under a progressive ratio schedule of reinforcement (wherein the response requirement increased with each successive injection or food pellet delivery; n=11). Data are expressed as means (± SEM) of the number of injections (break point, left y axis) and of the last ratio completed (in number of lever presses, right y axis) during regular self-administration (Baseline), animals being connected to the stimulator but not stimulated (Sham), stimulation of the insular region (Stim), or the substitution of nicotine infusions with saline (Saline). The Sham and Stim treatments were counterbalanced in both experiments. **p < .01; ***p < .001 vs. Sham; Bonferroni multiple comparisons following repeated-measures one-way ANOVA.
Effect of Stimulation on Nicotine-associated Cue-Induced Reinstatement of Nicotine Seeking

The ANOVA performed on the active lever presses indicated a main effect of treatment \([F_{(2,9)}=21.54, p < .001]\). Post hoc analyses showed that the cue presentation during the sham session induced a significant reinstatement of the presses on the active lever \((p < .001\) sham vs. extinction) and that stimulation in the insular region significantly decreased this cue-induced reinstatement \((p < .05\) stim vs. sham; Fig. 15A).

Effect of Stimulation on Nicotine-Induced Reinstatement of Nicotine Seeking

The ANOVA performed on the active lever presses indicated a main effect of treatment \([F_{(2,9)}=17.03, p < .001]\). Post hoc analyses showed that nicotine priming prior to the sham session induced a significant reinstatement of the presses on the active lever \((p < .001\) sham vs. extinction) and that stimulation of the insular region significantly decreased this cue-induced reinstatement \((p < .05\) stim vs. sham; Fig. 15B).
Figure 15. Effect of stimulation of the insular region on (A) nicotine-associated cue- (single non-contingent presentation at session start and subsequently or (B) nicotine priming-induced (.15 mg/kg SC, 10 min before the session) reinstatement of nicotine seeking tests after extinction (n=8 and 6, respectively). Data are expressed as means (± SEM) of the number of lever presses during extinction conditions (Extinction) and during sessions with animals being connected to the stimulator but not stimulated (Sham) or stimulation of the insular region (Stim). The Sham and Stim treatments were counterbalanced in both experiments. *p < .05 vs. Sham; Bonferroni multiple comparisons following repeated-measures one-way ANOVA.
Effect of Stimulation on Inactive Lever Presses

The ANOVAS performed for each experiment showed no significant effect of treatment on inactive lever responding (data not shown) indicating the effect of stimulation was specific to active lever responding.

Electrophysiology

Neurons in the insular cortex (Fig. 16A) had a resting membrane potential of -71.7 ± 1.8 mV (n=8), which is consistent with values described elsewhere (Stone et al., 2011). At rest and before stimulation, the cells do not exhibit spontaneous spiking activity in vitro. When high frequency stimulation (HFS) was applied to these cells, all of them initially exhibited hyper-excitability and fired bursts of action potentials, but then became quiescent during the stimulation period and returned to pre-HFS resting membrane potentials after stimulation was turned off (post-HFS= -69.7 ± 3.1 mV; n=8; p=0.646). Interestingly, we observed 2 different inactivating responses of insular neurons to HFS. In 3 neurons, HFS induced a burst of action potentials, which quickly inactivated concurrently with a rapid repolarization of the resting membrane potential; the inactivation of spike activity occurred within 1 second of HFS even though stimulation remained on (Fig. 16B). In contrast, 5 other recorded neurons became quiescent from HFS by undergoing a depolarization block phenomenon (Fig. 16C). These cells depolarized by 29.0 ± 2.9 mV during HFS and maintained this potential throughout the stimulation period. After the stimulation was terminated, insular neurons repolarized to pre-HFS values. All of these cells however had similar resting membrane potentials (-72.3 ± 1.0 mV for fast inactivating, -71.3 ± 1.9 mV for depolarization blocked cells, p=0.813), morphology under IR-DIC, regular spiking activity with current injection and input resistance (166.7 ± 18.7 mΩ for fast inactivating, 174.4 ± 28.7 mΩ for depolarization blocked cells, p=0.910; Fig. 16A).
Figure 16. Electrophysiological responses of insular neurons to electrical stimulation. (A) Responses to 500 pA negative and positive current injection to insular neurons show regular spiking activity at depolarized potentials. These cells were identified and recorded using IR-DIC optics and a representative neuron is shown on the right. The black asterisk represents an insular neuron whereas the red asterisk represents the position of the stimulating electrode. When HFS (130 Hz, 60 to 90 μsec, 30-120 seconds) was applied to insular neurons, all of these neurons became quiescent. However, the inactivation was induced by fast-inactivation in 3 cells (B) and by a depolarization block in 5 others (C). Traces represent a sample size of 8 insular neurons.
4.5 Discussion

The results of this study demonstrate that bilateral electrical stimulation of the insular region significantly decreases nicotine SA under two schedules of reinforcement and attenuates the reinstatement of nicotine seeking behavior induced by either nicotine-associated cues or nicotine priming injections. In contrast, there was no effect of stimulation on lever pressing for food self-administration and no effect of stimulation of the area surrounding the insular region on nicotine SA under an FR-5 schedule of reinforcement.

The decreases observed in both nicotine-taking and nicotine-seeking are similar to the effects noticed with the inactivation of the granular insular cortex using a baclofen/muscimol mixture (Forget et al., 2010a). This result suggests that a functional target inactivation may be one potential result of high frequency stimulation (HFS) in the insular region. The observed electrophysiological effects provide some correlative evidence in support of this suggestion, as all neurons examined showed inactivation, although two different responses were observed. At present, it is uncertain whether the two responses may to be related to two different populations of neurons. Given that all of these cells had similar resting membrane potentials, morphology under IR-DIC, regular spiking activity with current injection and input resistance, we speculate that the same type of insular neurons can respond to HFS by becoming inactive in different ways, depending on their ion channel composition. For instance, differences in the sensitivity of delayed inward-rectifying K\textsuperscript{+} (K\textsubscript{Dir}) channels or Ca\textsuperscript{2+}-dependent BK K\textsuperscript{+} channels to voltage potentials could underlie the fast inactivating responses. Conversely, the cells that underwent a depolarization block might have become quiescent due to the inactivation of Na\textsuperscript{+} voltage-gated channels. These neurons required longer durations of HFS to elicit this response. Further work
would be required to examine the respective ion channel compositions of the two neuronal populations observed here.

It must be noted that although decreases in nicotine SA and reinstatement were observed both in our previously published pharmacological inactivation of the granular insular cortex and in the electrical stimulation conducted here, the magnitude of this decrease appears to differ in the two cases. This suggests that the effect of stimulation in the insular region, specifically at the parameters conducted in this study, may not produce the same functional inactivation as baclofen/muscimol infusions. It should be noted that although the electrophysiology data suggest that insular inactivation is a potential result of in vivo stimulation in the region, it cannot be determined whether this is the actual mechanism occurring in our behavioral effects. Other brain areas may also have been influenced by high frequency stimulation and the behavioral effect observed may be consequent to changes at a distance from the stimulated target and not merely local inhibition (McCracken and Grace, 2007).

