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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inactivation of the Insular Cortex Increases Anxiety-Like Behavior in Rats:  
Relevance to Drug Abuse and Interoception

A Thesis submitted in partial satisfaction of the requirements for the degree  
Master of Science

in

Biology

by

Monica Wolfe

Committee in Charge:

Professor Gerhard Schulteis, Chair  
Professor P. A. George Fortes, Co-Chair  
Professor Kathleen French

2011



The thesis of Monica Wolfe is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

## Table of Contents

<b>Signature Page</b> .....	<b>iii</b>
<b>Table of Contents</b> .....	<b>iv</b>
<b>List of Figures</b> .....	<b>vi</b>
<b>List of Tables</b> .....	<b>vii</b>
<b>List of Abbreviations</b> .....	<b>viii</b>
<b>Acknowledgments</b> .....	<b>ix</b>
<b>ABSTRACT OF THE THESIS</b> .....	<b>x</b>
<b>1 Introduction</b> .....	<b>1</b>
<b>2 Materials and Methods</b> .....	<b>9</b>
<b>2.1 Animal Selection</b> .....	<b>9</b>
<b>2.2 Drugs</b> .....	<b>9</b>
<b>2.3 Insular cortex Bilateral Cannulation Surgeries</b> .....	<b>9</b>
2.3.1 Rostral Agranular Insular Cortex (RAIC) .....	10
2.3.2 Caudal Granular Insular Cortex (CGIC).....	10
<b>2.4 Cortical Inactivation</b> .....	<b>12</b>
2.4.1 Use of Lidocaine .....	13
<b>2.5 Histological Verification</b> .....	<b>13</b>
<b>3 Experiment 1: Effects of Reversible Inactivation of the Insular Cortex on Place Conditioning</b> .....	<b>17</b>
<b>3.1 Introduction</b> .....	<b>17</b>
<b>3.2 Apparatus</b> .....	<b>20</b>
<b>3.3 Experimental Design</b> .....	<b>21</b>
<b>3.4 Data Analysis</b> .....	<b>23</b>
<b>3.5 Results</b> .....	<b>23</b>
<b>3.6 Discussion</b> .....	<b>26</b>
<b>4 Experiment 2: The Effects of Insular Cortex Inactivation on Behavior in the Elevated Plus Maze</b> .....	<b>30</b>

4.1	Introduction.....	30
4.2	Apparatus.....	32
4.3	Experimental Design .....	33
4.4	Data Analysis .....	34
4.5	Results .....	35
4.6	Discussion .....	44
5	Experiment 3: Effects of Insula Inactivation on Acoustic Startle.....	51
5.1	Introduction.....	51
5.2	Apparatus.....	53
5.3	Experimental Design .....	53
5.4	Results .....	55
5.5	Discussion .....	57
6	Conclusions.....	59
	Reference List .....	63

## List of Figures

Figure 1-1. Overview of the Interoceptive Circuit.....	8
Figure 2-1. Cannulae and Injector Placement for RAIC.....	11
Figure 2-2. Cannulae and Injector Placement for CGIC .....	12
Figure 2-3. Histological Verification of RAIC Lidocaine Infusion Sites.....	15
Figure 2-4. Histological Verification of CGIC Lidocaine Infusion Sites .....	16
Figure 3-1. Place Conditioning Box Design.....	20
Figure 3-2. Dots vs. White Pre-conditioning Day Differences .....	24
Figure 3-3. Compartment Preference on Pre-Conditioning Day.....	24
Figure 3-4. Failure Rate for each Compartment .....	25
Figure 3-5. Total Distance during Pre-Conditioning Phase.....	25
Figure 3-6. Effect of Drug-Paired Compartment on Aversion Score .....	26
Figure 4-1. Schematic Drawing of the Elevated Plus Maze .....	32
Figure 4-2. Anxiety-like effect of Lidocaine Infusion into the Insular Cortex.....	37
Figure 4-3. Anxiety-like effect is specific to Insular Cortex Inactivation .....	40
Figure 4-4. Attenuation of Anxiety-like effect after repeated Lidocaine inactivation ...	41
Figure 4-5. Vehicle Pre-Infusion Attenuates Lidocaine Effect.....	43
Figure 4-6. Lidocaine Infusion into the Insular Cortex has no effect on Mobility.....	44
Figure 5-1. Startle reflex in the Rat.....	52
Figure 5-2. Effect of Infusion on Peak Startle Magnitude.....	56

## List of Tables

Table 3-1. Experimental Design for CPA.....	22
Table 4-1. Elevated Plus Maze Experimental Design .....	34
Table 4-2. Elevated Plus Maze Infusion Groups.....	34
Table 4-3. Summary of One-Factor ANOVAs for Lidocaine Infusion Volumes.....	36
Table 4-4. Summary of One-Factor ANOVAs for Control Site Infusions .....	38
Table 4-5. Summary of One-Factor ANOVAs for Repeated Lidocaine Infusions .....	39
Table 4-6. Summary of One-Factor ANOVAs for Vehicle Pre-Infusions .....	42

## **List of Abbreviations**

RAIC, Rostral Agranular Insular cortex  
CGIC, Caudal Granular Insular cortex  
S2, Somatosensory Cortex  
S1J, Secondary Somatosensory Cortex  
BLA, Basolateral Amygdala  
VEH, Vehicle  
LIDO, Lidocaine  
aCSF, artificial cerebral-spinal fluid  
CPP, Conditioned Place Preference  
CPA, Conditioned Place Aversion

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## **ABSTRACT OF THE THESIS**

Inactivation of the Insular Cortex Increases Anxiety-Like Behavior in Rats: Relevance to Drug Abuse and Interoception

By

Monica Wolfe

Master of Science in Biology

University of California, San Diego, 2011

Professor Gerhard Schulteis, Chair

Professor P. A. George Fortes, Co-Chair

The insular cortex is an integral component of the interoceptive circuit, and is thought to be important in regulating changes in hedonic valuation of interoceptive information. In this thesis, direct insular cortex manipulation or “silencing” of both the caudal granular insular cortex (CGIC) and Rostral Agranular insular cortex (RAIC) was used to examine the role of the insula in the interoceptive circuit as it relates to regulation of behavior. Insular inactivation resulted in behavioral modifications consistent with an increased anxiety-like state in the 1) place conditioning, 2) elevated plus maze, and 3) acoustic startle paradigms. In the conditioned place aversion test, RAIC and CGIC surgically altered animals showed an innate bias for the darker compartment which was not present in intact rats. Testing in the elevated plus maze revealed a significant anxiety-like effect following insula inactivation on the behavioral

measures of percent time, percent distance and number of entries in the open arms of the maze. Lastly, a trend for elevated startle magnitude was seen in RAIC and CGIC rats, although only CGIC rats receiving infusions showed significantly higher startle magnitude. These anxiety-like effects detected after insular manipulation in each of the three behavioral paradigms indicate that the insula plays an important role in the interoceptive circuit responsible for coordinating the integration of external and internal stimuli as a means to generate behavior responses appropriate to the current state of the body, and thus may indicate a role for the insula in regulating drug addiction, anxiety, and other related indications.

## 1 Introduction

Drug addiction and recreational use of prescription drugs, especially opioids, is a serious ongoing problem in the US with substantial growth occurring within the last decade (Compton and Volkow 2006). Although drug abuse costs the health care system billions of dollars a year, effective treatments are few and most prove ineffective at preventing future relapses. Addiction is defined as “compulsive drug use despite negative consequences” including failure in life roles, medical problems, risk of injury and trouble with the law (Hyman 2005). Drugs have both direct and conditioned effects, however, the most defining characteristic of addiction is its persistent nature. Although some individuals are able to stop compulsive use of drugs without assistance, addiction for most people proves to be a chronic and relapsing condition (Hyman 2005). Withdrawal symptoms can be intensely unpleasant, variable, and include a wide range of bodily systems. For opioid dependence, withdrawal symptoms can include various autonomic and somatic symptoms such as increased blood pressure, diarrhea, sweating, as well as anxiety, dysphoria, depression, and irritability (O'Brien et al. 1976; Zhang and Schulteis 2008). While the symptoms of withdrawal can cause escalation to compulsive use, maintenance and relapse, one of the main causes of relapse is craving caused by exposure to environmental cues (including people, places, and drug paraphernalia) previously associated with drug use (Hyman 2005; Zhang and Schulteis 2008).

Investigators have long sought answers to the neural mechanisms of drug addiction, and now believe that addiction represents a pathological hijacking of the neural mechanisms normally used for learning, memory, and reward. Normally, internal motivational states (such as hunger and thirst) cause an increase in the value of external stimuli (such as sight or odor of food) and can strengthen motivational

states related to achieving the goal (such as eating or drinking) and maintaining internal homeostasis. As a result, motivational behaviors can be initiated by the presence of goal-related cues predictive of some form of “reward” (achieving the goal or avoiding a negative consequence) (Hyman 2005). Addictive drugs can elicit similar behavior patterns. Neuroadaptive responses to opioids are influenced by conditioned associations between drug-use and environmental cues, and are consistent with recreational use of drugs where addicts often experience intoxication and withdrawal under similar conditions and in specific surroundings (Koob and Le 2005; Amitai et al. 2006; Koob and Le 2008). Even after a period of prolonged sobriety, cues associated with previous drug use (such as people, places, or drug paraphernalia) can activate drug-craving, seeking, and consumption (Stolerman 1993; Wang et al. 1999). Basically, conditioned stimuli can trigger a neural cascade manifesting as a physical desire to use a drug. This becomes a motivational goal for the body and actions are deployed to satisfy the urge (Davenport 2008; Paulus et al. 2009).

Interoception can be defined as the “sense of the physiological condition of the body” (Craig 2003). This includes three important aspects: 1) sensory information input (temperature, itch, pain, hunger, thirst, etc), 2) an evaluation of the homeostatic state of the individual, and 3) a motivational response appropriate to the situation (Craig 2002; Craig 2009). These interoceptive body states are distinct from somatic sensations in that they have intrinsic hedonic (pleasant or unpleasant) value. One example, given by Paulus and Stein, explains how the properties of a stimulus can be viewed differently depending on the internal state of the individual. For example, the thermal sensation of heat experienced when tissue is in contact with an object that is hot often creates a strong withdrawal action. However, the motivational component of the signal (movement away from the heat) depends on the homeostatic evaluation of

the individual and determines whether a stimulus is rewarding or harmful depending on the current state of the body (thus, heat may be favorable to a person experiencing hypothermia) (Craig 2002;Paulus and Stein 2006;Paulus et al. 2009;Naqvi and Bechara 2009). Overall, integration of interoceptive information creates an internal representation of the entire body at a given point in time.

Interoception is important for understanding drug addiction because the physiological changes induced by drugs change the overall interoceptive representation of the body. The central body state of the individual is relevant to how the constructs of reward, craving, and urges are perceived. Thus, the interoceptive state is crucial for determining propensity for drug addiction; whether an individual will likely want to take a drug again or be unable to resist despite negative consequences (Paulus et al. 2009;Paulus and Stein 2010). In addition, nearly all drugs of abuse have interoceptive properties (sensory stimuli with intrinsic hedonic value such as tastes, smells, sensations, pain, and autonomic changes) that impart distinct subjective qualities to drug-use rituals. With repeated exposure, interoceptive and environmental cues become associated and begin to reinforce conscious ongoing drug use and increase the likelihood of relapse (Naqvi and Bechara 2009). In laboratory studies, exposure to drugs and/or drug-conditioned cues have been shown to produce physiological responses and activation of certain brain regions including the amygdala, insular cortex, nucleus accumbens, and other related structures (Wang et al. 1999;Hyman 2005;Miller and Marshall 2005). Activation of these regions in response to drug-conditioned stimuli suggests they may be involved in encoding a representation of the interoceptive effects of drug use, in a classical “pavlovian” conditioning mechanism. The result is a subjective feeling of urge that is tied to the memory of the drug and drug-use ritual, and puts many at risk for relapse even years

after “quitting”. This understanding of interoception’s role in addiction provides an important starting point for understanding, and possibly treating, the neural basis of drug addiction.

The interoceptive “circuit” consists of an extensive network of afferent and efferent connections through many brain regions. Interoceptive sensory information travels by small diameter C-fiber afferents and terminates on lamina-I neurons in the spinal dorsal horn (Craig 2007). With a series of projections through a thalamic-cortical relay, signals end up projected onto the posterior insular cortex, a hidden region of the frontal cortex that is bounded by the orbital and sensory cortices (Shi and Cassell 1998; Craig 2007; Butti and Hof 2010). Once inputs are received, the posterior insula provides time and location-specific information to the anterior insular cortex, where it is integrated further with homeostatic information from the body. Interoceptive states are also centrally generated (independent of direct sensory input, for example, remembering a feeling or sympathizing with the feelings of others) within the temporal and parietal cortex and projected onto the anterior insular cortex (Craig 2002; Craig 2003; Gray and Critchley 2007; Lerner et al. 2009; Paulus et al. 2009). Within the anterior insular cortex, a multi-faceted representation of the current body state is presented as a “global moment in time” which allows the individual a sense of awareness for itself and surroundings (Craig 2009). Efferent pathways lead from the anterior insular cortex to other subcortical, limbic and executive regions capable of controlling salience and attention (such as the amygdala), reward and motivation (such as the nucleus accumbens and ventral striatum), valuation of environmental stimuli (such as the orbitofrontal cortex), and motor output (such as the anterior cingulate cortex, ACC) (Craig 2002; Craig 2003; Paulus et al. 2009; Naqvi and Bechara 2009; Paulus and Stein 2010). (See Figure 1.1 for schematic). Thus, the posterior granular insular cortex is

more involved with basic visceral, motor, and somatosensory information while the anterior agranular insula is better positioned for higher order processes such as integration of autonomic and visceral information into relevant emotional and behavioral correlates (Craig 2002; Craig 2003). Functional imaging studies have confirmed insula activation during a wide array of sensory (such as sensual touch, thirst, pain, itch, hunger, temperature, and metabolic state) and emotional (such as urges, salience, awareness, self-recognition, risk, intent, attention, and perception) processes relevant to interoceptive circuitry. See and (Craig 2003; Craig 2009) for review.

Interestingly, disruption of the interoceptive circuit reverses drug-conditioning processes causing the interoceptive effects of drug use and withdrawal to lose their motivational value. Individuals may be able to quit “easily, immediately, without relapse and without the urge”, which is what has been reported in individuals with lesions that included the insular cortex (Naqvi et al. 2007). Naqvi et al (2007) showed that patients who experienced lesions in the insula (as a result of stroke) had diminished addictive behavior for smoking. They suggest this effect might be due to a reduced ability to detect interoceptive states linked to craving or a reduced ability to recognize the hedonic feelings induced by smoking a cigarette, thus implicating the insula as a “critical neural substrate in the addiction to smoking” (Gray and Critchley 2007; Naqvi et al. 2007). Recently, animal behavioral studies have also shown that the granular region of the Insula is critically involved in nicotine drug addiction in rats; inactivation of the region decreased nicotine seeking behavior and reinstatement (Forget et al. 2010) while blockage of insular hypocretin-1 receptors (but not somatosensory hypocretin-1 receptors) decreased nicotine self-administration (Hollander et al. 2008). Additionally in rats, insula inactivation was shown to block

expression of amphetamine-conditioned place preference (Contreras et al. 2007). These studies are in line with an interoceptive model of addictive behavior in which exposure or recollection of an experience with a drug reactivates an “interoceptive memory state” (Jones et al. 2010; Naqvi and Bechara 2010). When the Insula is not functioning, it is unable to represent that memory state and therefore is unable to suggest an output signal (i.e. an urge to use drugs) (Gray and Critchley 2007). This is consistent with patients in Naqvi’s study (2007) who “forgot the urge” to smoke (Naqvi et al. 2007). In addition, the cytoarchitecture of the agranular insula shows a high density of D1 dopamine receptors, type-1 corticotrophin-releasing hormone receptors, and mu-opioid receptors which also support its central role in mediating the effects of drug use (Hurd et al. 2001; Baumgartner et al. 2006; Naqvi and Bechara 2009).

Aberrant interoceptive processing isn’t confined solely to addiction. Changes in interoceptive processing can alter the body state representation of an individual (with regard to awareness of self and environment) leading to erroneous belief-based decision making (Paulus and Stein 2010). Both anxiety and depression have been shown to alter interoceptive processing such that there is an increased bias towards negative self-view (depression) or an increased attention bias towards threat (anxiety). The result is an abnormal body state and sense of “self” from which interoceptive signals are interpreted (Paulus and Stein 2010). Anxiety disorders consist of a group of conditions including generalized anxiety disorder, panic disorder, post-traumatic stress disorder (PTSD), social phobia, and specific phobias associated with object-related anxiety (Amstadter 2008). Common to all of these pathologies is an extreme avoidance of an event, object, or situation that has become associated with an intense negative emotional state (Paulus and Stein 2010). Compared to normal subjects, patients with these disorders have been shown to have increased insula activation

during processing or anticipation of aversive stimuli (Wright et al. 2003; Simmons et al. 2006; Stein et al. 2007), and may be more sensitive to interoceptive signals in general, but less able to differentiate interoceptive noise from relevant rewarding or aversive afferents (Paulus and Stein 2010).

As seen in many studies, the insula plays a significant role in regulating interoceptive processes important for a variety of functions and implicated in a variety of disorders. Although the insula has been linked to interoceptive function in drug addiction, the precise role of this structure as it relates to onset, development, and maintenance of drug dependence is still unclear. Very few animal models of drug reward and withdrawal have studied the insula exclusively. Understanding of this brain region and its role in regulating drug-induced changes in interoceptive processing may provide novel targets for addiction treatment and therapy.

In this thesis, the role of the insular cortex in mediating the hedonic processing of interoceptive stimuli will be examined. Direct insular cortex manipulation or “silencing” of both the caudal granular insular cortex (CGIC) and rostral agranular insular cortex (RAIC) through inhibition with the Na<sup>+</sup> channel blocker lidocaine will be used to examine the function of the insula as a component of the interoceptive circuit, specifically with regard to drug addiction. By differentiating the region into anterior and posterior components, we hope to identify any regulatory/pathway differences between the two regions (i.e. posterior insula is involved in processing the interoceptive effects of drug use while anterior insula is involved in drug-related urges leading to relapse). If the insula plays a critical role in the experience of drug use, as previous studies have indicated, silencing of the region should diminish behaviors associated with drug addiction.

The results of these experiments should help to identify the role the insular cortex plays in regulating interoceptive states, and more generally, the effects of insula inactivation (and disruption of the interoceptive circuit) on behavior. Based on the results, modulation of insula activity through non-invasive approaches may be considered as a therapeutic target for addiction (and other related conditions) as a means to ease cravings and withdrawal symptoms and prevent relapse.

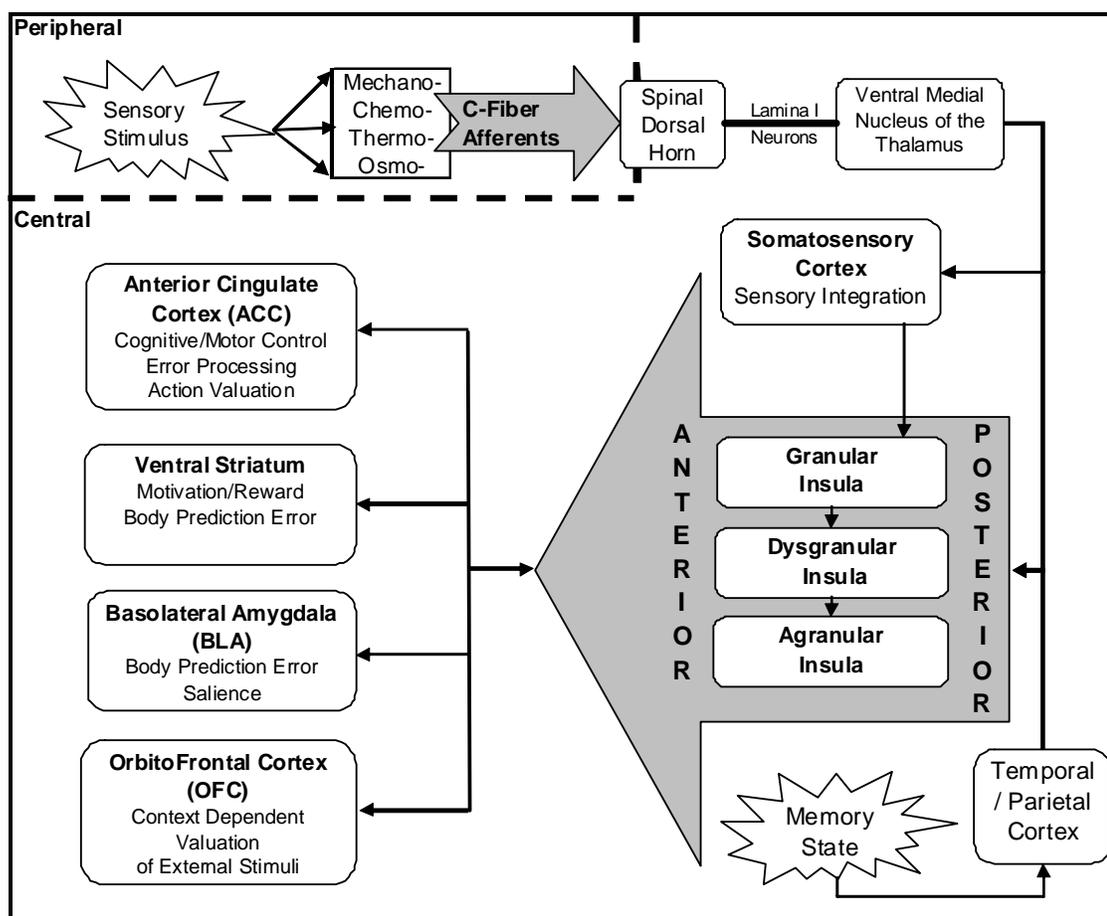


Figure 1-1. Overview of the Interoceptive Circuit

## **2 Materials and Methods**

### **2.1 Animal Selection**

Male Wistar rats (n=503) purchased at a weight of 200-225g (Harlan, Livermore facility) were pair housed in a controlled environment with a 12 hour light/dark cycle (lights on at 6am), and had *ad libitum* access to food and water. At the time of surgery, all rats weighed between 300g and 350g, and had been acclimated to the colony for at least one week. All experimental procedures were approved by the Subcommittee on Animal Studies of the VA San Diego Healthcare System, an AAALAC-accredited facility, and were carried out in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals" (revised 1996). All efforts were made to minimize animal suffering and to reduce the number of animals required.

### **2.2 Drugs**

Drugs for injection were prepared using physiological saline (0.9%) and all injections were made subcutaneously (SC) in a volume of 0.1ml/100g body weight. Doses of drugs are expressed as the salt. Morphine Sulfate (Research Resources Drug Supply System of the National Institute on Drug Abuse, Bethesda, MD, USA) was administered SC at a dose of 10mg/kg. Naloxone HCL (Sigma, St. Louis, MO, USA) was administered SC at a dose of 1.0 mg/kg.

### **2.3 Insular cortex Bilateral Cannulation Surgeries**

Rats were anesthetized by inhalation of 3-4% isoflurane (plane 2) and the surgery site was shaved and cleaned with a combination of alcohol and betadine. Rats were placed in a stereotaxic apparatus in order to chronically implant the guide cannulae . The incisor bar was set to "flat skull" at a level of -3.3mm. Cannulae (26 gauge, Plastics One, VA) were 9mm long, stainless steel, and sterilized in 70% EtOH. They

were fixed to the skull with stainless steel screws (Plastics one, VA) and dental acrylic. In order to maintain cannulae patency, a 9mm stylet occluder was inserted immediately following surgery and kept in place throughout recovery and between infusions. Antibiotics and local analgesic were administered to the site of the incision at the end of surgery and rats were allowed at least 5 days to recover before entering any behavior tests.

Guide cannulae were aimed at the following regions of the Insular cortex:

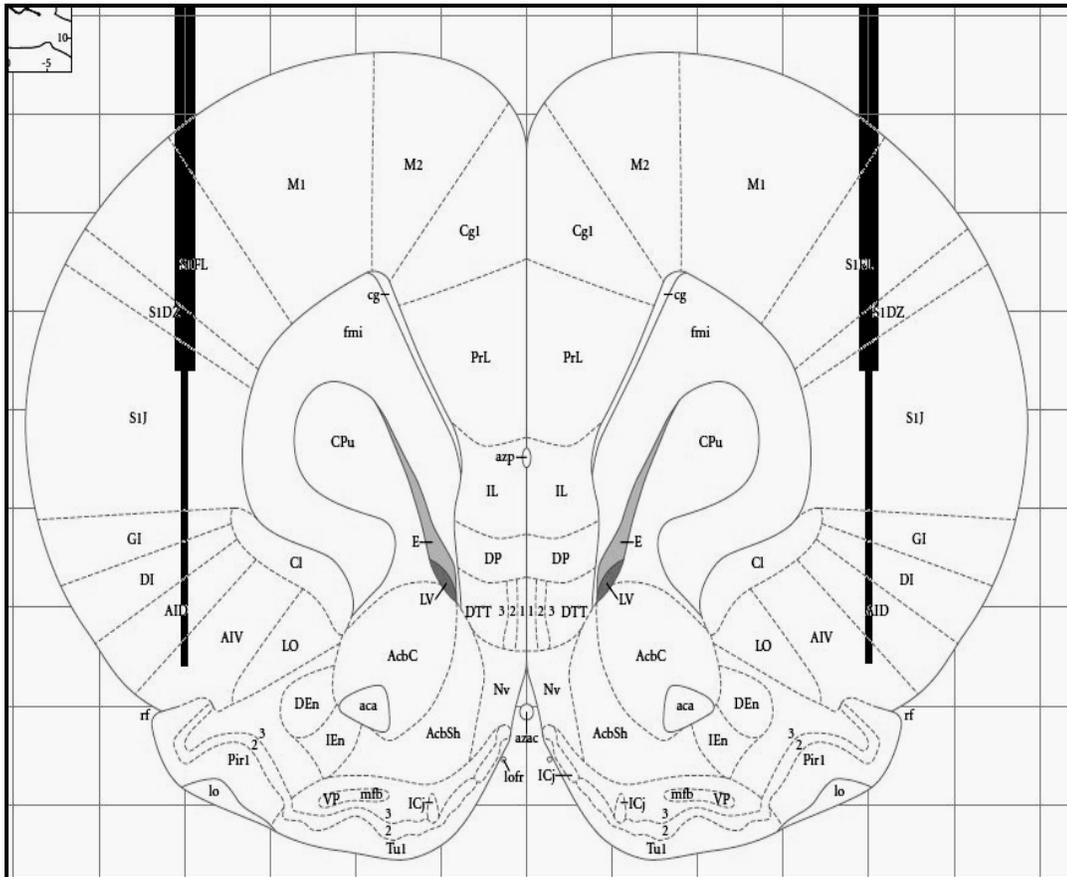
### **2.3.1 Rostral Agranular Insular Cortex (RAIC)**

In order to target the rostral agranular region of the insular cortex, guide cannulae were aimed at the following coordinates of the Paxinos and Watson Rat Brain Atlas (6<sup>th</sup> edition, 2007); relative to bregma +2.5mm anteroposterior, +/- 4mm medial lateral, -3.6mm dorsoventral from cranial surface. Injectors (33 gauge, Plastics One, VA) extended +3mm from the end of the cannula to a depth of -6.6mm from the cranial surface to terminate in the agranular insular cortex. See Figure 2-1 for cannulae placement.

### **2.3.2 Caudal Granular Insular Cortex (CGIC)**

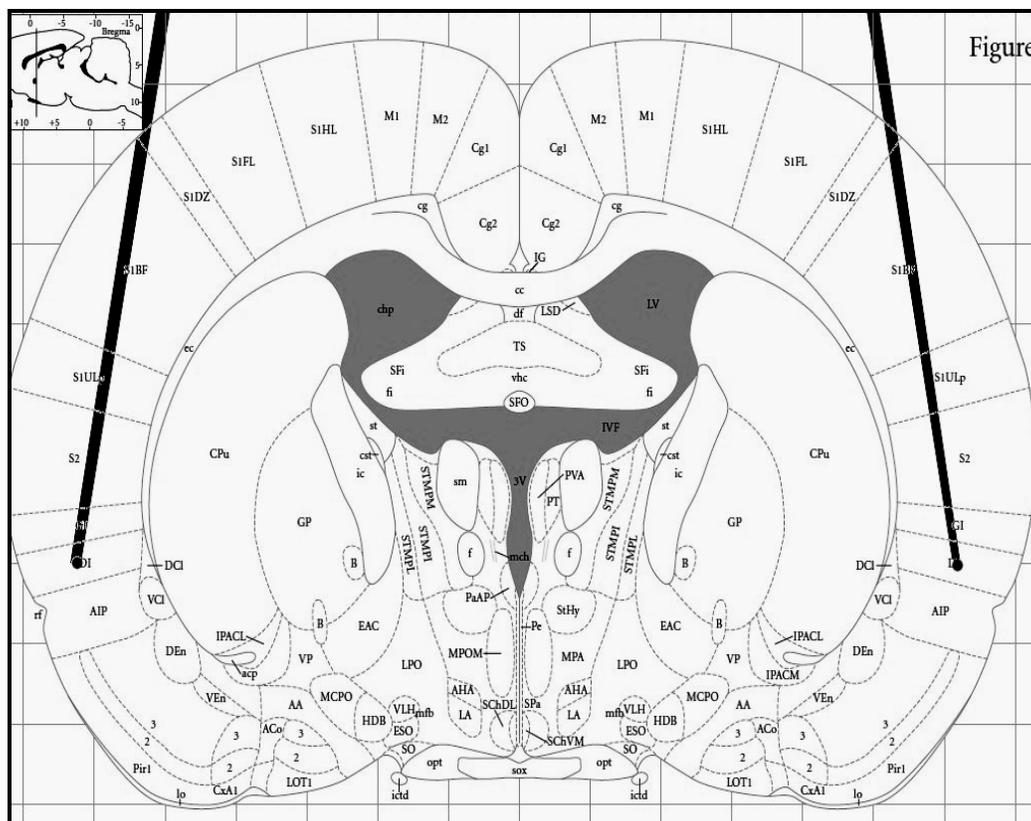
In order to target the caudal granular region of the insular cortex, guide cannulae were aimed at the following coordinates of the Paxinos and Watson Rat Brain Atlas (6<sup>th</sup> edition); relative to bregma -1.0mm anteroposterior, +/- 5mm medial lateral, -4mm dorsoventral from cranial surface. The guide cannulae were placed at a divergent direction of 10 degrees from vertical. Injectors extended +3mm from the end of the cannula to a depth of -7mm from the cranial surface to terminate in the granular insular cortex. See Figure 2-2 for cannulae placement.