The neurocircuitry underlying the insula’s involvement in addiction has only recently begun to be explored. The granular insular cortex’s primary function is to map affective bodily feelings, specifically those critical to homeostasis and survival (Craig, 2009b), which include the bodily sensations produced by nicotine. The insula has interconnections with the amygdala (Allen et al., 1991), the orbitofrontal cortex (OFC) and the ventromedial prefrontal cortex (vmPFC; Hurley et al., 1991) which also receive dopaminergic input from the ventral tegmental area that can be released by nicotine’s central effects. The theory proposed by Naqvi and Bechara (2010) is that both the central and peripheral effects of nicotine are involved in the conscious pleasure produced by smoking. Smoking-related cues are posited to result in interconnections from the amygdala and OFC/vmPFC triggering a representation in the insula of the bodily sensations
produced by nicotine and subsequently resulting in insular projections to the nucleus accumbens
motivating drug-seeking behavior (Naqvi and Bechara, 2010). It is through these two pathways
that the insula is posited to be involved in both nicotine-taking and -seeking behaviors.
However, it should be noted that recent evidence suggests the lateral habenula may also be
involved in conveying information from the anterior insular cortex to midbrain monoaminergic
centres (Kim and Lee, 2012).

Limited work has been done to identify specific receptor populations or molecular
changes in the insula associated with nicotine self-administration. Antagonism of hypocretin-1
receptors present on insular neurons has been demonstrated to decrease nicotine taking
(Hollander et al., 2008) in a rodent self-administration model. Additionally, incubation of
nicotine seeking has been associated with enhanced protein kinase A-regulated signaling of
dopamine- and cAMP-regulated phosphoprotein of 32 kDa in the insula suggesting that
amplified dopaminergic signaling in this area is critical to the incubation (Abdolahi et al., 2010).
This is of significant interest as the insula has been noted as an extra-striatal site of unusually
high dopamine transmission (Jones et al., 1986). Further work should be conducted to better
understand the various receptor populations and molecular changes involved in the insula’s role
in nicotine self-administration.

Regardless of the underlying neurocircuitry involved, the fact that electrical stimulation
in the insular region attenuates behaviors relevant to nicotine abuse is of considerable interest
both from a basic and from a clinical perspective. Deep brain stimulation is a technique that is
widely used in Parkinson’s Disease (Bronstein et al., 2011) and a few groups have reported
positive effects of DBS of the nucleus accumbens for smoking cessation (Kuhn et al., 2009;
Mantione et al., 2010). Other potential methods for electrically modulating insular activity, such as repetitive transcranial magnetic stimulation (rTMS), should also be considered.

The role of the insula in addiction has only recently begun to be explored, yet the findings thus far point towards it being a crucial structure for different addictive behaviors across various addictive substances. More work is required to uncover other potential treatments, pharmacological or otherwise, which may target this critical brain area.
CHAPTER 5. General Discussion

5.1 Summary of Critical Findings

The work presented here was conducted to investigate the effects of modulating activity in two insular subregions, the CGIC and RAIC, through the use of multiple modalities (chemical lesions, pharmacological inactivation or electrical stimulation), across a variety of behaviours relevant to addictive disorders. A greater understanding of the addiction-specific role of the lower-order, sensory-focused CGIC in comparison to the downstream, cognition and affect-focused RAIC, will allow us to determine how best to target these insular cortex subregions, individually or together, for various behavioural aspects of addictive disorders.

To this end, we pursued the following step-wise objectives:

Objective 1: Understanding the role of the insular cortex in decision-making under risk would allow us to determine whether therapeutically targeting this region can be expected to result in adverse effects (eg. increase risky decision-making), additional positive impacts (eg. decreased risky decision-making), or no significant clinically-relevant impact on behaviour. In addition, our particular focus on the individual subregions, CGIC vs. RAIC, was to determine whether the lower-order sensory-focused CGIC would also be involved in addiction-relevant behaviour not previously reinforced by addictive substances, or whether only the higher-order cognitive and affect-focused RAIC would mediate said behaviours.

Objective 2: Having clearly established differential roles of the CGIC vs. RAIC in decision-making behaviour, we sought to determine whether such differences were also present in behaviours reinforced by addictive substances. Already having established the critical role of the CGIC across nicotine-taking and seeking behaviours (Forget et al., 2010a), we repeated the same experimental protocol to determine whether pharmacologically inhibiting the RAIC would produce similar or differential effects as that of the CGIC.

Objective 3: Having demonstrated the involvement of both subregions of the insular cortex in substance-based addictive disorders (Forget et al., 2010a; Pushparaj et al., 2015a), the next obvious objective was to explore the potential of inhibiting insular cortex activity as a therapeutic approach for addictive disorders. That said, direct brain cannulation cannot be
translated to the clinical situation, thus we utilized a viable therapeutic approach, DBS targeting the CGIC, and examined the effects on drug taking and seeking behaviours.

I will summarize here the critical findings of each study before moving on to address the validity and limitations of the methodological approaches used and thus the questions that may arise in deciding whether to move these findings forward into human subjects.

5.1.1 Critical Findings from "Differential Involvement of the Agranular vs. Granular Insular Cortex in the Acquisition and Performance of Choice Behaviour in a Rodent Gambling Task"

Having known from existing literature that the insular cortex played a role in decision-making under risk we conducted this study to better understand whether therapeutically targeting this region would result in adverse effects (eg. increase risky decision-making), additional positive impacts (eg. decreased risky decision-making), or no significant clinically-relevant impact on behaviour. Our particular focus on the individual subregions, CGIC vs. RAIC, was to determine whether the lower-order sensory-focused CGIC would also be involved in addiction-relevant behaviour not previously reinforced by addictive substances, or whether only the higher-order cognitive and affect-focused RAIC would mediate said behaviours.

Lesions of the RAIC, but not the CGIC, prior to any behavioural training, resulted in the animals demonstrating increased preference for optimal (P1+P2) choices over suboptimal (P3+P4) choices when compared to sham-lesion control animals. Overall, there was also a consistently greater proportion of optimally responding animals (ie. those who made >50% optimal choice in a session) throughout the acquisition period in the group of animals who received RAIC, but not CGIC, lesions when compared to sham-lesion control animals.

Pharmacological inactivation using a mixture of GABA agonists (baclofen/muscimol) of the RAIC, but not the CGIC, following the acquisition and establishment of stable responding on
the rGT resulted in a significant change in choice behaviour when compared to bilateral local infusions of sterile saline as a control condition (within-subjects design). When examined as an overall group, RAIC inactivated animals demonstrate a significant increase in P1 choice behaviour and a significant decrease in P3 choice behaviour, resulting in an overall increase in optimal (P1+P2) choice behaviour and decrease in suboptimal (P3+P4) choice behaviour.

However, if the RAIC inactivated group of animals are further dissected into two groups, optimal vs. suboptimal, based on their choice behaviour during their vehicle control session (ie. optimal = >50% choice of P1+P2), we see differential effects of the RAIC inactivation on each group. Specifically, the optimal group of animals demonstrated a significant decrease in P2 choice with a corresponding increase in P1 choice while the suboptimal group demonstrated a significant decrease in P3 choice with corresponding significant increases in both P1 and P2 choice.

Overall, these results demonstrate the involvement of the RAIC, but not CGIC, in decision-making under risk in rodents, utilizing a task with contingencies similar to those utilized in the IGT. Thus far, I have only identified two other studies utilizing rodent models of decision-making under risk to examine the involvement of the insular cortex. One study utilized a reward-based probabilistic discounting task (St Onge and Floresco, 2010) while the other utilized a differing variation of reward-based probabilistic discounting task and a delay-based probabilistic discounting task (Ishii et al., 2012). It is important to note that both of these studies only examined the RAIC using inactivations and that they both utilized tasks in which decisions were made between two available options, where one was small/certain and the other large/risky.

St. Onge and Floresco (2010) found no significant effects of RAIC inactivation on choice behaviour in their task, while Ishii and colleagues (2012) found a significant decrease in large/risky choice behaviour in both the reward and delay-based tasks. To a degree, the present findings can be said to align with the conclusions of Ishii and colleagues (2012), though the
unique contingency structure of the rGT allows us to postulate on whether this finding is more than the simple dichotomy of decreased risk taking being "good" and increased risk taking being "bad". Such a conclusion, being based on the validity and limitations of the rGT, will be discussed further in the section below.

The findings of this study demonstrate the role of the RAIC, but not CGIC, in the addiction-relevant decision-making behaviour as observed in the rGT. With the CGIC already having been demonstrated to be involved in substance-based addictive behaviour, the lack of involvement in non-substance based behaviours is supported by its sensory-focused functionality. The involvement of the higher-order RAIC in decision-making is also understandable given its cognitive and affect-focused functionality. Yet the question of whether it would also be critically involved in drug taking and seeking behaviour remained unclear.

5.1.2 Critical Findings from "Involvement of the Rostral Agranular Insular Cortex in Nicotine Self-Administration in Rats"

Having established a differential role of the RAIC vs. CGIC in decision-making behaviour, our next objective was to identify whether the RAIC was involved in drug taking and seeking behaviour, as previously demonstrated for the CGIC.

Bilateral inactivation of the RAIC was demonstrated to cause significant attenuation of nicotine taking behaviour under the FR5 schedule of reinstatement, similar to that demonstrated by the positive control session where saline was substituted for nicotine in the drug infusion line. In a control experiment, RAIC inactivation had no significant effects on food self-administration under an FR5 schedule of reinforcement. Finally, RAIC inactivation was demonstrated to significantly attenuate the reinstatement of nicotine seeking induced by a nicotine associated light cue.
Overall, these findings appear straightforward given the cytoarchitecture of the insular cortex, which appears to consolidate information moving from caudal to rostral, with the highest order multimodal thalamic inputs being sent directly to the RAIC (Krettek and Price, 1977; Allen et al., 1991; Van der Werf et al., 2002). Given that the CGIC has limited projections to other brain regions compared to the RAIC yet has several reciprocal connections with the RAIC (Shi and Cassell, 1998a; Shi and Cassell, 1998b), it would be reasonable to assume that the findings of RAIC involvement in nicotine taking and seeking behaviour in the present work are just an extension of our prior findings of CGIC involvement.

Considering the stepwise approach taken within this thesis, the finding of this study should be examined within the broader context of the prior work. The prior study demonstrated a differential involved of RAIC vs. CGIC in decision-making under risk as assessed using the rGT (Pushparaj et al., 2015b) while the findings of the present study, when combined with our laboratories prior work examining the CGIC (Forget et al., 2010a), suggest that there is a similar behavioural phenotype regardless of whether RAIC or CGIC are inactivated in nicotine taking and seeking rodent models. These findings raise the question as to the potential explanation for differential involvement of the CGIC in nicotine addiction-relevant behaviours vs. decision-making behaviour under risk.

Assuming that there are no particular limitations of the methodologies utilized in these studies which may be confounding these findings (potential limitations discussed further in section below), it would be safe to postulate a number of potential rationales for the differential involvement of the CGIC in these two paradigms. One such explanation would be the idea that the CGIC receives critical interoceptive cues during nicotine self-administration which, given the relatively extensive training utilized in our model, eventually becomes part of the complete
reinforcing effect of a nicotine delivery. Thus, when the CGIC is inactivated, the complete reinforcing effects of nicotine are not fully perceived by the animal and thereby operant behaviour and motivation for the reinforcer is decreased. In the reinstatement paradigm, the cue induced reinstatement of nicotine seeking could be presumed to require a recall of the interoceptive state produced by a nicotine infusion, which may require CGIC activity. This idea is supported by the findings of Contreras and colleagues (2012) who demonstrated that bilateral infusions of the protein synthesis inhibitor, anisomycin, into the CGIC immediately following amphetamine place preference conditioning, blocked the expression of amphetamine-conditioned place preference examined on a later date. As such, it may be that the full reinstatement of nicotine seeking behaviour requires that the nicotine-associated light cue is able to cause the recall of the nicotine-associated interoceptive state.

Finally, it should be noted that our prior findings examining CGIC inactivation included demonstration of significant attenuation of motivation for nicotine taking as assessed using a progressive ratio schedule of reinforcement as well as significant attenuation of nicotine seeking reinstated by nicotine priming injection. Without confirming similar findings with RAIC inactivation, we cannot at this point claim identical behavioural outcomes of RAIC vs. CGIC inactivations on nicotine taking and seeking. Of course, it should also be noted that neither the RAIC or CGIC have been evaluated for their involvement in the reinstatement of nicotine seeking by stressors.

The findings of this study demonstrated the similarity of involvement between the CGIC and RAIC in drug taking and seeking behaviour. Thus, given the clear potential for cognitive effects when targeting the RAIC, as demonstrated by its involvement in decision-making
behaviour, the rationale for preferentially targeting the CGIC during initial therapeutic trials appears strong.

5.1.3 Critical Findings from "Electrical Stimulation of the Insular Region Attenuates Nicotine-Taking and Nicotine-Seeking Behaviors"

The final study presented within the body of this thesis was intended to examine the potential of a viable therapeutic approach for addictive disorders, deep brain stimulation, to inhibit insular cortex activity and thereby attenuate drug taking and seeking behaviours. In this regard, we chose to specifically target the CGIC, as the effect of its pharmacological inhibition was as effective at attenuating drug taking and seeking behaviours as inhibition of the RAIC, but without effects on decision-making behaviour.

This study utilized bilaterally implanted electrodes to apply high frequency electrical stimulation to the insular region. Several behaviours were examined to determine whether they were affected by said electrical stimulation including nicotine taking under both FR5 and progressive ratio schedules of reinforcement, along with the reinstatement of nicotine seeking induced by either nicotine-associated light cues or nicotine priming injections. In order to determine the particular neuronal response to such high frequency electrical stimulation, an in vitro electrophysiological approach was utilized to study insular neurons.

High frequency electrical stimulation of the insular region was capable of attenuating nicotine taking under an FR5 schedule of reinforcement, yet in a control experiment it had no significant effect on food taking behaviour under the same FR5 schedule. Similarly, high frequency electrical stimulation of the insular region attenuated motivation for nicotine, as demonstrated by decreased reinforcements and breakpoint under a progressive ratio schedule, yet again in a control experiment it had no significant effect on food taking behaviour under the
same progressive ratio schedule. High frequency electrical stimulation of the insular region was also capable of attenuating nicotine seeking behaviour reinstated by either nicotine-associated light cues or nicotine priming injection.

Finally, in vitro electrophysiological examination of insular neurons under high frequency electrical stimulation was able to confirm functional inactivation of these neurons. Particularly, there were two different responses to high frequency electrical stimulation observed, either fast-inactivating or depolarization block. This finding suggested the possibility of two different neuronal population; however, it should be noted that all cells had similar resting membrane potentials, morphology under infrared differential interference contrast, regular spiking activity with current injection, and input resistance. Thus it is reasonable to speculate that the same type of neurons could respond to high frequency electrical stimulation in different ways, potentially dependent on their ion channel composition.