In order to show that the behavioral effects observed were result of inactivation of the insular cortex, guide cannulae were also aimed at two regions +1mm above the Insular Cortex to serve as diffusion controls. These are referred to as RAIC-ctrl and CGIC-ctrl surgeries and are identical to the corresponding RAIC and CGIC surgeries, except that they stopped 1 mm shorter in the dorsoventral direction, relative to cranial surface.



**Figure 2-1. Cannulae and Injector Placement for RAIC**

**Schematic showing proposed location of cannulae and injectors targeting the RAIC (AP+2.5mm). Images adapted from the atlas of Paxinos and Watson (6th edition). GI, granular insula; DI, dysgranular insula; AID, dorsal agranular insula; AIV, ventral agranular insula; SIJ, secondary somatosensory cortex; LO, lateral orbital; rf, rhinal fissure**



**Figure 2-2. Cannulae and Injector Placement for CGIC**

**Schematic showing proposed location of cannulae and injectors targeting the CGIC (AP-1.0mm). Images adapted from the atlas of Paxinos and Watson (6th edition). GI, granular insula; DI, dysgranular insula; AIP, posterior agranular insula; S2, somatosensory cortex; rf, rhinal fissure**

## 2.4 Cortical Inactivation

The injectors were attached to a 10 $\mu$ l Hamilton Syringe by polyethylene tubing (Plastics One, VA), filled with 4% Lidocaine or artificial CSF (for control groups) and inserted into the guide cannulae after removal of the stylet. 1  $\mu$ l of lidocaine (or aCSF) was infused over the period of two minutes on each side using a Harvard micro-infusion pump, 5 minutes prior to testing. Following infusion, the injectors were left in place an additional 60 seconds to prevent backflow into the cannulae and the stylets were immediately replaced.

### **2.4.1 Use of Lidocaine**

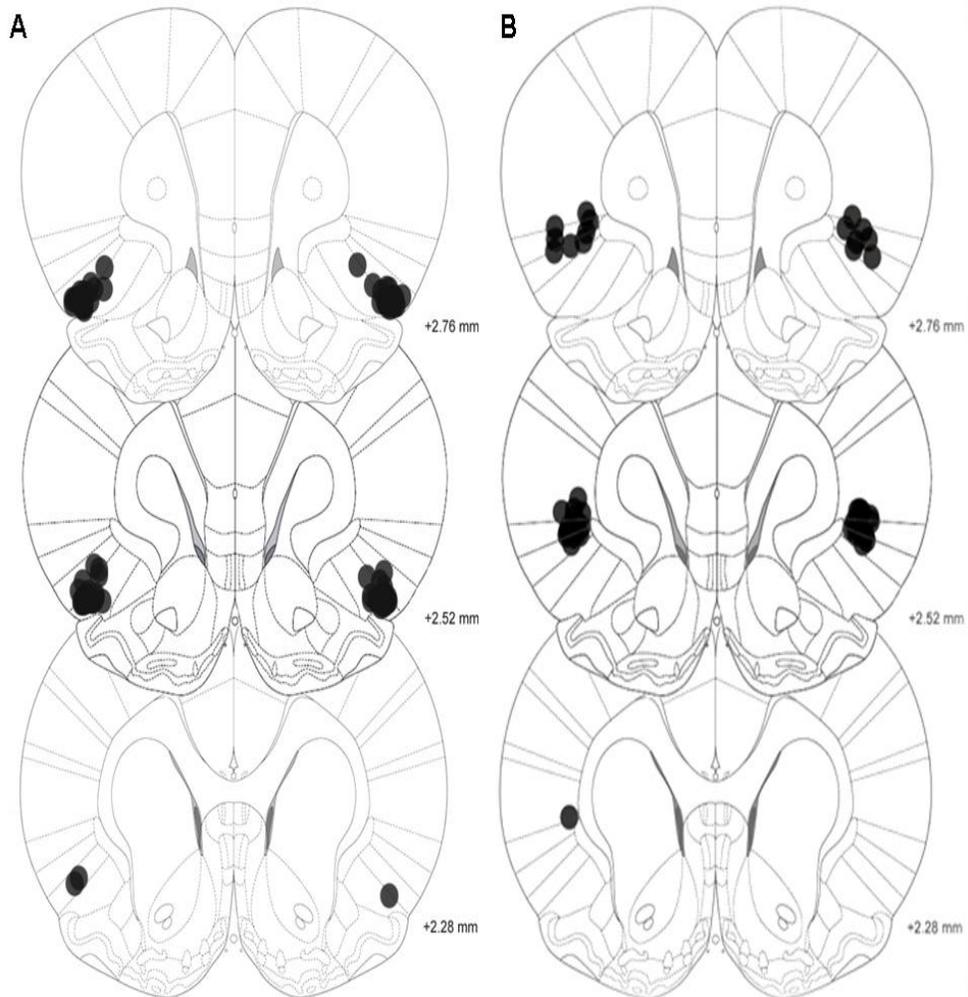
Lidocaine, a voltage-dependent sodium channel blocker, was used for reversible cortical inactivation of the insular cortex because it has been found to reduce neural activity by 50% of baseline for up to 20 minutes after infusion, and has a radial diffusion of <1mm (Tehovnik & Sommer, 1997; Boehnke & Rasmusson, 2001). Maximum inactivation is achieved within 5 minutes, and neural activity gradually returns so that 50% of baseline activity is recorded at 20-30 minutes post infusion (Tehovnik & Sommer, 1997). Muscimol, a GABA<sub>A</sub> agonist, was also an option for reversible inactivation of the insula, however, lidocaine was chosen over muscimol as a long lasting inhibition was not necessary for the desired behavioral tests (Van Duuren et al. 2007). Also, preliminary tests showed fluorescent-labeled muscimol had a much larger and uneven radial diffusion, and seemed to be unable to pass through certain brain tissues – causing, in some cases, unilateral/unidirectional diffusion (data not shown). Both drugs inhibit neuronal activity, but lidocaine also blocks fibers of passage (Van Duuren et al. 2007).

### **2.5 Histological Verification**

Following completion of behavior testing, permanent injector stylets (12mm long, extending +3mm past the end of the cannula) were inserted and left in place for a minimum of 5 days. Animals were then injected with euthasol (10ml/kg) and perfused through the left ventricle with a saline flush followed by 10% formalin. The brains were removed and post-fixed in 10% formalin for 24 hours, followed by 30% sucrose in phosphate buffered saline (PBS) until they sank. Brains were cut frozen at a thickness

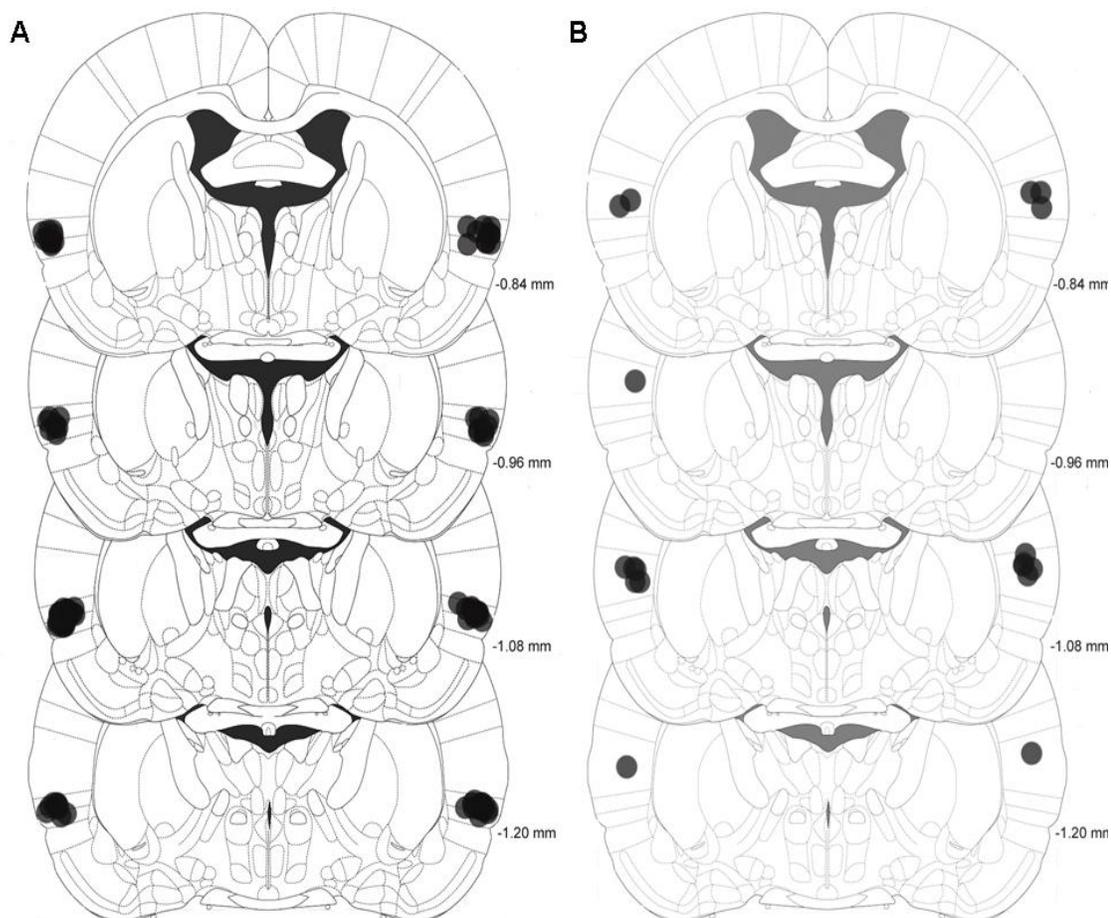
of 50  $\mu\text{m}$  along the coronal plane using a sliding microtome/cryostat. Sections were then stained with cresyl violet for proper cannulae visualization.

Inclusion of rats in statistical analyses was based on the histological verification of cannula and injector placement, without knowledge of the behavior data for individual rats. Only animals where the injectors clearly terminated in the Insular Cortex were used in the study. In the case of the RAIC, the injectors had to terminate bilaterally in the rostral agranular insular cortex within the region extending from AP +2.28 to +2.8 mm in front of bregma (Figure 2-3). In the case of the CGIC, the injectors had to terminate bilaterally in the caudal granular or dysgranular insular cortex within the region extending from AP -0.8 to -1.2mm behind bregma (Figure 2-4). Inclusion of diffusion control infusion sites for each region required that the injector terminate +1mm above the respective region of interest (Figure 2-3 (b), Figure 2-4 (b)).



**Figure 2-3. Histological Verification of RAIC Lidocaine Infusion Sites**

**Injector termination for a) RAIC lidocaine infusions and b) RAIC-ctrl lidocaine infusions. Numbers represent distance from bregma, anteroposterior. Dots represent location of injector termination. Images adapted from the atlas of Paxinos and Watson (6<sup>th</sup> edition).**



**Figure 2-4. Histological Verification of CGIC Lidocaine Infusion Sites**

**Injector placement for a) CGIC lidocaine infusions and b) CGIC-ctrl lidocaine infusions. Numbers represent distance from bregma, anteroposterior. Dots represent location of injector termination. Images adapted from the atlas of Paxinos and Watson (6<sup>th</sup> edition).**

### **3 Experiment 1: Effects of Reversible Inactivation of the Insular Cortex on Place Conditioning**

#### **3.1 Introduction**

Place conditioning is a behavioral test used to assess the rewarding or aversive nature of an unconditioned stimulus. Briefly, animals are placed in a distinct neutral environment paired to a rewarding or aversive unconditioned stimulus (UCS). Later, animals are given the choice to spend time in the environment paired with the UCS or in another environment paired with a neutral control stimulus. During the course of conditioning, the distinct stimuli in the UCS paired environment gain motivational properties such that they can act as conditioned stimuli (CS) to elicit behavioral responses. An increase or decrease in the time spent in the UCS-paired environment post-conditioning reflects the treatment's reinforcing or aversive properties, respectively. For example, if the UCS is perceived to be negative in nature, avoidance of the UCS-paired environment will occur and the animal will spend less time in contact with cues associated with the negative UCS (Mucha et al. 1982; Tzschentke 2007). The place conditioning design is advantageous for many reasons; 1) it is very sensitive to motivational variables and is likely to measure aversive behavior at very low levels that may not be detected by other procedures, 2) it allows use of an acute model of drug exposure since there is no pre-training or conditioning required, 3) detection of motivational changes is achieved with very minimal stress to the animal, 4) testing occurs when the animal is OFF drug, and 5) it allows for differentiation between the development (acquisition) and expression of a conditioned behavior by targeting either the conditioning or testing phase of the experiment.