Overall, this study demonstrated the capability of high frequency electrical stimulation to result in functional inactivation of insular neurons as demonstrated by in vitro electrophysiology as well as by the similar affect on nicotine taking and seeking behaviour as observed with pharmacological inactivation in our prior studies (Forget et al., 2010a; Pushparaj et al., 2015a). Of course, with the rationale behind this study being a proof-of-concept for further research into the potential of utilizing electromagnetic technologies (deep brain stimulation or transcranial magnetic stimulation) to modulate insular activity as a novel therapeutic approach for smoking cessation, this study indeed demonstrated the potential of this concept.

Overall, the work presented within this thesis supports the targeting of the CGIC for substance-based addictive behaviours and targeting of the RAIC for non-substance reinforced addictive behaviours (eg. gambling disorder). Due to its higher-order position, targeting of the
RAIC may result in cognitive side effects; however, these may be tolerable depending on the severity of the addictive disorder. Finally, deep brain stimulation is a potentially viable option for therapeutic targeting of the insular cortex.

5.2 Validity and Limitations with Regards to the Clinical Situation

5.2.1 Animal Models of Behaviour

The use of rodent behavioural models in the present work, in order to elucidate a clearer understanding of a role for the insular cortex in the neurobiological underpinnings of addiction, has allowed this work to progress at a rate which could not have been achieved through the use of human neuroimaging and/or laboratory studies or even with non-human primate behavioural models. The direct lesion, inactivation and electrical modulation approaches utilized here are most quickly and easily conducted in rodents compared to non-human primates, and are ethically impossible to conduct in human subjects. This of course is not meant to suggest that human studies are inferior to rodent models, as the very basis for the work presented here were observational findings of behavioural differences in human subjects with neuroimaging confirmation of stroke-induced damage to the insular cortex compared to patients with damage in other brain regions and/or to healthy controls (Naqvi et al., 2007; Clark et al., 2008). Yet the rodent models utilized here have allowed us to form and validate experimental hypotheses which could not reasonably have conducted without them. Thus, having established the underlying rationale for the use of rodent behavioural models in the present work, it remains critical to identify the limitations of said models, particularly with regards to their interpretation in light of the human clinical situation and the development of therapeutic approaches.
5.2.1.1 The Intravenous Drug Self-Administration Model

Intravenous drug self-administration, with nicotine specifically being used in the present research, has been suggested to have high predictive validity with regards to abuse liability of the substance being self-administered (Balster, 1991). Essentially, the reliability with which a drug is self-administered in rats is generally correlated with both self-reports of positive subjective effects and scheduling status by the Drug Enforcement Agency of the United States (O'Connor et al., 2011). This model has clear face validity as animals have been shown to perform increasing behaviours (ie. progressive ratio schedules) or even withstand aversive stimuli to obtain drug, as can be related to individuals with drug addiction devoting increasing time and resources, often at high financial and social costs, to continue to consume their drug of abuse.

But what is the predictive validity of this model for the development of novel therapeutics for substance-related and addictive disorders? Is it necessary for an efficacious therapeutic for smoking cessation to directly decrease the reinforcing effects of nicotine in an animal model, or even in human smokers for that matter? Importantly, for the case of nicotine addiction examined here, the most effective therapeutic available - varenicline - was brought to clinic following its promising effects in the nicotine self-administration model in rats (Rollema et al., 2007). Yet at the same time, another approved therapeutic - buproprion - has had conflicting effects on responding for nicotine under FR schedules of reinforcement and no significant effect under the progressive ratio (Bruijnzeel and Markou, 2003; Rauhut et al., 2003; Shoaib et al., 2003; Liu et al., 2008b). Rather, buproprion has been shown to significantly reverse both the somatic and affective (increased brain reward threshold) aspects of withdrawal from nicotine in rats (Cryan et al., 2003). More importantly, there are numerous therapeutics which have demonstrated some ability to decrease nicotine self-administration behaviour and have either
been shown to have significant adverse effects or not to have been clinically meaningful in human smoking cessation trials.

Our prior findings with CGIC inactivation (Forget et al., 2010a) and present findings with RAIC inactivation (Pushparaj et al., 2015a), appear to lend support to the conclusions of Naqvi and colleagues (2007) that stroke-induced damage to the insular cortex was in fact responsible for the disruption of smoking addiction. Specifically, our demonstration of the ability of insular cortex inactivations to attenuate FR5 and progressive ratio schedules of reinforcement lend support to the particular findings of immediate and easy smoking cessation in their insula-damaged patients.

Thus, though the work presented here also demonstrates the ability of electrical modulation of insular activity to decrease self-administration of nicotine under both fixed and progressive ratio schedules of reinforcement, this only suggests its potential to become a clinically meaningful therapeutic approach for smoking cessation. There remain several other behavioural measures which could further suggest its potential, such as measures of withdrawal or reinstatement.

5.2.1.2 The Reinstatement Paradigm

Relapse is known among both clinicians and patients to be the most insidious aspect of drug addiction (Hunt et al., 1971; O'Brien, 2005) and thus the animal model of relapse, the reinstatement paradigm, is noted to be one of the most critical methods for preclinical assessment in the development of novel therapeutics (O'Brien and Gardner, 2005). The model has excellent face validity as the same triggers of craving and relapse in human subjects with substance dependence, also having being verified in human laboratory models, are known to demonstrate reliable reinstatement of drug seeking in animals: acute re-exposure to the self-administered drug
or "drug priming" (de Wit and Stewart, 1981; De Wit, 1996), drug-associated cues/contexts (O'Brien et al., 1992; Meil and See, 1996; Weiss et al., 2000; Crombag et al., 2002), and stressors (Shaham et al., 2000; Sinha and Li, 2007).

In terms of predictive validity of the reinstatement model in the development of novel therapeutics for relapse preventions, this model appears to be well correlated with those drugs currently approved for substance dependence, including: varenicline (O'Connor et al., 2010), naltrexone (Shaham and Stewart, 1996; Le et al., 1999), buprenophine (Sorge et al., 2005), methadone (Leri et al., 2004), and acomprosate (Bachteler et al., 2005). However, once again buproprion appears to be the exceptional case (Liu et al., 2008b).

Our prior findings with CGIC inactivation (Forget et al., 2010a) and present findings with RAIC inactivation (Pushparaj et al., 2015a), appear to lend support to the conclusions of Naqvi and colleagues (2007) that stroke-induced damage to the insular cortex was in fact responsible for the disruption of smoking addiction. Specifically, our demonstration of the ability of insular cortex inactivations to attenuate reinstatement induced by nicotine-associated cues or nicotine priming lend support to the particular findings of smoking cessation without craving or relapse in their insula-damaged patients. Importantly, our work did not include an examination of stress-induced reinstatement of nicotine seeking which is a major trigger of relapse and would be presumed to have been disrupted in Naqvi and colleagues (2007) insula-damaged patients.

The work presented here also demonstrates the ability of electrical modulation of insular activity to attenuate the reinstatement of nicotine seeking behaviour by nicotine-associated cues and nicotine priming injections; however, again it should be noted that we did not examine reinstatement by stressors. The ability of electrical modulation of the insular cortex should be
examined in that regard to demonstrate a robust potential of this therapeutic approach for relapse prevention.

5.2.1.3 The Rat Gambling Task

Unfortunately, as there are no current medications approved for the treatment of gambling disorder, nor specifically for the improvement of cognitive or decision-making aspects in addictive disorders as a whole, the predictive validity of the rGT or even the IGT cannot be established. However, I will speak briefly to the face validity of the rGT, as the idea of "gambling" rats likely seems somewhat absurd to many individuals. The first question to be addressed is whether the IGT has face validity as a simulation of gambling or merely a decision-making task. The most obvious aspect of gambling, the risking of one's own finances (or financial future, if credit is used), is not an aspect of the IGT. Yet it is worth questioning the importance of this aspect in any model of gambling disorder, as pathological gamblers still perform poorly on the IGT compared to healthy controls. Thus we should question whether the need or desire to actually risk one's finances may not necessarily be a critical driving or differentiating trait of pathological gamblers.

Therein arises the question of the true differentiation and underlying similarities between gambling disorder and substance dependence. Both individuals with gambling disorder and those with drug use demonstrate impaired decision-making behaviour on the IGT, as outlined in the introduction of this thesis. If one attempts to examine the behaviour of these patients in the real world, they should not be surprised to observe what society deems as inappropriate decision-making behaviour. Though it is an overgeneralization, they do seem to appear to have an innate preferential disposition to make high risk (financial, health and social risks), high reward (financial and/or euphoria-based reward) decisions. The key difference appears to be in the
timing of which the detrimental effects of the risk may be experienced. For gamblers, the risk of a negative outcome seems highly apparent and quite nearly immediate while for addicts the risk of a negative outcome may be somewhat apparent and often quite delayed. However, it should be noted that if one views a gamble as not a single independent risk but as part of the overall risk of numerous gambles, the negative outcome now can be understood to be subjectively delayed until the conclusion of the gambling session. Importantly in this regard, pathological gamblers are well noted to have cognitive biases, one of such known as the gambler's fallacy, in which they link multiple independent events together to create a perceived dependent relationship between these events (also observed in healthy controls to a lower degree). Thus, pathological gamblers not view the loss after a single gamble as a negatively as a healthy individual, as they perceive it to be part of a necessary process in obtaining their large future win.

Returning to an examination of the present findings, the study of Ishii and colleagues (2012) concluded that the RAIC contributes to increase risk taking behaviour in rodents, and thus the bilateral inactivation of the RAIC decreased risk taking behaviour. The results presented in this thesis could conclude the same; however, that would be a simplification of these findings. Though risk taking behaviour (ie. suboptimal choice behaviour) was decreased in RAIC inactivated rats, this finding is complicated when the group is dissected into optimal and suboptimal subgroups based on their responding during the vehicle control session. The suboptimal group demonstrated a clear decrease in risk-taking/suboptimal choice (P3 choice specifically) and corresponding increases in optimal choice (both P1 and P2 choice) when their bilateral RAIC was inactivated; however, the optimal subgroup demonstrated a decrease in the most optimal choice (P2) and a corresponding increase of the second most optimal choice (P1). It is important to note, that P2 has been denoted the most "optimal" choice not based solely on its "risk" or probability of punishment (which is 20% compared to only 10% with P1 choice), but
rather due to the fact that if chosen consistently throughout the session, it will produce the largest expected outcome (411 pellets). Though P1 has a lower risk (10% probability of punishment), if chosen consistently throughout the session, it will only yield an expected outcome of 295 pellets.

Thus, in the rGT task, akin to most situations in everyday human life, the most "optimal" option is not the least risky. As such, there is the question of the clinical value of this finding and whether it should be translated into therapeutic approaches to inhibiting RAIC activity for gambling disorder and addiction in general. In this regard, I can only begin to make the argument that based on the findings of high risk preference in pathological gamblers and individuals with substance dependence on tasks of decision-making under risk (Murch and Clark, 2015), it is not unreasonable to presume that these individuals are somewhat analogous in their behaviour to the suboptimal subgroup of rats observed in the present work. Thus, in such individuals already predisposed to high risk taking behaviour, it is reasonable to assume that attempting to modulate activity within their insular cortex could potentially decrease such high risk taking behaviour to that within a clinically healthy range. More importantly, given the serious health, social, and economic consequences both personally and to those around them, there is a high need for novel therapeutic approaches for individuals with substance-related and addictive disorders.

5.2.2 Critical Differences in the Rodent vs. Human Insular Cortex

Clearly, it is impossible to fully model the human situation of any addictive disorder utilizing a laboratory paradigm; however, individual aspects, such as drug taking and seeking behaviours can be isolated for examination and show some predictive value as discussed above. Yet our ability to examine functional aspects of specific brain regions, particularly cortical areas, is also limited by the high degree of evolution in complexity between the rodent and human brain. The
general cytoarchitecture of the insular cortex, with the posterior granular subregion receiving the majority of input from the thalamus and projecting primarily to the dysgranular midinsula and agranular anterior subregion, remains similar overall between rodents to primates, including humans (Craig, 2002).

However, there are some degrees of complexity within the primate brain that are not observed in rats, including the posterior and basal ventromedial nuclei of the thalamus as well as a distinct lateral sulcus and insular lobe (Van Essen, 1997; Craig, 2011). Among primates, only macaque, great apes, and humans are observed to possess specialized von Economo neurons, which are large bipolar neurons with an atypical spindle- or corkscrew-shaped soma and thick basal and apical dendrites, in the anterior insula and anterior cingulate cortices, with their number being greatest in the human brain (Allman et al., 2010). In post mortem examinations of subjects with a history of alcoholism, reduced anterior insular volume has been attributed to loss of von Economo neurons (Senatorov et al., 2015). Their selective destruction during early frontotemporal dementia correlate with behavioural changes such as loss of empathy, social awareness, and self-control (Allman et al., 2011).

Interestingly, these von Economo neurons also appear to be greater in number in the right hemisphere compared to the left (Allman et al., 2011). This finding of hemispheric differences is consistent with prior studies where patients with right insular lesions demonstrated neglect of tactile, visual and auditory stimuli (Berthier et al., 1987; Manes et al., 1999b) along with more frequent subjective anergia, hypoactivity, and fatigue (Manes et al., 1999a) compared with non-insular or left insular lesion patients, while left insula dysfunction has been observed to more likely result in aphasia (Shuren, 1993; Marshall et al., 1996; Nestor et al., 2003).
Recent work has attempted to define whether there is a particular influence of the right or left (or dominant/non-dominant) insular areas in this finding of smoking disruption; however, preliminary findings suggest little difference between unilateral dominant insular damage over non-dominant damage in regulating urge, withdrawal, and smoking cessation (Abdolahi et al., 2015a). Another study has observed similar null findings when examining the effect of unilateral right vs. left basal ganglia-insular lesions on incentive motivation, though there was an interesting observation in right, but not left, lesion patients of reduced preference and viewing of stimuli they rated as only slightly positive, and particularly an avoidance of images with sexual content (Vijayaraghavan et al., 2013).