Previous studies have shown the place conditioning test to be a highly sensitive index of the aversive motivational consequences of withdrawal from both chronic and acute states of opioid dependence. Acute opioid dependence is defined as the “precipitation of withdrawal-like signs by opioid antagonists following a single dose or a short-term infusion of opioid agonists” (Martin and Eades 1964). Studies have demonstrated acute withdrawal symptoms across a broad range of somatic, physiological, and subjective symptoms similar to those observed upon withdrawal from a chronic state of opioid dependence, including emotional disturbances such as dysphoria-like and anxiety-like behavior (Haertzen et al. 1970; West and Gossop 1994; Tehovnik and Sommer 1997; Aston-Jones and Harris 2004; Koob and Le 2008). For example, after a single dose of morphine, naloxone (an opioid antagonist) will precipitate withdrawal symptoms in rats including elevations in ICSS thresholds and conditioned place aversion (Azar et al., 2003; Liu and Schulteis, 2004; White et al., 2005) decreased operant responding for food (Schulteis et al., 1997; Schulteis et al. 2009), and decreased open arm exploration in the elevated plus maze (Zhang and Schulteis, 2008).

In the place conditioning paradigm, the memory of the association between naloxone-induced withdrawal symptoms and novel environment is crucial for the acquisition and expression of a conditioned place aversion. By reversibly inactivating the insular cortex at either the acquisition or expression phase of testing and measuring changes in place conditioning, it is possible to determine which motivating properties of opiate addiction and withdrawal the insula is important in regulating. Using an acute model of drug dependence, it is possible to circumvent many of the complications that arise with repeated exposure (including tolerance, somatic

symptoms and physical dependence), while at the same time limiting the amount of damage to the brain during infusion.

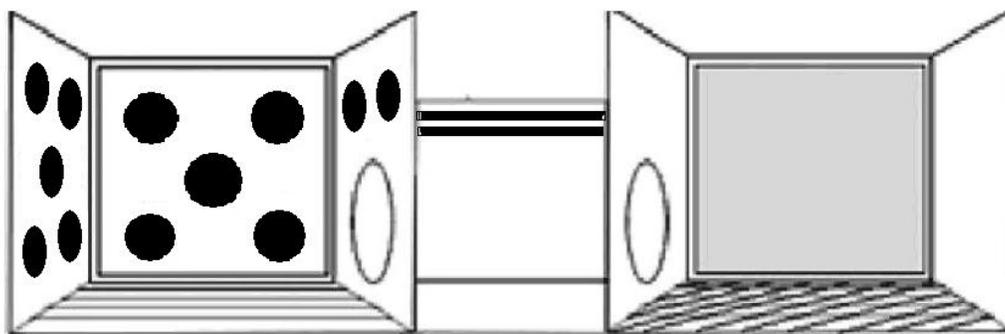
Place conditioning has been used previously in the study of the Insula. Contreras, et al (2007) showed that inactivation of the insula disrupts conditioned place preference to amphetamine drug treatment. Rats exposed to amphetamine develop a preference for a naturally avoided white compartment, however, infusion of 2% lidocaine into the insula temporarily reverses this preference. Subsequent testing without lidocaine inactivation showed that the animals continued to prefer the white compartment, suggesting that inactivation of the insula disrupted the expression of drug conditioned place preference (Contreras et al. 2007).

For this thesis, place conditioning was chosen as an appropriate means for measuring withdrawal from acute opioid dependence for the following reasons; 1) as mentioned above, prior work on the role of the insula in animal models of drug reward and dependence has focused almost exclusively on place conditioning and 2) previous work on withdrawal-induced CPA from acute morphine dependence provided a strong one-trial conditioning paradigm that had already been validated in reversible inactivation studies of the basolateral amygdala (BLA), providing a positive control against which to compare initial insula inactivation results. In those studies, both the CPP to morphine and the CPA to naloxone-precipitated withdrawal were completely reversed by bilateral infusion of lidocaine (1  $\mu$ l of 4% solution) into the BLA (Schultheis and Chiang 2010).

The purpose of this experiment was to determine whether reversible inactivation of the RAIC or CGIC could abolish acquisition and/or expression of a conditioned place aversion following acute opioid withdrawal.

### 3.2 Apparatus

Setup of the place conditioning apparatus was accomplished using an unforced design that was previously shown to be un-biased in naïve, uncannulated animals (Schulteis and Chiang 2010). The apparatus consisted of two square boxes (30x30x30 cm each) accessible via a rectangular center compartment (15x30x30 cm) all aligned in a linear fashion. Visual (solid white or black dots on walls) and tactile (rough or smooth plastic flooring) cues distinguished each of the conditioning compartments (referred to hereafter as the “dots” or “white” compartments) and the specific combination used was shown to be unbiased in naïve animals. The narrow center compartment had no special characteristics (except for increased light intensity) and was not paired with a drug treatment; dividers between each compartment were inserted in order to confine the animal to the desired area during conditioning. Positioning of the animal was recorded (distance, time, and number of entries for each box) using photocell beams in each of the compartments. The apparatus was located in a dimly lit testing room equipped with a white noise generator to control for differences in noise throughout the testing period.



**Figure 3-1. Place Conditioning Box Design**

**Visual and textual cues were varied between the two conditioning compartments (dots vs. white walls, rough vs. semi-smooth floors). Image modified from (Prus et al. 2009)**

### 3.3 Experimental Design

Testing was accomplished as previously described (Azar et al. 2003;Schulteis and Chiang 2010). Briefly, conditioned place aversion testing consisted of three phases: 1) a pre-conditioning phase, 2) a conditioning phase, and 3) a test phase. Prior to the beginning of testing and after at least 5 days recovery from bilateral insular cortex cannulation surgery, rats were handled 5 minutes each and housed in the holding room for at least 1 hour to acclimate and overcome their tendency to freeze when placed in a novel experiment.

Day 1 - Pre-Conditioning Phase: Naïve RAIC and CGIC surgery rats were given a vehicle injection before being placed into the narrow center compartment of the three-compartment conditioning box and allowed to freely roam the box for 20 minutes (1,200 seconds). This phase allows the animals to habituate to the apparatus and provides a baseline measurement of distance, time and entries for each compartment. Rats spending more than 700s or less than 300s in either of the two conditioning compartments were said to have “failed” the pre-conditioning phase and were not used in the study (roughly 10% failure rate is typical). At this point, one compartment of the apparatus was chosen to be paired with drug-treatment and the other with vehicle treatment during the conditioning phase.

Days 2 and 3 - Conditioning Phase: During the conditioning phase, a drug treatment (morphine/naloxone) and a control treatment (vehicle/vehicle) are paired with different sets of stimuli in each of the conditioning boxes. To measure a CPA, rats were injected with morphine (10mg/kg, SC), followed four hours later by an injection of naloxone (1.0mg/kg, SC). Immediately following the naloxone injections, animals were confined in either of the pre-selected compartments (dots or white) for 20 minutes. On the alternate conditioning day, animals received vehicle injections only and were

confined in the other compartment (white or dots) for 20 minutes. Both the day of drug-treatment and the drug-paired compartment were randomly assigned and evenly distributed between animals and test groups. To test the role of the insula in acquisition of place conditioning, animals receive mock, vehicle, or lidocaine infusions 5 minutes prior to being placed in the drug-paired compartment.

Day 5 - Test Phase: After a day of rest (to prevent any possible withdrawal hangover from the conditioning phase) all rats were given a vehicle injection before being placed in the testing apparatus and allowed free exploration for 20 minutes. Again, distance, time, and entries for each compartment were measured. To test the role of the insula in expression of place conditioning, animals receive mock, vehicle, or lidocaine infusions 5 minutes prior to being placed in the conditioning apparatus.

Data are expressed as an “Aversion Score”, defined as the difference between time spent in the drug-paired compartment on the Test Day and the time spent in the same compartment on the Pre-Conditioning Day. The aversion score is used to evaluate how much the animal avoided the drug-paired compartment; a larger negative number represents a more aversive conditioning stimulus.

**Table 3-1. Experimental Design for CPA**

Treatment Day	Treatment Phase	Injection T=0	Infusion T=235mins	Injection T=240mins	Conditioning Apparatus
D1	Pre-Conditioning	VEH	N/A	VEH	Open
D2 or D3	Drug Conditioning	Mor 10	Lidocaine, Vehicle or Mock	Nal 1.0	Dots or White
D3 or D2	Vehicle Conditioning	VEH	N/A	VEH	White or Dots
D4	Rest Day	N/A	N/A	N/A	N/A
D5	Test	VEH	Lidocaine, Vehicle or Mock	VEH	Open

Assuming the insula is involved in the acquisition or expression of drug conditioned aversion, it is expected that lidocaine infusion prior to sessions on conditioning day or test day should reduce acquisition or expression, respectively, of withdrawal conditioned aversion to the drug-paired compartment

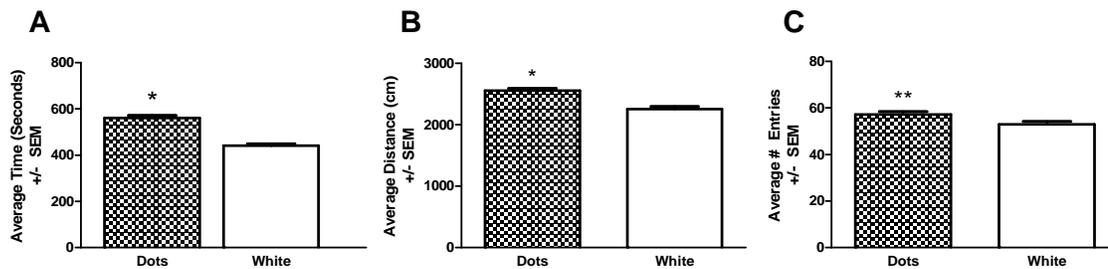
### **3.4 Data Analysis**

CPA was demonstrated by rats spending less time in the withdrawal-paired compartment on test day. This was quantified using the aversion score (the time spent in the drug-paired compartment on test day minus the time in the same compartment during the pre-conditioning phase). All behavioral data are presented as average +/- SEM. Student's t-tests were used to compare averages between the two compartments.  $P < 0.05$  was set as the level of statistical significance for all statistical analyses. Statistical calculations were performed using GraphPad Prism Software.

### **3.5 Results**

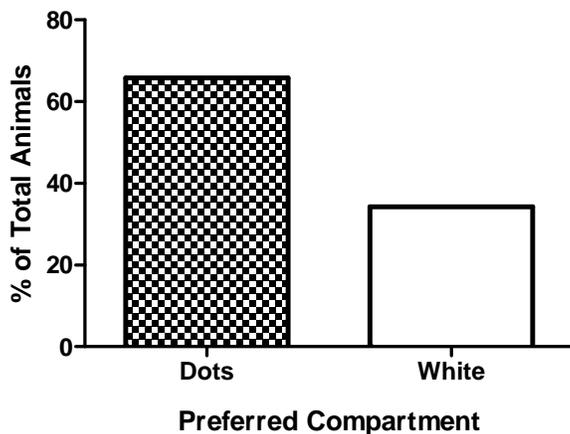
An analysis of pre-conditioning day data indicated a significant bias towards the dots compartment in RAIC and CGIC animals, as measured by time, distance, and entries. Rats spent an average of 562 +/- 10.08 seconds spent in the dots compartment compared to an average of 441 +/- 8.69 seconds spent in the white compartment (Figure 3-2, a), with roughly 2/3 of the animals showing a preference for the dots side compared to 1/3 preferring the white side (Figure 3-3). These rats showed a significant increase in time spent in the dots compartment compared to naïve uncannulated animals which had been previously shown to spend an average of 452 +/- 11 seconds in the dots compartment and 470 +/- 9 seconds in the white compartment (Schulteis and Chiang 2010). Distance and number of entries was also significantly higher in the dots compartment compared to white (Figure 3-2 b, c).

In addition, day one failure rates reflected this same bias. 15.98% of all animals spent more than 700s in the dots compartment compared to only 2.28% of all animals on the white side (Figure 3-4); normally an 8-10% total failure rate for both compartments is typical.



**Figure 3-2. Dots vs. White Pre-conditioning Day Differences**

Animals showed significantly higher a) time b) distance and c) entries in the dots compartment compared to the white compartment on day one of the pre-conditioning phase. Data represent mean +/- SEM for all RAIC and CGIC animals (n=219). Student's t-test \*p<0.0001, \*\*p<0.05 vs. white compartment



**Figure 3-3. Compartment Preference on Pre-Conditioning Day**

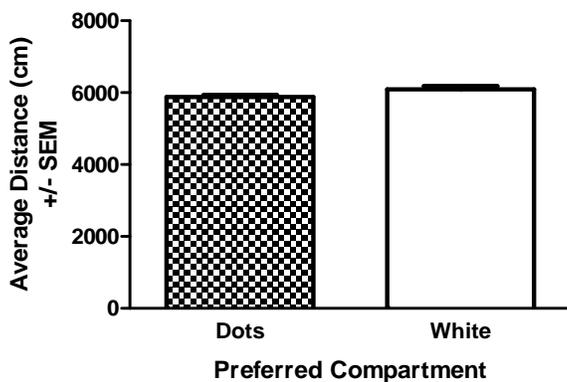
Percentage of total animals preferring either the dots or the white compartment after day one of testing. Data represent percentage of all RAIC and CGIC animals tested (n=219)



**Figure 3-4. Failure Rate for each Compartment**

Data represent percentage of total animals (n=219) that spent more than 700s in either the white or dots compartment, and were classified as “failing”.

Total distance traveled for both groups of rats (those preferring the dots compartment and those preferring the white compartment) was not significantly different, suggesting this bias was not due to an effect on motor ability (Figure 3-5).

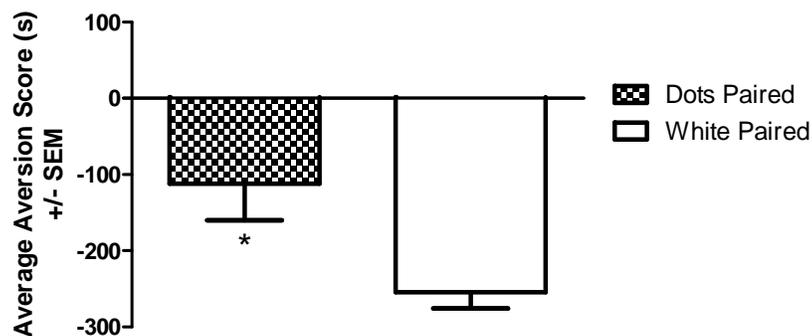


**Figure 3-5. Total Distance during Pre-Conditioning Phase**

Average total distance travelled by RAIC and CGIC animals preferring the dots compartment was not significantly different from animals preferring the white compartment. Student's t-test  $p=0.05$

Aversion scores in Mor10/Nal1.0 Mock and VEH infusion groups on test day were greater in animals with white as the drug-paired compartment (-245.5 +/- 21s) and smaller for animals with dots as the drug-paired compartment (-111.7 +/- 48s) (Figure 3-6), suggesting the initial bias was influencing the effects of drug conditioning as well. Previously, naïve uncannulated rats were shown to produce a significant, reliable aversion score of 149 +/- 33 seconds after treatment with Mor10/Nal1.0, with no main effect of drug-paired side (Schulteis and Chiang 2010).