There is some evidence to suggest that though left insula cortical thinning is consistent with substance dependence regardless of sex, there is an interaction of sex in bilateral insula volume and right insula cortical thickness with females with substances dependence having lower volumes/thickness than gender-matched healthy controls while males with substance dependence have greater volumes/thickness (Tanabe et al., 2013). Additionally, a recent finding in adolescents receiving substance use treatment, demonstrated increased left insular white, but not gray, matter being linked with drug enhancement motives (eg. reported using drugs in order to "get high") and thereby with frequency of binge drinking while right insula white matter was correlated with obsession/craving for alcohol (Chung and Clark, 2014). Another contrary finding in young adult amphetamine-related stimulant abusers observed a positive correlation between amphetamine use history and grey matter volume in the left mid-insula (Mackey et al., 2014). This last finding may represent the possibility that higher left insula volumes predispose individuals to drug taking behaviours.
Hemispheric selective findings of functional connectivity and activity in the insular cortices are very common across the human imaging literature, as reviewed in the introduction of this thesis. Yet there have yet to be any studies indicating hemispheric differences in insular function in rodents. Within the work presented in this thesis, cannulae that were misplaced outside of the insular cortex in one hemisphere but not the other were generally excluded from analysis; however, simple examination of this small group of animals showed no apparent behavioural effects of single hemisphere inactivations, though these animals were grouped together regardless of whether the misplaced cannulae was in the left or right hemisphere (data not shown). As such, it remains unclear as to whether functional hemispheric differences exist within the insular cortices of rodents; however, their existence is clear within the human brain and thus should be evaluated further. Additionally, there are clearly aspects of insular complexity involved in addictive disorders which cannot be studied in rodents, such as the loss of von Economo neurons in the brains of individuals with alcohol dependence (Senatorov et al., 2015).

5.3 Integration of the Present Findings into the Broader Literature around the Insular Cortex in Addictive Disorders

5.3.1 Differential Involvement of the Caudal Granular vs. Rostral Agranular Subregions in Addictive Disorders

Despite the lack of any findings of hemispheric differences of the insular cortex in animal models, there remain findings of differential involvement of the caudal granular vs. rostral agranular subregions in a variety of behaviours, including in decision-making under risk examined in the present thesis (Pushparaj et al., 2015b). Importantly, this differential finding correlates with human imaging studies where certain findings have been localized to the anterior or posterior regions of the insular cortex, as shall be discussed further below.
It should be noted that though this thesis did not directly compare CGIC vs. RAIC manipulations for their effects on nicotine-self administration behaviour, a more general comparison of the nature of the effect of each manipulation was made between the present work (Pushparaj et al., 2015a) and the previous findings of our laboratory (Forget et al., 2010a). In this regard, for the behaviours examined in both studies (nicotine or food taking under FR5 and the reinstatement of nicotine seeking by nicotine-associated cues), both inactivation of the CGIC and RAIC produced attenuations of nicotine-, but not food-, relevant behaviours. Unfortunately, the work presented here examining the RAIC did not examine nicotine or food taking behaviour under a progressive ratio schedule of reinforcement nor priming-induced reinstatement of nicotine-seeking behaviour. Future work should examine whether the RAIC plays a similar role as the CGIC in these behaviours as well.

In contrast to our own findings, RAIC, but not CGIC, inactivation attenuated the cue-induced, but not priming, reinstatement of cocaine seeking behaviour (Cosme et al., 2015). The level of cue-induced reinstatement of cocaine seeking behaviour has also been correlated to Fos protein expression, a marker of neuronal activity, in the RAIC (Kufahl et al., 2009), though the CGIC was not included as a region of interest in that study. Similar to our own findings with food taking behaviour, the work of Cosme and colleagues (2015) also demonstrated no significant effects of RAIC inactivation on food seeking behaviour induced by cues or priming. Cocaine-associated contextual odor cues have also been demonstrated to cause a greater fMRI BOLD response in the insula, particularly the agranular subregion, when compared to neutral odor in rats trained to self-administer cocaine (Johnson et al., 2013).

A prior study found that local infusion of the protein synthesis inhibitor anisomycin into either the CGIC or RAIC shortly after memory retrieval resulted in a loss of amphetamine-
induced CPP; however, without memory retrieval, only anisomycin infusions into the CGIC, not the RAIC, could facilitate the extinction of amphetamine-induced CPP (Contreras et al., 2012). Another study in rats also found that inactivation of the CGIC, but not RAIC, was capable of blocking the acquisition of morphine-induced CPP (Li et al., 2013). Yet the same study demonstrated that both inactivation of the CGIC or RAIC were capable of blocking the acquisition of CPA induced by naloxone-precipitated morphine withdrawal. Another study examining the difference in zif268 expression between the acquisition of CPP induced by cocaine vs. social interaction in rats, demonstrated significantly greater expression in both the CGIC and RAIC of the cocaine group (El Rawas et al., 2012). Finally, a study in mice examining lesions spanning both the CGIC and RAIC observed involvement of the insular cortex only in nicotine-induced CPP and not for CPA induced by mecamylamine-precipitated nicotine withdrawal (Scott and Hiroi, 2011).

Thus, the differential involvement of the CGIC vs. RAIC in relapse and withdrawal remains unclear. The seemingly contradictory findings in these various studies may be due to differences in insular involvement for the particular addictive drugs being studied. Yet with the findings of the insular involvement in the actions of a range of addictive drugs (Naqvi et al., 2014) it is more likely that the contradictory findings are a result of other, potentially subtle, methodological differences. Regardless of the contradictory findings for particular subregions, all of this animal evidence, along with the human imaging and lesion studies described in the introduction of this thesis, make a clear case for the role of the insular cortex as a whole in the aspects of withdrawal from and relapse to addictive drugs.

5.3.2 Insular Cortex Activity as a Promoter vs. Preventer of Addictive Behaviour

A) Evidence from animal model studies
The work presented within this thesis, particularly those studies examining nicotine self-administration, provide a basis for exploring the potential of technologies capable of modulating insular activity as a therapeutic strategy for smoking cessation. Specifically, the work suggests that inhibiting insular cortex activity would be beneficial in preventing drug taking and relapse, and thus indirectly suggests the possibility that insular cortex activity promotes nicotine taking and seeking behaviour. Of course, the current work did not directly examine whether increasing activation of the insula would promote relapse or nicotine-taking behaviours under fixed or progressive ratio schedules. It should be noted that during the experimental work conducted for our prior study examining inactivation of the CGIC (Forget et al., 2010a), we attempted to pilot the infusion of GABA antagonists (bicuculline/saclofen) prior to an extinction session; however, the infusion resulted in motor stereotypy, specifically a nodding of the head and movement of the jaw which lasted approximately 2 hrs (data not reported).

Few others have directly attempted to examine whether enhancing insular activity promotes drug taking or seeking behaviours in animals. Only one study examined the role of muscarinic acetylcholine receptors in morphine-induced CPP and found opposing roles of the M1 and M4 receptor subtypes, with an agonist of the M1 and antagonist of the M4 both enhancing CPP expression while an antagonist of the M1 and agonist of the M4 both inhibited CPP expression (Wu et al., 2014). It should be noted that the precise signaling cascade induced by those receptor subtypes in that neuronal population was unconfirmed; however, this is one of the only pieces of evidence in which insular modulation was demonstrated to increase drug seeking behaviour.

Other strong, yet still indirect, evidence supporting insular activity promoting drug-taking behaviour comes from a study examining rats self-administering alcohol where aversive stimuli
(either bottle alcohol adulterated with quinine or footshocks paired with alcohol delivery during operant self-administration) were used to specifically examine aversion-resistant alcohol seeking (Seif et al., 2013). This study utilized optogenetics to inhibit glutamatergic projections from the RAIC to the NAcc core demonstrating that glutamate release from these RAIC projections was required for aversion-resistant alcohol intake, though interestingly it did not play a role in alcohol intake without the presence of aversive stimuli. Thus, there is evidence to suggest that insular activity promotes drug taking and seeking behaviour, though the precise neuronal populations involved in the particular aspects of these behaviours is only beginning to be studied using selective approaches such as optogenetics.

To my knowledge, only one rodent study exists which demonstrated increased drug seeking with decreased insular activity (Pelloux et al., 2013). This study observed an increase in cocaine seeking behaviour in rats with RAIC lesions during re-introduction to the self-administration chamber following a forced abstinence period of 7 days. It should be noted that prior to their 7 day abstinence period, these rats were tested for their cocaine seeking behaviour under intermittent punishment (50% of cocaine infusion reinforcements being delivered while the other 50% resulted in delivery of footshock with no cocaine); therefore, the possibility exists that sham control animals displayed hesitation in reinitiating cocaine-seeking behaviour and that RAIC integrity is required for the acquisition, storage or retrieval of the memory underlying this hesitation. Regardless, this finding presents a situation in which insular activity is involved in preventing drug seeking behaviour and supports the idea that insular activity is not limited to aspects promoting addictive behaviour.

B) Evidence from human imaging studies
The evidence from human imaging and lesion literature is generally highly supportive of a role for insular activity promoting addictive behaviour (see Naqvi and Bechara, 2009) for review; however, more recent studies have observed some seemingly contradictory results. Thus I will review here data on both sides before discussing a possible framework for integrating these findings into our current understanding of the role of the insular cortex in addictive disorders.

Firstly, the majority of the findings with regards to the effects of addictive substances on brain volume suggest loss of gray matter volumes across particular brain regions (Buttner, 2011), with the insular cortex decreases found across all major drugs of abuse (Franklin et al., 2002; Makris et al., 2008a; Makris et al., 2008b; Schwartz et al., 2010; Durazzo et al., 2011; Lopez-Larson et al., 2011; Weller et al., 2011; Gardini and Venneri, 2012; Mackey and Paulus, 2013; Battistella et al., 2014; Fritz et al., 2014; Hanlon et al., 2014; Morales et al., 2014; Senatorov et al., 2015) and importantly in internet addiction as well (Zhou et al., 2011b; Weng et al., 2013; Yuan et al., 2013; Zhu et al., 2015). At first glance, this gray matter volume loss with addictive disorders may appear to be contradictory to the finding of insular damage resulting in a disruption of addictive behaviour (Naqvi et al., 2007; Suner-Soler et al., 2012; Yousefzadeh-Fard et al., 2013; Gaznick et al., 2014; Abdolahi et al., 2015c; Abdolahi et al., 2015b). Though some drugs of abuse, such as methamphetamine (Yu et al., 2015) and alcohol (de la Monte and Kril, 2014), are well known to be highly neurotoxic, others such as nicotine have both neurotoxic and neuroprotective effects (Ferrea and Winterer, 2009) while internet addiction is obviously not directly neurotoxic at all.

Interestingly, cortical gray matter thinning is thought to be critical for cognitive improvements during childhood/adolescent development (Sowell et al., 2004; Casey et al., 2005; Tau and Peterson, 2010), and is thought to result from both synaptic pruning and myelination.
(Sowell et al., 2001; O'Donnell et al., 2005; Toga et al., 2006; Dosenbach et al., 2010). Thus, it may be reasonable to suggest that the widespread findings of decreased gray matter insular volumes across a variety of addictive disorders can be explained as a selective synaptic pruning and myelination which focuses insular activity on addiction-relevant neuronal connectivity. If so, it would be expected that addiction-relevant cognitive processes and behaviours would be enhanced while all other processes and behaviours would be impaired.

Examining the studies of rsFC, we see that withdrawal from addictive substances results in an increased insular connectivity with the DMN (Sutherland et al., 2013a; Huang et al., 2014), amygdala (Gu et al., 2010; Xie et al., 2011) and even general global connectivity (Wang et al., 2014). Individuals with internet gaming disorder (Petry et al., 2014) have also been observed to have a large range of insula centered increases in rsFC, including amygdala-insula (Ko et al., 2015), right anterior insula-ACC, anterior insula-DMN, left anterior insula-right putamen, and posterior insula-somatosensory/sensorimotor cortices (Zhang et al., 2015). Increased rsFC of the right anterior insula-ACC was correlated with the duration of internet gaming disorder, while increased rsFC of the anterior insula-DMN and posterior insula-somatosensory/sensorimotor cortex was correlated with the severity of internet gaming disorder. Finally, resting state cerebral blood flow has been observed to be increased in the bilateral insula (Feng et al., 2013) of adolescents with internet gaming disorder compared to control subjects.

Importantly, several of the studies with addictive substances demonstrated that resumption of drug taking or the administration of replacement pharmacotherapies (eg. vareneline) were capable of attenuating these increases in rsFC (Gu et al., 2010; Sutherland et al., 2013a; Wang et al., 2014) though not necessarily reaching the insula rsFC levels observed in healthy controls (Huang et al., 2014). Additionally, decreased rsFC of the insula-DMN has also
been correlated with current cocaine levels in individuals with cocaine dependence (Liang et al., 2015).

The functional connectivity data described above supports a role for increased insular connectivity during resting state which can be attenuated by the resumption of drug taking or the administration of replacement pharmacotherapies. Thus, it has been suggested that this hyperconnectivity of the resting brain network is responsible for allowing the addicted individual quickly and subconsciously execute cognitive processes and behaviours toward drug related goals along with a loss of normal inter-regional communications which may result in deficits of cognitive control and inhibition (Wang et al., 2015).

With regards to drug related cues, contexts or stress and their ability to induce craving, these have consistently been reported to correlate with increased insular activity (Sell et al., 1999; Wang et al., 1999; Garavan et al., 2000; Kilts et al., 2001; Wexler et al., 2001; Bonson et al., 2002; Brody et al., 2002; Kilts et al., 2004; Myrick et al., 2004; Tapert et al., 2004; Lee et al., 2005; McClernon et al., 2005; McBride et al., 2006; Franklin et al., 2007; Wang et al., 2007; Filbey et al., 2009; Engelmann et al., 2012; Lou et al., 2012), with similar findings for gambling-related cues in individuals with gambling disorder (Goudriaan et al., 2010) and for highly palatable food images in obese subjects (Killgore et al., 2003; Geliebter et al., 2006; Grill et al., 2007; Rothemund et al., 2007; Stoeckel et al., 2008; Brooks et al., 2013; Goldman et al., 2013). In fact, insula reactivity to cues has been described as a potential biomarker of addiction with it being correlated to dependence severity for both alcohol (Claus et al., 2011a) and nicotine (Franklin et al., 2009b; Franklin et al., 2011a). The intensity of craving evoked by smoking cue exposure during withdrawal has been observed to correlate with insula-precuneus connectivity, suggesting that the greater the conscious awareness of the current bodily state (ie. lack of drug),
the greater the craving induced by cues (Moran-Santa Maria et al., 2015). Additionally, the cue reactivity of the dorsal striatum has been associated with the rsFC of the mPFC-left insula in smokers, suggesting the possibility that this rsFC may itself cause the heightened dorsal striatum cue reactivity (Janes et al., 2012).