The day one pre-conditioning bias prevented any further progress with this experiment, as accurate and consistent aversion scores from each compartment were unattainable.



**Figure 3-6. Effect of Drug-Paired Compartment on Aversion Score**

RAIC and CGIC animals received Mor10/Nal1.0 in combination with a Mock or Vehicle infusion and were paired with either the dots (n=27) or white (n=24) compartment on conditioning day. Aversion scores are expressed as the mean time (s) spent in the drug-paired compartment on test day minus the mean time (s) spent in the same compartment on the pre-conditioning day. Student's t-test \*p<0.05

### 3.6 Discussion

Applying previously established techniques to examine CPA following inactivation of the insular cortex revealed a significant bias in many rats. Following pre-

conditioning on Day 1, about 15% of all rats were excluded on the basis of spending too much time in the dots compartment (up to 10% total failure rate is typical for this behavior test), but even the animals that remained in the study clearly preferred the dots compartment over the white. This bias carried on throughout the experiment as aversion scores were significantly greater for animals that were paired with the white compartment. Conversely, animals initially preferred the dots compartment and were less likely to avoid it even after conditioning (aversion scores were smaller for the dots compartment).

It is important to have an unbiased apparatus because the magnitude of the aversion score depends on the initial baseline preference of the animals (Tzschentke 2007). When naloxone-precipitated withdrawal is paired with the dots compartment, the negative effects of the withdrawal treatment need to be stronger than the unconditioned bias for the compartment in order to see an aversion. On top of that, changes in preference on test day cannot be fully attributed to effects of the treatment. An unbiased design is capable of detecting these small changes in behavior that may not be interpreted correctly in a biased design.

To be defined as an unbiased apparatus, time averages for each compartment on day one should differ by less than 50s (average 20s difference between compartments in naïve animals), and aversion scores should average about -150s regardless of the drug paired compartment (Schulteis and Chiang 2010). This bias was unexpected as the visual and textural cue combination for each compartment required to maintain neutral preferences across a group of naïve and cannulated (targeting the BLA) rats had already been established in the laboratory. Since the only factor different in this experiment is the placement of the cannulae to target the Insular cortex, it can be

speculated that the brain regions being damaged as a result of the surgery are involved in generating the bias.

The regions dorsal to the insular cortex (and damaged by surgery) are the primary and secondary somatosensory cortices (S2 and S1J), and while initially unexpected, these results are consistent with what is known about these brain areas. Damage to the somatosensory cortices as a result of lesions from the cannulae placement could cause impairment of sensory input identification and interpretation (pain, touch, temperature, itch, proprioception, etc) (Carey 2006). Activation of the somatosensory cortices are also associated with the euphoric experience of opiate drug use. Meng, et al showed that lesions of the somatosensory cortices prevented acquisition of morphine-conditioned place preference, possibly by negating the rewarding somatosensory effects of drug use (Meng et al. 2009). Even though the somatosensory cortices have only been implicated in the rewarding sensory experiences of drug use, their afferent connections with the insula and interoceptive circuit could explain both the initial change in bias towards the dots compartment and the blunted effect of conditioning after naloxone-precipitated withdrawal. Damage to the somatosensory cortex may make the animals more sensitive to changes in texture or light intensity, which could explain why they prefer the dots compartment over the white. Additionally, they may be unable to fully process the negative sensory experience associated with withdrawal, thus allowing persistence of the initial bias towards the dots compartment.

Alternatively, the change in preference towards the darker of the two compartments could be a reflection of increased anxiety levels in these rats. The insula (as a part of the interoceptive circuit) has been implicated in regulating the negative affective states of abnormal psychological disorders, such as anxiety and

depression (Ibanez et al. 2010). Thus, changes to interoceptive processing (even through damage to the somatosensory cortices) may alter the body representation of the animal causing it to react with fear and anxiety when placed in the novel conditioning apparatus. This is confirmed with evidence from human studies; Paulus and Stein (2006) suggest that the tendency for certain individuals to view interoceptive sensations as dangerous or threatening is mediated through a neural circuit that features the insular cortex in a central role (Paulus and Stein 2006; Stein et al. 2007) and Weller, et al. (2009) demonstrate that patients with insula lesions were more cautious in decision-making tasks and risk-taking behavior compared to healthy controls (Weller et al. 2009).

Moving forward, a two-fold course of action was possible: 1) the examination of different combinations of cues in order to establish neutral conditioning apparatus in rats implanted with cannulas so that the experiment could be continued as planned, and 2) the possibility of studying the role of the insula in mediating withdrawal-induced anxiety using the elevated plus maze as an alternate behavioral paradigm. Due to time constraints for this thesis, only the latter possibility was examined.

It is important to consider the effects of somatosensory cortex damage on future place conditioning studies. Even if a set of neutral cues can be established, damage to the somatosensory cortex during cannulation surgery may prevent acquisition of drug associative memory, and may therefore limit conclusions as to the role of the insular cortex in mediating those same pathways (Meng, 2009). Thus, it may be necessary to target the insula from a different angle to avoid damage to the somatosensory cortices.

## **4 Experiment 2: The Effects of Insular Cortex Inactivation on Behavior in the Elevated Plus Maze**

### **4.1 Introduction**

Anxiety is a state of diffuse arousal following the perception of a real or imagined threat. Anxiety disorders, while being a diverse set of phenotypes, are alike in that they all involve excessive negative affect typically in the form of fear and avoidance of an event, object or situation (Amstadter 2008). In addition to its many functions, insula activation has been shown in patients with anxiety disorders (Paulus and Stein 2006; Stein et al. 2007), and suggests that altered insula function and perception of interoceptive stimuli may be an important component for generating an anxious state (Paulus and Stein 2010).

In addition to anxiety as a component of mental illness (i.e. Generalized Anxiety Disorder), anxiety also plays an important role in the maintenance of drug addiction. Abstinence in drug addicts can result in a wide range of autonomic and somatic symptoms of withdrawal, including anxiety (Hyman 2005; Zhang and Schulteis 2008). The negative emotional states arising during withdrawal are often reported to be more aversive than some of the somatic signs and are thought to contribute to the continuation of use and relapse (Aston-Jones and Harris, 2004; Schulteis and Koob, 1996). As a result, a number of animal models have been used to study the neurological substrates involved with the negative emotional signs of withdrawal. Anxiety-like behavior has been shown in both spontaneous and antagonist-precipitated withdrawal in studies such as the defensive probe-burying task and the elevated plus maze (Schulteis et al, 1998). Withdrawal from acute opioid dependence (with or without an opioid antagonist such as naloxone) has been shown to elicit symptoms

similar to those observed during withdrawal from chronic opioid exposure (Azar et al., 2003; Liu and Schulties, 2004; Schulteis et al., 1997).

The elevated plus maze is a novelty-based approach-avoidance task that measures the rodents' natural tendency to explore a novel environment against the aversive properties of an open, brightly lit, elevated space. The instinct to explore the open, unprotected arms of the p-maze weighed against the avoidance of open spaces for protection from potential predators creates an internal conflict for the rat. Rats will tend to spend more time within the enclosed arms of the maze after administration of an anxiogenic drug or during an "anxiety-provoking" state such as withdrawal; whereas rats will tend to explore the open arms when administered an anxiolytic drug (Lapiz-Bluhm et al. 2008).

The elevated plus maze has been shown to be a sensitive index for measuring anxiety-like behavior accompanying antagonist-precipitated withdrawal from acute opioid exposure (Schulteis et al. 1998; Zhang and Schulteis 2008). Acute naloxone-precipitated withdrawal from opioids causes a significant dose-dependent expression of anxiety-like behavior in the elevated plus maze as measured by a decrease in the percent of time spent in the open arms and percent open arm entries (Zhang and Schulteis 2008).

The goal for this experiment was to understand the role of the insular cortex in mediating the negative emotional state of anxiety induced after acute naloxone-precipitated morphine withdrawal. If the insula is important for the interoceptive effects of withdrawal (including anxiety), inactivation of the region should attenuate these effects.

## 4.2 Apparatus

The elevated plus maze apparatus used for this experiment is the same as described previously (Zhang and Schulteis 2008). Briefly, the apparatus is an automated system obtained from Kinder Scientific (Poway, CA) consisting of two opposing “open” arms (50cm x 10.8cm) bounded by 4-mm high ledges on the sides and end, and two opposing “closed” arms of equal width and length except being bounded by 33.5cm high walls on all sides except at the entrance to the center of the maze. The center of the maze is a 10.8x10.8 cm square area from which the animal can access any of the four arms (connected at 90 degree angles). The maze is elevated 85 cm from the floor. Position of the rat in the maze is continually tracked using photo beam arrays embedded along the entire base of each arm and entry point to all arms.

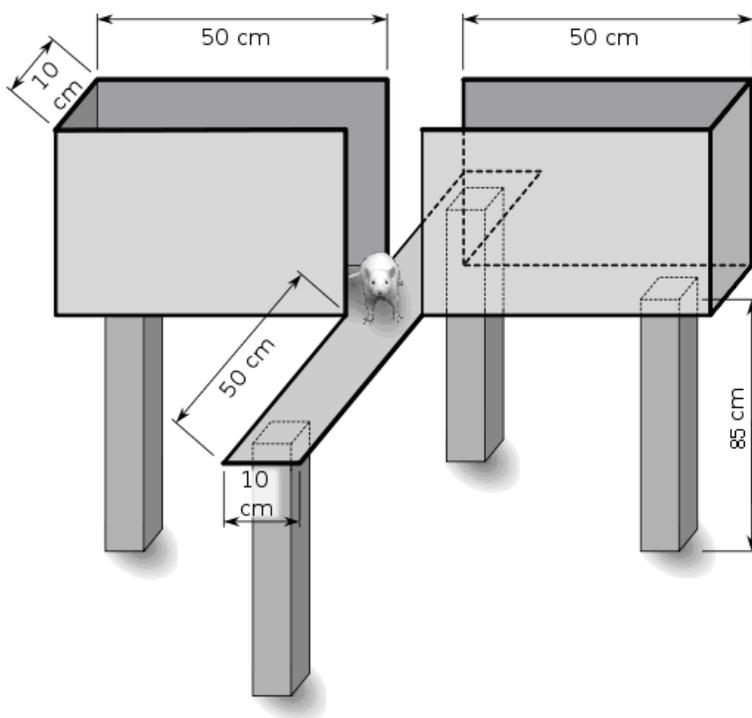


Figure 4-1. Schematic Drawing of the Elevated Plus Maze

Testing is conducted in a quiet room with a white noise generator producing about 65dB background noise. The room is illuminated by two 25-W light bulbs illuminating the walls behind each closed arm of the maze. To begin a test session, rats are placed in the center of the maze facing towards one of the enclosed arms. After testing the maze is cleaned with water and dried with paper towels.

#### **4.3 Experimental Design**

Experimental design for this experiment is similar to that previously described (Zhang and Schulteis 2008). A minimum of 5 days after recovery from surgery (cannulae targeting RAIC or CGIC), animals were taken to the testing suite consisting of a front “holding” room and a back “testing” room with the maze. After initial transport, rats were gently handled for 5 minutes, weighed, injected with vehicle (saline), and returned to their cage in the holding room for at least four hours (to allow sufficient acclimatization to the lighting and noise conditions) before being returned to the animal facility. This procedure was repeated for three consecutive days. Typically, on the fourth day (when testing takes place) all rats are moved to the holding room, weighed, and injected with vehicle or morphine (10mg/kg), then placed back into their home cages. 8 hours later, animals receive an infusion (Vehicle or 4%Lidocaine) into the RAIC or CGIC (0.5 or 1  $\mu$ l at a flow rate of 30  $\mu$ l /hour), followed by an injection of Vehicle or Naloxone (1.0mg/kg) 10 minutes prior to onset of the test. See Table 4-1 for experimental design.

**Table 4-1. Elevated Plus Maze Experimental Design**

Day	D1	D2	D3	D4
<i>Injection T=0h</i>	VEH	VEH	VEH	VEH or Mor
<i>Injection T=8h</i>				VEH or Nal
<i>Infusion</i>				
<i>T=4h</i>	VEH, Lido or None	VEH, Lido or None	None	None
<i>T=8h</i>	N/A	N/A	N/A	VEH or Lido

Based on preliminary results, testing with morphine/naloxone was discontinued and additional groups with repeated infusions were added (See Table 4-2). Animals in repeated infusion groups received infusions on day 1 and 4 (for two infusion groups) and day 1, 2, and 4 (for three infusion groups). Separate groups of rats (RAIC and CGIC) were divided into treatment groups as follows. All animals received vehicle injections only.

**Table 4-2. Elevated Plus Maze Infusion Groups**

<i>Infusion Group:</i>	<i>D1 Infusion</i>	<i>D2 Infusion</i>	<i>D4 Infusion</i>	<i>RAIC (n)</i>	<i>CGIC (n)</i>
1µl Vehicle	N/A	N/A	Vehicle	10	11
0.5µl Lidocaine	N/A	N/A	Lidocaine	6	11
1µl Lidocaine				12	15
1µl Lidocaine x 2	Lidocaine	N/A	Lidocaine	10	10
1µl Lidocaine x 3	Lidocaine	Lidocaine	Lidocaine	10	13
1µl Vehicle x 2 + Lidocaine	Vehicle	Vehicle	Lidocaine	10	9
1µl Lidocaine Ctrl Infusion Sites	N/A	N/A	Lidocaine	13	9

#### 4.4 Data Analysis

Data was collected and analyzed by a windows-XP computer system using MotorMonitor Software (Kinder Scientific, Poway, CA). The following measures were

computed for each rat: 1) time spent in the open arms as a percentage of the total time spent in both the open and closed arms (% Time Open); 2) number of entries to the open arms as a percentage of the total number of entries into both open and closed arms (% Entries Open); 3) distance traveled in the open arms as a percentage of the total distance traveled in both open and closed arms (% Distance Open); 4) total number of entries into the closed arms (# Closed Entries); and 5) total distance traveled in both the open and closed arms of the maze (Total Distance). All behavioral data are presented as average  $\pm$  SEM. One-factor ANOVAs were used to compare averages between groups.  $P < 0.05$  was set as the level of statistical significance for all statistical analyses. Statistical calculations were performed using GraphPad Prism Software.

#### **4.5 Results**

In this experiment, RAIC and CGIC surgery rats were tested in the elevated plus maze to evaluate the effect of insula inactivation on acute opioid withdrawal-induced anxiety. It is important to note, however, that when baseline testing for this experiment began, there was a significant anxiety-like effect produced by lidocaine infusion independent of any exposure to drug treatment.