Finally, the inferior frontal cortex, a region identified to play a role in self-regulation across motor, affective and craving (Berkman et al., 2009; Tabibnia et al., 2011), has shown negative functional connectivity with the insula during a craving self-control task in abstinent smokers (Tabibnia et al., 2014), suggesting that the inferior frontal cortex inhibition of the insula may be critical to maintaining self-control during craving. Mindful attention has also been shown to reduce self-reported cue-induced craving and reduce functional connectivity between the ACC and bilateral insula in smokers (Westbrook et al., 2013). A positive correlation was found between smoking cue-induced insula activation and lapse during a 8-week smoking cessation in a clinical trial utilizing nicotine replacement therapy (Janes et al., 2010a) with lapsing individuals also showing decreased functional connectivity between the insula and inhibitory control regions such as the dorsal anterior cingulate cortex and the dorsal lateral prefrontal cortex. Thus, while insular functional connectivity appears to be increased for certain regions such as the DMN, amygdala and striatum, it appears to be decreased for others such as the inferior frontal cortex, ACC and dorsolateral PFC. These latter regions appear to play a role in inhibiting the insular projections to the former regions and so this evidence overall also lends support to the idea that insular activity promotes addictive behaviour.

In conflict with the simple conclusion that increased insular activity correlates to relapse, a recent prospective study demonstrated greater anterior insula activation during reward contingency learning predicted abstinence of cocaine-dependent individuals at 1 year follow-up
A study examining the stop signal task in cocaine-dependent individuals under fMRI found that in male participants, decreased stop error-related activations of the left insula predicted relapse and earlier time to relapse (Luo et al., 2013). Similar results have been obtained in treatment-engaged, methamphetamine-dependent individuals where a negative correlation was observed between right insular activation during a simple decision making task performed during early recovery and the likelihood of relapse at 1 year follow-up (Paulus et al., 2005). Those authors had similar findings using a risky decision making task, where abstinent methamphetamine-dependent patients had lower insula activation during safe decisions than risky decisions while the subjects who relapsed showed similar insula activation during safe and risky decisions (Gowin et al., 2014). A combination of cocaine and methamphetamine dependent patients also showed a negative correlation between right insular activation during a selective attention task and relapse (Clark et al., 2014b). Insula activation during the cognitive-control Stroop task has also been associated with better treatment outcome - lower cotinine levels - in young adult smokers (Krishnan-Sarin et al., 2013) which has recently been demonstrated to be enhanced by treatment with the noradrenergic α2a agonist guanfacine (3mg/day) (McKee et al., 2015).

Critically, those studies did not involve addiction-related cues or stress suggesting that individuals prone to relapse may have their insular activity focused on addiction relevant stimuli and thus learning for non-addiction stimuli may be compromised. This interpretation is supported by a study in which heavy drinkers showed significantly greater activity in the insula relative to light drinkers during NoGo trials when the NoGo signal was represented by images of beer bottles (Ames et al., 2014). The authors of that study suggested that the heavy drinkers may have had to exert increased working memory demand and control efforts to withhold a response due to poorer inhibitory control from enhanced salience of alcohol cues on the beer NoGo trials.
In contrast, importantly in a study in which no addiction-relevant stimuli were used, there was an association between right insula activation with inhibition success and increased abstinence duration (Bell et al., 2014).

From these findings, we can conclude that increased insular activity during resting state and addiction-relevant tasks predispose individuals to relapse, while increased insular activity during unrelated tasks, particular those involving inhibitory cognitive control, are predictive of successful abstinence. As such, modulation of insular activity as a therapeutic approach to addictive disorders should prove most beneficial when paired with such tasks in an attempt to decrease insular activity during extinction training for addiction-relevant stimuli and/or increase insular activity during inhibitory cognitive control training tasks.

5.4 Conclusions

The current body of work demonstrated the involvement of the RAIC, but not CGIC, in cognitive aspects of addictive disorders, such as gambling-relevant behaviours. Yet both regions were demonstrated to be implicated in substance-based addictive disorders. Thus, therapeutics using electrical modulation to target the CGIC may be preferable for substance dependence, with limited cognitive side effects.

Overall, the clinical value of the work is tempered by the limitations in the methodological approaches utilized to demonstrate these findings; however, it is important to note that these findings still represent some of the best evidence cementing a critical role for the insular cortex within the neurobiological framework of addiction and for further examining the potential of modulating insular cortex activity as a therapeutic approach for addictive disorders.
The insular cortex and the role of interoception in addictive disorders, as well as a large range of other psychiatric disorder, is still being studied and better understood. However, the work presented herein, along with that of my betters in the field, have clearly demonstrated to me the crucial role of this brain region and its cognitive function in the behaviour of both rats and men.

As such, I would like to note here that there remains an oversimplification of the insular cortex within the field of addiction, one of which I have likely contributed to throughout the text of this thesis. In our attempts to form a neat and simple diagrammatic neurocircuitry underlying addiction, we should come to realize that it has always been obvious to us all that the true picture is so overwhelmingly complex that it likely can never be visualized in any way meaningful to human perception.

5.5 Future Directions

The obvious future direction for this work lies in its translation towards the modulation of insular cortex activity becoming a therapeutic approach for substance-related and addictive disorders. In this regard, much work has already begun both amongst my colleagues in the laboratory and those abroad. Though it is unlikely, at least with the current state of the technology, that there will be many patients with nicotine addiction who opt for DBS of the insular cortex, or any brain region for that matter, as a therapeutic approach to smoking cessation. As such, the fact that rTMS has the potential to modulate long-term brain activity in localized regions is fortunate (Fitzgerald, 2011). Thus far, 19 studies have examined the effects of rTMS targeting different brain regions on drug craving and consumption (Gorelick et al., 2014).

One study has examined deep rTMS, using the relatively novel H-coil, targeting both the lateral PFC and insular cortex bilaterally for smoking cessation (Dinur-Klein et al., 2014). High
frequency deep rTMS treatment significantly reduced cigarette consumption and nicotine
dependence though it had no significant effects on reported craving. The combination of rTMS
treatment with exposure to smoking cues enhanced reduction in cigarette consumption leading to
an abstinence rate of 44% at the end of the treatment and an estimated 33% at 6 month follow-
up.

Work amongst my laboratory colleagues has already begun to explore the effects of deep
rTMS targeting the bilateral insular cortex on striatal basal dopamine levels in healthy control
subjects, using positron emission tomography (PET) with [(11)C]-(+)-PHNO. Future studies
will begin to explore the effect of deep rTMS targeting the insula cortex on cigarette smoking-
induced dopamine release in individuals with nicotine dependence, again using PET with
[(11)C]-(+)-PHNO. Finally, the long term aim will be to conduct a pilot clinical trial of deep
rTMS of the insular cortex as a potential therapeutic aid for smoking cessation.

Of course, though the work presented here, along with that of others in the field, has
cemented a critical role for the insular cortex in the neurocircuitry of addiction, further work
should be conducted to delineate the potentially differential involvements of the three major
subregions, individual neuronal populations within said subregions, particular neuronal receptor
populations, and the critical projections through which the insular cortex establishes it role.
There are several interesting avenues to proceed in this regard, such as glutamatergic projections
of the RAIC to the NAcc which appear selectively involved in aversion-resistant alcohol intake
(Seif et al., 2013), the hypocretin-1 receptors within the CGIC who antagonism attenuates
nicotine self-administration (Hollander et al., 2008), the reciprocal connections of the RAIC and
BLA which appear critical to guiding choice between goal-directed actions (Parkes and Balleine,
2013), and the role of insular DARPP-32 in the incubation of nicotine-seeking behaviour.
(Abdolahi et al., 2010). Of course, these are only a few interesting avenues which may be pursued to further elucidate the functioning and role of the insular cortex in the neurobiological underpinnings of addiction.


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Title: Differential Involvement of the Agranular vs Granular Insular Cortex in the Acquisition and Performance of Choice Behavior in a Rodent Gambling Task

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