Reversible inactivation of the RAIC produced anxiety-like behavior as measured by significant decreases in time spent in the open arms of the maze (% Time Open), number of entries into the open arms (% Entries Open), and distance travelled in the open arms (% Distance Open) (Figure 4-2a and Table 4-3 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle Group for %Time Open, % Entries Open and % Distance

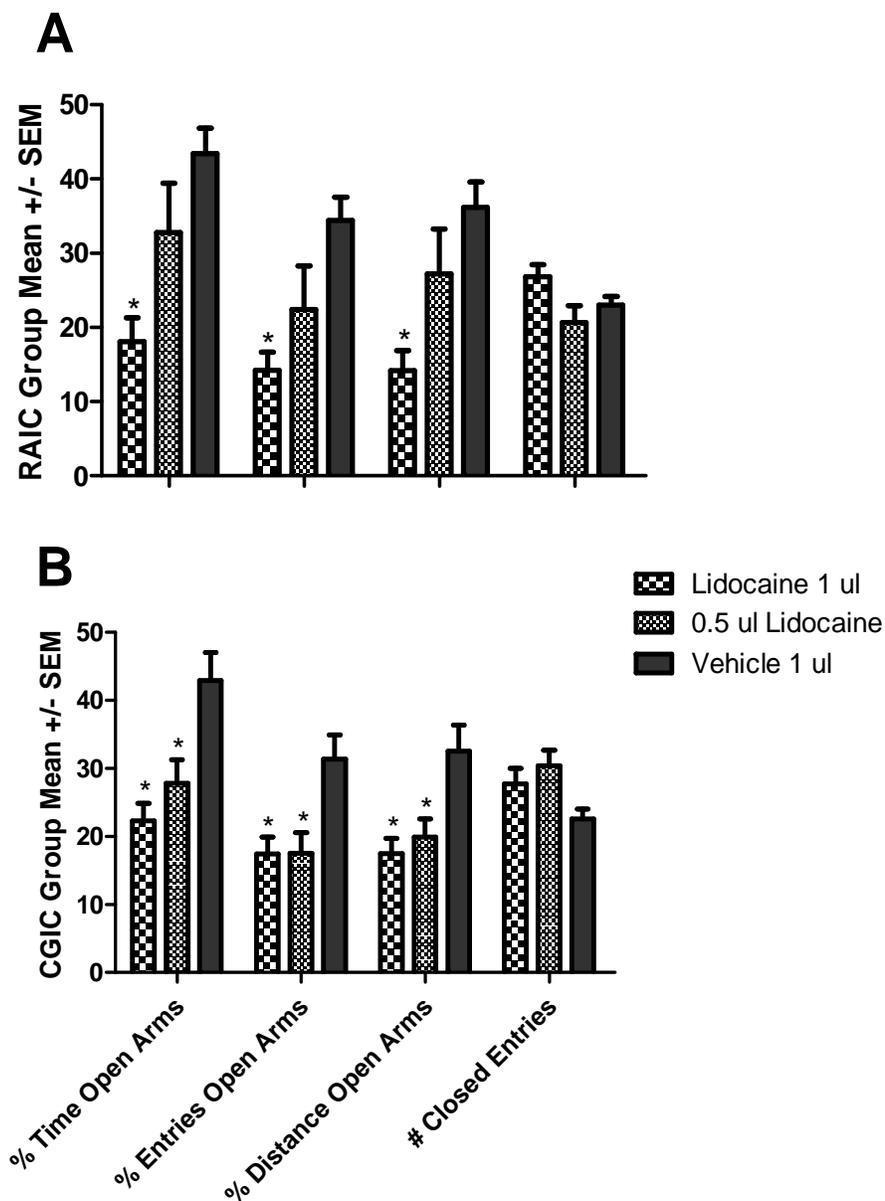
Open). As seen in Figure 4-2a, a smaller lidocaine infusion volume (0.5  $\mu$ l) showed an intermediate dose-dependent effect in the RAIC.

Reversible inactivation of the CGIC produced a similar effect on behavior in the plus maze (Figure 4-2b and Table 4-3 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect for both the 0.5  $\mu$ l and 1  $\mu$ l Lidocaine groups ( $p < 0.05$  vs. Vehicle Group for %Time Open, % Entries Open and % Distance Open) (Figure 4-2b).

Based on the initial finding that lidocaine infusion alone was causing an anxiety-like effect in the plus maze, further studies utilizing lidocaine inactivation of the insula to reverse withdrawal-induced anxiety were postponed until follow-up studies could determine what factors were contributing to the effect of inactivation alone.

**Table 4-3. Summary of One-Factor ANOVAs for Lidocaine Infusion Volumes**

Plus Maze Measure	Site	One-Factor ANOVA	Graph
<i>Percent Time Open</i>	RAIC	(F[2,27]=11.87, $p < 0.001$ )	Figure 4-2a
	CGIC	(F[2,36]=10.39, $p < 0.0005$ )	Figure 4-2b
<i>Percent Entries Open</i>	RAIC	(F[2,27]=10.24, $p < 0.001$ )	Figure 4-2a
	CGIC	(F[2,36]=6.858, $p < 0.005$ )	Figure 4-2b
<i>Percent Distance Open</i>	RAIC	(F[2,27]=10.79, $p < 0.001$ )	Figure 4-2a
	CGIC	(F[2,36]=7.634, $p < 0.01$ )	Figure 4-2b
<i># Closed Entries</i>	RAIC	(F[2,27]=3.427, $p < 0.05$ )	Figure 4-2a
	CGIC	(F[2,36]=3.083, N.S.)	Figure 4-2b



**Figure 4-2. Anxiety-like effect of Lidocaine Infusion into the Insular Cortex**

Acute infusion of (a) 1  $\mu$ l Lidocaine into the RAIC and (b) 0.5  $\mu$ l and 1  $\mu$ l Lidocaine into the CGIC produce a significant anxiety-like effect in the elevated plus maze as measured by % Time, % Entries, and % Distance (\* $p$ <0.05 vs. Vehicle Groups). There was no effect on general motor activity (Closed Entries). Data represent mean (+/- SEM) for each measure.  $n = 6-12$ /group (a);  $n = 11-15$ /group (b)

**Table 4-4. Summary of One-Factor ANOVAs for Control Site Infusions**

<b>Plus Maze Measure</b>	<b>Site</b>	<b>One-Factor ANOVA</b>	<b>Graph</b>
<i>Percent Time Open</i>	RAIC	(F[2,34]=24.72, p<0.0001)	Figure 4-3a
	CGIC	(F[2,34]=9.714, p<0.0005)	Figure 4-3b
<i>Percent Entries Open</i>	RAIC	(F[2,34]=23.39, p<0.0001)	Figure 4-3a
	CGIC	(F[2,34]=6.208, p<0.01)	Figure 4-3b
<i>Percent Distance Open</i>	RAIC	(F[2,34]=21.75, p<0.0001)	Figure 4-3a
	CGIC	(F[2,34]=7.161, p<0.005)	Figure 4-3b
<i># Closed Entries</i>	RAIC	(F[2,34]=2.611, N.S.)	Figure 4-3a
	CGIC	(F[2,34]=2.086, N.S.)	Figure 4-3b

To verify that this anxiety-like effect was specific to inactivation of the insular cortex, and not a result of lidocaine diffusion up the cannulae tract or a general effect of lidocaine infusion into any brain region, control sites +1mm above both the RAIC and CGIC were included (RAIC-ctrl and CGIC-ctrl). As seen in Figure 4-3a, inactivation of the RAIC-ctrl region (lidocaine control group) prior to plus maze testing produced no anxiety-like effect (See Table 4-4 for statistics). Post-hoc comparisons for each infusion group revealed the only significant effect was in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle and 0.5  $\mu$ l Lidocaine Group for %Time Open, % Entries Open and % Distance Open) (Figure 4-3a).

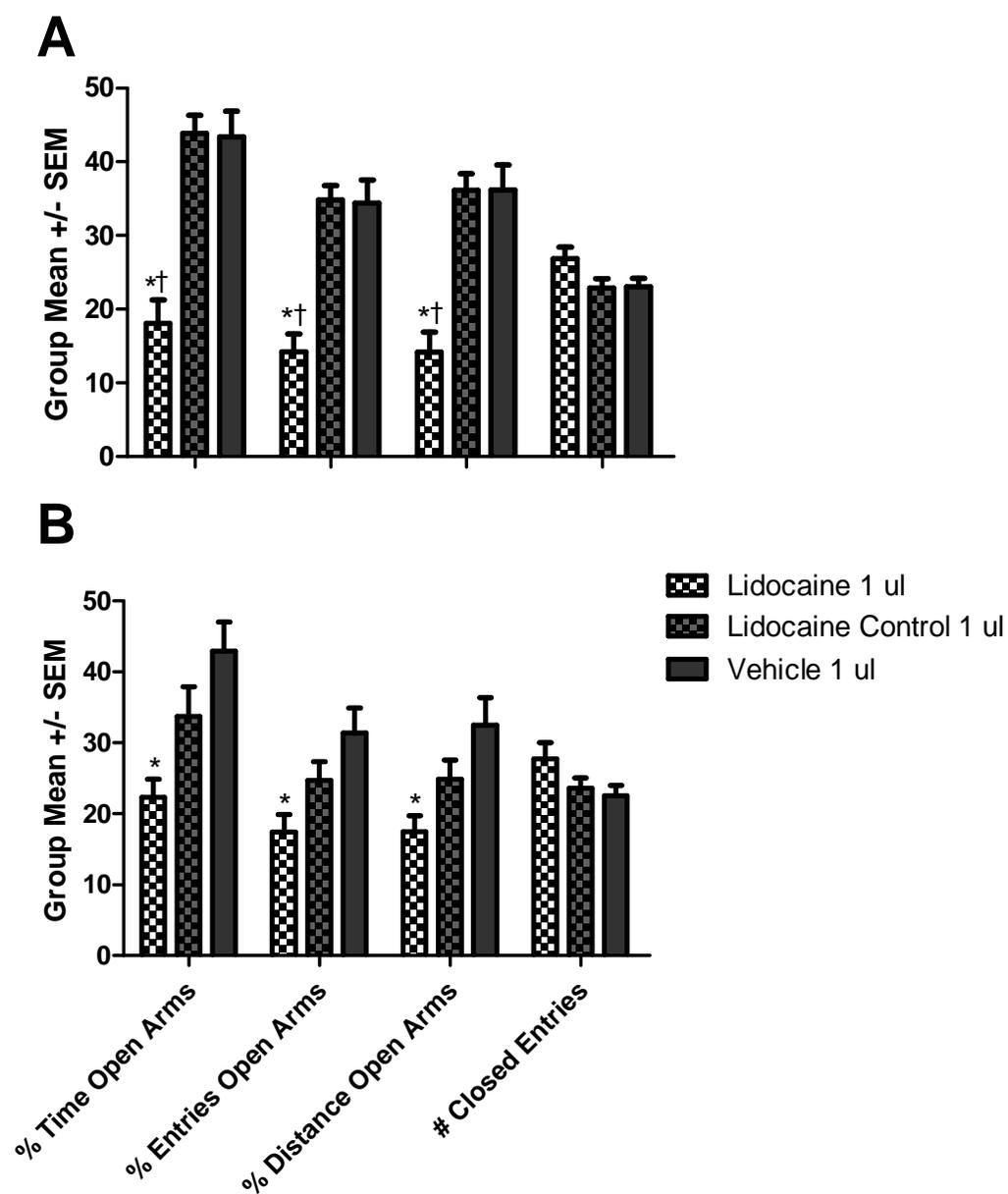
Lidocaine infusion into the CGIC-ctrl region (Lidocaine control group) produced an intermediate anxiety-like effect on the plus maze behaviors (Figure 4-3b and Table 4-4 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle Group for %Time Open, % Entries Open and % Distance Open) (Figure 4-3b).

Multiple lidocaine infusions were tested to determine if the effect was dependent on the novelty of inactivation, or if it would persist through multiple infusions. As seen in Figure 4-4a, repeated infusions of lidocaine into the RAIC over the 3 days prior to plus maze testing (see experimental design) progressively diminished the effect of insular inactivation on anxiety-like behavior (See Table 4-5 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle Group for %Time Open, % Entries Open and %Distance Open; and  $p < 0.05$  vs. Lidocaine x 3 Group for %Time Open and % Distance Open) and 1  $\mu$ l Lidocaine x 2 Group ( $p < 0.05$  vs. Vehicle Group for % Entries Open and % Distance Open) (Figure 4-4a).

A similar tendency for diminished anxiety-like behavior was seen after repeated lidocaine infusions into the CGIC (Figure 4-4b and Table 4-5 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle and Lidocaine x 3 Group for %Time Open, % Entries Open and %Distance Open) (Figure 4-4b).

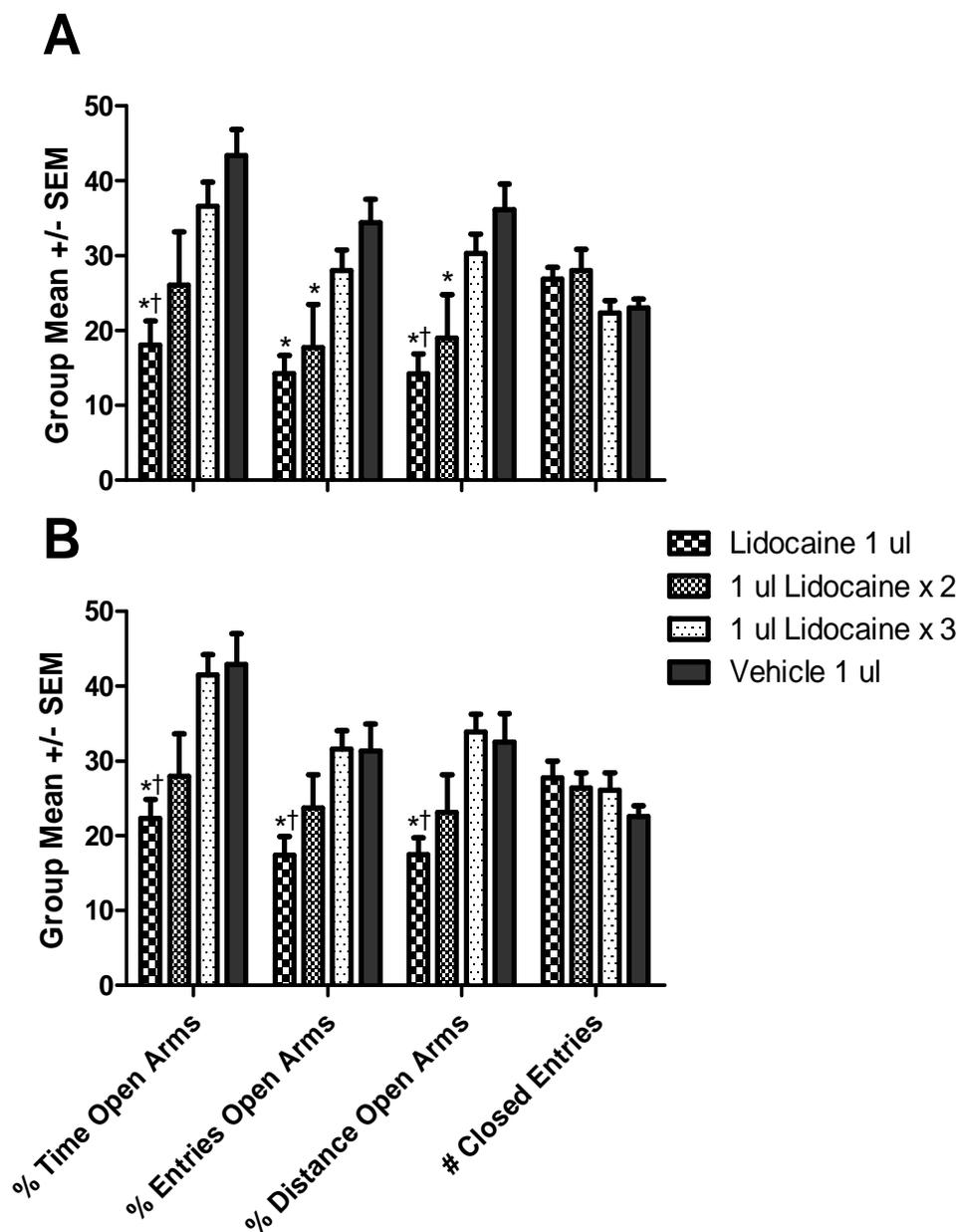
**Table 4-5. Summary of One-Factor ANOVAs for Repeated Lidocaine Infusions**

<b>Plus Maze Measure</b>	<b>Site</b>	<b>One-Factor ANOVA</b>	<b>Graph</b>
<i>Percent Time Open</i>	RAIC	(F[3,41]=6.594, $p < 0.01$ )	Figure 4-4a
	CGIC	(F[3,48]=8.249, $p < 0.0005$ )	Figure 4-4b
<i>Percent Entries Open</i>	RAIC	(F[3,41]=6.613, $p < 0.001$ )	Figure 4-4a
	CGIC	(F[3,48]=5.192, $p < 0.005$ )	Figure 4-4b
<i>Percent Distance Open</i>	RAIC	(F[3,41]=7.440, $p < 0.001$ )	Figure 4-4a
	CGIC	(F[3,48]=6.234, $p < 0.005$ )	Figure 4-4b
<i># Closed Entries</i>	RAIC	(F[3,41]=2.123, N.S.)	Figure 4-4a
	CGIC	(F[3,48]=1.035, N.S.)	Figure 4-4b



**Figure 4-3. Anxiety-like effect is specific to Insular Cortex Inactivation**

Acute infusion of 1  $\mu$ l Lidocaine (a) into a control region +1mm above the RAIC; (b) into a control region +1mm above the CGIC. (\* $p$ <0.05 vs. Vehicle Group; † $p$ <0.05 vs. Lidocaine Control Region). There was no effect on general motor activity (Closed Entries) for any group. Data represent mean (+/- SEM) for each measure,  $n$  = 10-13/group (a);  $n$  = 9-15/group (b).



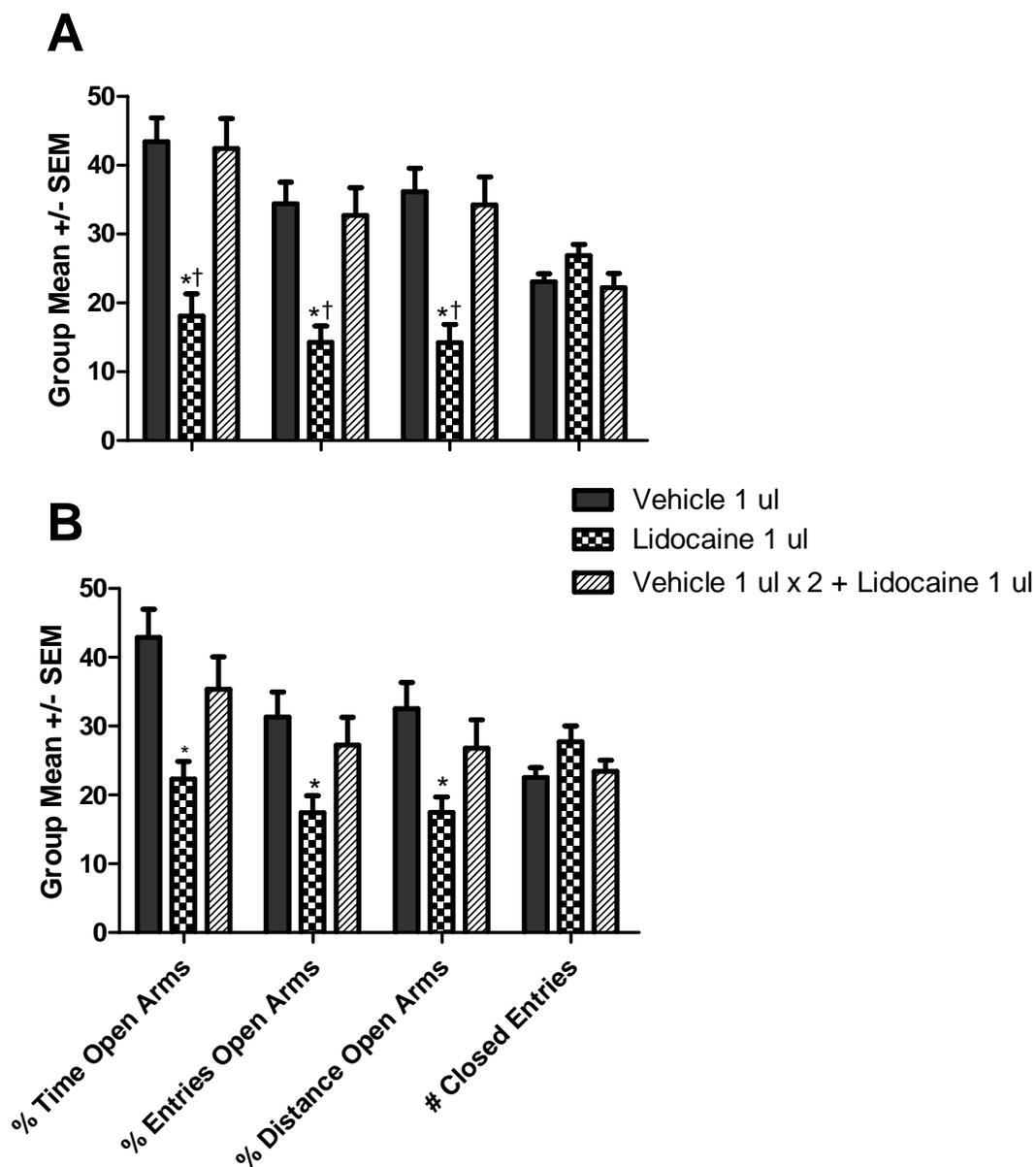
**Figure 4-4. Attenuation of Anxiety-like effect after repeated Lidocaine inactivation**

One or two Infusions of 1  $\mu$ l Lidocaine into the (a) RAIC and (b) CGIC significantly decreased % Time, % Entries, and % Distance in the open arms of the maze ( $p < 0.05$  vs. Vehicle Group;  $\dagger p < 0.05$  vs. Lidocaine x 3 Group). Three lidocaine infusions was not different than vehicle treatment. There was no effect on general motor activity (#Closed Entries). Data represent mean ( $\pm$  SEM) for each measure.  $n = 10-12/\text{group}$  (a),  $n = 10-15/\text{group}$  (b)

**Table 4-6. Summary of One-Factor ANOVAs for Vehicle Pre-Infusions**

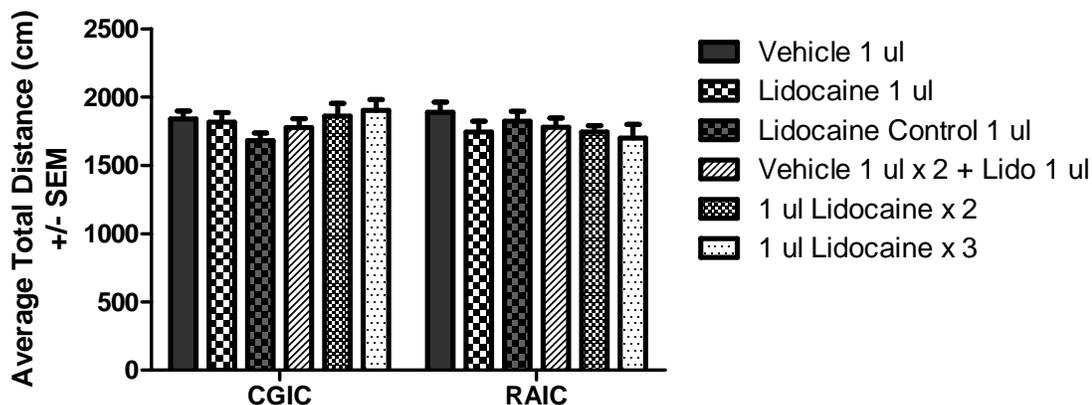
<b>Plus Maze Measure</b>	<b>Site</b>	<b>One-Factor ANOVA</b>	<b>Graph</b>
<i>Percent Time Open</i>	RAIC	(F[2,31]=16.45, P<0.0001)	Figure 4-5a
	CGIC	(F[2,34]=9.355, p<0.005)	Figure 4-5b
<i>Percent Entries Open</i>	RAIC	(F[2,31]=12.91, P<0.0001)	Figure 4-5a
	CGIC	(F[2,34]=5.435, p<0.05)	Figure 4-5b
<i>Percent Distance Open</i>	RAIC	(F[2,31]=13.96, p<0.0001)	Figure 4-5a
	CGIC	(F[2,34]=6.098, p<0.05)	Figure 4-5b
<i># Closed Entries</i>	RAIC	(F[2,31]=2.306, N.S.)	Figure 4-5a
	CGIC	(F[2,34]=2.079, N.S.)	Figure 4-5b

Showing that the effect was reversible with repeated lidocaine infusions, it was unclear as to whether this was an effect of habituation or due to damage caused by repeated injection. To test this, vehicle pre-infusions were tried followed by lidocaine infusion on test day. As shown in Figure 4-5a, two prior infusions of vehicle into the RAIC were capable of eliminating any effect of lidocaine infusion on test day (See Table 4-6 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle and Vehicle x 2 + Lidocaine Group for %Time Open, % Entries Open and %Distance Open) (Figure 4-5a). This effect was consistent with vehicle pre-infusions into the CGIC as well (Figure 4-5b and Table 4-6 for statistics). Post-hoc comparisons for each infusion group revealed a significant effect in the 1  $\mu$ l Lidocaine group ( $p < 0.05$  vs. Vehicle Group for %Time Open, % Entries Open and %Distance Open) (Figure 4-5b).



**Figure 4-5. Vehicle Pre-Infusion Attenuates Lidocaine Effect**

Two prior infusions of 1  $\mu$ l Vehicle attenuates the effect of Lidocaine infusion into the (a) RAIC and (b) CGIC in the elevated plus maze as measured by % Time, % Entries, and % Distance (\* $p$ <0.05 vs. Vehicle Group; † $p$ <0.05 vs. Vehicle x 2 + Lidocaine Group). There was no effect on general motor activity (Closed Entries). Data represents mean (+/- SEM) for each measure,  $n = 10-12$ /group.



**Figure 4-6. Lidocaine Infusion into the Insular Cortex has no effect on Mobility**

Infusion of Lidocaine or Vehicle into the Insular Cortex (RAIC or CGIC) produces no significant effect on general motor activity as measured by total distance traveled in the open and closed arms of the elevated plus maze. Data represent mean (+/- SEM) for each measure, n = 10-15/group.

Overall, there was no effect on general motor activity with any combination of infusion treatment in either the RAIC or CGIC region, as measured by total distance travelled in the plus maze (Figure 4-6). Two-way ANOVA revealed no significant effect of Infusion ( $F[5,120]=0.5135$ , N.S.) or brain region ( $F[5,120]=0.5597$ , N.S.) on total distance travelled.

#### 4.6 Discussion

The original goal of this project was to assess the role of the insular cortex in mediating the state of anxiety induced after acute morphine withdrawal, by reversibly inactivating the insular cortex and measuring changes in plus-maze behavior compared to vehicle-infused controls. It is important to note, however, that when testing with lidocaine began there were some unexpected, yet interesting, side effects of insula inactivation on anxiety-like behavior in the plus maze. Specifically, rats

infused with lidocaine showed anxiety-like behavior independent of any drug exposure. This was seen as significant decreases in time spent in the open arms of the plus maze (% Time Open), open arm entries (% Entries Open), and distance traveled in the open arms (% Distance Open). Overall motor activity (measured by both number of closed entries and total distance travelled) was not affected by lidocaine infusion.

Since lidocaine alone was producing anxiety-like behavioral changes, it would be difficult, if not impossible, to abolish a withdrawal-induced anxiety state by using lidocaine to inactivate the insular cortex. Thus, follow-up experiments were conducted to further determine the role of the insula in anxiety-like behavior in the elevated plus maze. First, decreased lidocaine infusion volumes were tested to determine if the effect was dependent on dose. This experiment revealed different results for the RAIC and the CGIC. 0.5  $\mu$ l lidocaine infusion into the RAIC showed an intermediate effect on anxiety-like behavior, however, 0.5  $\mu$ l lidocaine infusion into the CGIC was equivalent to infusion of the 1.0  $\mu$ l lidocaine volume (Figure 4-3). This pattern of results may be indicative of the roles each of these regions play in the interoceptive circuit. Previous findings suggest that sensory information enters into the posterior (caudal) insula and is progressively organized into an emotionally relevant output that is sent to other cortical areas by the anterior (rostral) insula (Craig 2002; Craig 2003; Craig 2009). However, the posterior insula also has its own reciprocal projections to other cortical and amygdaloid regions involved with the processing of interoceptive stimuli, including the dorsolateral striatum and basolateral amygdala (Chikama et al. 1997; Shi and Cassell 1998). Blockage of the interoceptive circuit at the point where signals enter the caudal insula essentially cuts off the entire flow of information, even at smaller infusion volumes. After entering the CGIC, interoceptive signals are combined with other homeostatic information before being fully integrated

in the RAIC (Craig 2003). Inactivation of the rostral insula prevents output of the signals generated, but information has already been received and processed in the caudal region. While 1  $\mu$ l lidocaine infusion is capable of blocking the circuit, a smaller volume of lidocaine does not inactivate a large enough volume of the RAIC to block transmission of all efferent signals.

To confirm that this effect was specific to inactivation of the insula, and not a general effect of lidocaine diffusion into any nearby brain regions, two control sites 1mm above both the RAIC and CGIC were included. Inactivation of the RAIC-ctrl site (Rostral granular/dysgranular insular cortex and secondary somatosensory cortex) produced no anxiety-like effects. This finding indicates that the anxiety-like effect is specific to the rostral agranular insula, and confirms the caudal to rostral (granular to agranular) hierarchical organization of the insula where inputs come into the caudal granular insula and are progressively integrated into the rostral agranular insula (such that the rostral granular insula begins to lose significance in interoceptive processing) (Craig 2003). Lidocaine inactivation of the CGIC-ctrl site (somatosensory cortex) showed an intermediate effect of reduced exploration of the open arms, with the magnitude falling between lidocaine and vehicle-infused rats. While not anticipated, this pattern of results is consistent with what is known about S2 and also what was shown in the initial place aversion data where animals with cannula damage in S2 showed a bias for the darker dots compartment of the conditioning apparatus. Inactivation of the somatosensory cortices has been shown to reverse conditioned place preference to morphine and is important for processing visual, auditory, and sensory information (Meng et al. 2009). Damage or inactivation to the S2 may cause the animal to prefer the dark, enclosed arms over the open arms of the maze due to a change (or lack) of sensory processing. Neither site resulted in changes to mobility.

Additionally, the anxiety-like behavior produced with the 0.5  $\mu$ l infusion of lidocaine into the CGIC suggests that this region is particularly sensitive to inactivation. Although the spread from the 1.0  $\mu$ l lidocaine infusion into the CGIC-Control site should only diffuse 1 mm (Tehovnik and Sommer 1997), the pattern of results suggests that if some diffusion occurred, the small amount of lidocaine reaching the CGIC may have been sufficient to produce anxiety-like behavior in the plus maze. Further studies examining control sites farther from the insula (i.e. +2mm above the CGIC) could confirm this hypothesis. Overall, the anxiety-like effect seems to be specific to insular inactivation, especially in the rostral region where integration and motivational output are known to occur.

To test whether this anxiety-like effect was resulting from the novelty of insula inactivation (and the subsequent lack of interoceptive processing) repeated lidocaine infusions into the insula were administered in an attempt to habituate the animals to the experience of inactivation. Repeated 1 $\mu$ l lidocaine infusions prior to plus maze testing resulted in a gradual reversal of the anxiety-like effect, such that three total infusions into either region produced no significant effect of anxiety-like behavior in the plus maze. Thus, habituation to insular cortex inactivation led to a decrease in the amount of anxiety-like behavior in rats. These results suggest that the novel lack of interoceptive feeling may be aversive to the animal but that repeated lack of interoceptive information may reduce the initial aversion. On the other hand, the abolition of the effect with 3 infusions of lidocaine may also be explained by a more general effect of repeated infusions; there is potentially a significant amount of brain damage occurring after three infusions such that there is little insula tissue remaining to inactivate on test day. In order to differentiate the effects of "lidocaine habituation" after repeated infusion from damage caused by infusion, vehicle pre-infusions were

given prior to lidocaine infusion on test day. Surprisingly, two infusions of vehicle produced enough damage to block the effect of lidocaine inactivation on test day. This possibility had not been considered in prior insula inactivation studies that infused as many as 6 times (Forget et al. 2010), and calls for appropriate experimental design to minimize and control for this possible non-specific damage from repeated injection.

Further testing is required to determine if smaller volumes of lidocaine would alter the above effects. If the attenuation of the anxiety-like effect is related to cumulative damage caused by repeated infusion, a smaller infusion volume (causing less damage to the brain) might show preservation of the effect over multiple lidocaine infusions. Similarly, if multiple 1 $\mu$ l vehicle infusions are causing significant brain damage, vehicle infusions alone may cause an anxiety-like effect similar to a single lidocaine infusion. These control groups were not included in the initial design of the study. Histological verification to show location and quantity of damage would be required to verify these speculations. This follow-up would help to differentiate the role of insula inactivation from potential infusion damage, and would be beneficial in choosing appropriate parameters for future experiments.

Nevertheless, in naïve animals, lidocaine inactivation consistently reduced percent time in the open arms of the elevated plus maze. There are a few explanations as to why this effect is occurring: 1) inactivation of the insula disrupts behaviors dependent on interoceptive processing; 2) the insular cortex is a component of pathways mediating emotional states such as anxiety. Either of these reasons could explain why animals are tending to spend more time in the closed arms of the maze instead of exploring the open arms.

1) The insula is involved with integrating internal and external sensory information into an emotionally relevant state of the body (Craig 2002). It is believed that the

insula receives somatosensory input, processes the information with regard to the current body state, suggests an appropriate motor response and recruits the appropriate motor cortices through its afferent connections with regions such as the ACC (Craig 2003;Lerner et al. 2009). A lack of interoceptive integration and appropriate motor output may leave the rat in a state where it reacts to the novel plus maze environment with fear and avoidance, as appropriate behavioral responses are unclear. Actions resulting from this potentially aversive state would cause the rat to avoid the open arms of the plus maze (prefer the enclosed arms) and increase avoidance behavior, which is consistent with the results generated in this experiment.

Along the same lines, the decreases in open arm exploration may represent an interruption of the interoceptive circuit important for regulating motivational behaviors and natural urges, such as exploration of novelty.

2) It is also possible that the insula plays a part in a circuit important for emotional regulation. As mentioned earlier, aberrant insula processing has been shown in patients with anxiety disorders (Ibanez et al. 2010;Jones et al. 2010;Paulus and Stein 2010). In these cases, the insula may contribute to an erroneous representation of the body and changes to interoceptive signal interpretation which may manifest as an abnormal physiological state and inappropriate affective responses, such as anxiety (Ibanez et al. 2010). Consistent with this finding, Clarke, et al (2008) and Weller, et al. (2009) showed that patients with insular lesions showed altered risk-taking behavior and more cautious decision-making even under advantageous situations (Clark et al. 2008;Weller et al. 2009), indicating that a lack of insula function was capable of effecting emotional decision-based processes. Similar to humans, insula inactivation and altered interoceptive function could result in an altered body representation and negative emotional state (such as anxiety) in rats, which in turn would cause changes

to behavioral responses (such as the decreased exploration of the open arms of the maze).

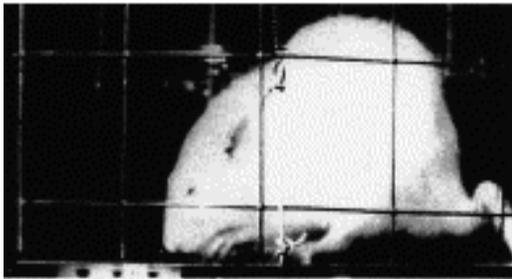
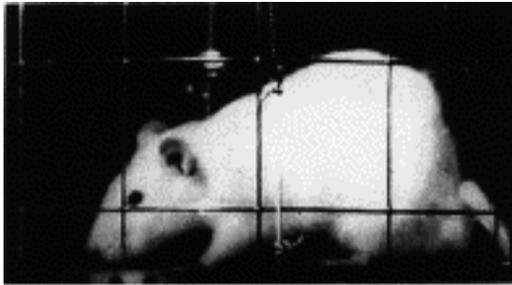
Using the elevated plus maze to measure anxiety utilizes an approach-avoidance task that measures changes in an animal's anxiety level, reflected as an increase or decrease in avoidance (of the open arms). This task allows the animal to choose where it spends its time, and therefore, reflects an active decision making process. Based on the results generated in the elevated plus maze, it is likely the insula is involved in mediating behaviors and emotional states related to interoceptive processing. To approach the effect from a different direction and confirm the anxiety-like effect of insula inactivation, the acoustic startle reflex was used to as an alternate measure of anxiety processing.

## **5 Experiment 3: Effects of Insula Inactivation on Acoustic Startle**

### **5.1 Introduction**

Following the discovery of a lidocaine-induced anxiety-like effect in the elevated plus maze, there was uncertainty as to whether this effect would carry over to different measures of anxiety. The acoustic startle reflex has been used as a measure of unconditioned anxiety in both human and animal models of anxiety, including studies of opiate withdrawal ((Risbrough 2010) for review).

The acoustic startle paradigm measures anxiety differently than the elevated plus maze. Instead of an approach-avoidance task where the animal makes a decision about where it spends its time, the startle paradigm measures changes in an unconscious/unconditioned startle reflex after presentation with a sudden intense stimuli and is therefore not primarily influenced by intentional control (Grillon and Baas 2003). The reflex is highly conserved across species, including rodents, and consists of skeletal muscle contraction leading to extension of the forepaws and hind paws followed by muscle flex into a hunched position (Figure 5-1) and is generally considered to be facilitation of a defensive posture, possibly to allow the fight or flight response or allow protection of the body from attack (Yeomans et al. 2002;Risbrough 2010). The acoustic startle reflex is mediated by a specific neural pathway where acoustic information enters the CNS through auditory nerve input on the cochlear nucleus. Resulting motor outputs are generated and project to the ventral spinal horn for control over skeletal muscle (Curzon et al. 2009).



**Figure 5-1. Startle reflex in the Rat**

**Animal depicted before (top) and after (bottom) presentation with an acoustically startling stimuli. Image adapted from Horlington, 1970.**

Although it is an unconscious reflex, the magnitude of the response is dependent on the internal state of the animal. Startle is increased by threatening stimuli and during states of negative emotional valence, such as anxiety, panic disorder, and PTSD as well as states of drug use and withdrawal (Grillon and Baas 2003). The negative emotional states associated with both chronic and acute opioid withdrawal can elicit emotional symptoms such as depressed mood, irritability, and anxiety in addition to many well characterized somatic signs (Schulteis et al. 1998). The acoustic startle reflex has been used as a measure of unconditioned anxiety and hyperarousal in studies of opioid withdrawal in humans and animals (Kalinichev and Holtzman 2003; Harris and Gewirtz 2004).

Based on the anxiety-like effects seen in the elevated plus maze experiment, inactivation of the insular cortex may be causing an elevation of general anxiety.

Anxious individuals tend to show sustained elevations in startle reactivity (Grillon and Baas 2003) and startle is potentiated in the presence of aversive or threatening stimuli (Hebb et al. 2003; Davis 2006; Lang and Davis 2006) so the goal of this experiment is to evaluate the effects of lidocaine infusion into the insular cortex on acoustic startle response. Animals used in the elevated plus maze (Experiment 2) were tested again in the acoustic startle test.

## **5.2 Apparatus**

All behavioral testing occurred in Kinder Scientific Startle Monitor chambers from Kinder Scientific (SM100, Poway, CA, USA). Apparatuses consisted of a clear nonrestrictive Plexiglas enclosure (18 cm x 9.5 cm x 12.5 cm) resting on a platform inside a ventilated, sound-attenuated, chamber. A high-frequency loud speaker mounted inside the chamber 24 cm above the animal produced both a continuous background noise of 70dB and the various acoustic stimuli. The whole-body startle responses of the animal caused vibrations of the Plexiglas box which was converted into analog signals by a piezoelectric accelerometer attached to the platform. These signals were then rectified and stored by a microcomputer and interface unit. Calibrations were performed on the chambers to ensure the accuracy of sound levels and measurements. Sound levels were measured using the dB(A) scale.

## **5.3 Experimental Design**

Naïve (no-surgery), RAIC, and CGIC animals were used for this experiment. Following behavior testing in the elevated plus maze (RAIC and CGIC) or after two weeks arrival in the animal facility (Naïve), all rats underwent two startle/PPI testing sessions (baseline and test) separated by at least 2 days. On the baseline day, rats were placed in the startle chamber for a brief baseline startle/prepulse inhibition (PPI)

session of 24 trials in which two types of acoustic stimuli were presented (8 PULSE-ALONE trials of 115 dB white noise pulse; and 16 PREPULSE+PULSE trials in which a 8 dB above background noise was presented 100 ms before the onset of the 115 dB pulse). In this session and the subsequent test session, the background noise (70 dB) was presented alone for 5 min and then continued throughout the remainder of the session. Trials were presented in a pseudo-random order. The mean startle amplitude across startle stimuli on the baseline day was used to assign rats into mock, vehicle, or lidocaine groups with similar means. On test day, animals were placed in the same startle chamber they were in for the baseline test. The test session contained five different trial types: three different PULSE-ALONE trials in which a 40ms 95, 105, or 115 dB stimuli was presented; two PREPULSE+PULSE trials in which 20ms stimuli (78dB) were presented 100ms before the onset of either a 105 or 115 dB stimulus. The session also included NO STIMULUS trials which included only the background noise. All trial types were presented several times in a pseudo-random order for a total of 119 trials (12 of each trial type plus hidden NO STIMULUS trials between each stimulus trial). An average of 18.9s (ranging from 13 to 23s) separated consecutive trials. The total duration of the session was approximately 25 minutes. House lights were on in the startle chambers during testing.

To evaluate the effects of lidocaine inactivation of the insular cortex on startle magnitude, RAIC and CGIC rats were placed into one of three infusion groups; Mock, 1 $\mu$ l Vehicle (aCSF) or 1 $\mu$ l Lidocaine (as previously described). Naïve non-surgery animals were used as baseline controls and were treated the same as the Mock group.

Data Analysis Startle magnitude was calculated as the average response (N) +/- SEM to each of the PULSE-ALONE trials. PPI data were not reported for this thesis. Startle magnitude was analyzed in a 2-factor ANOVA with group as a between-subject

factor and intensity as a within subject factor using Bonferroni post-hoc tests to assess group differences from control. A statistical significance of  $p < 0.05$  was set for all experiments. All statistical analyses were done using GraphPad Prism Software.

#### 5.4 Results

Startle magnitude responses in naïve, non-surgery rats were compared to rats with mock, vehicle, or lidocaine infusions into either the RAIC or the CGIC. In CGIC animals (Figure 5-2, b) a 2-factor ANOVA showed a main effect of pulse intensity ( $F[2,144]=113.04$ ,  $p < 0.0001$ ) and a group x intensity interaction ( $F[3,144]=2.96$ ,  $p < 0.05$ ) driven by significant increases in startle magnitude at the 115dB intensity in vehicle and lidocaine infusion groups, compared to non-surgery control animals. Mock infused rats were not significantly different than non-surgery control, vehicle-infused or lidocaine-infused rats. For the RAIC rats (Figure 5-2, a), there was a main effect of pulse intensity, as expected ( $F[2,126]=89.97$ ,  $p < 0.0001$ ), however there were no significant differences between the infusion groups.

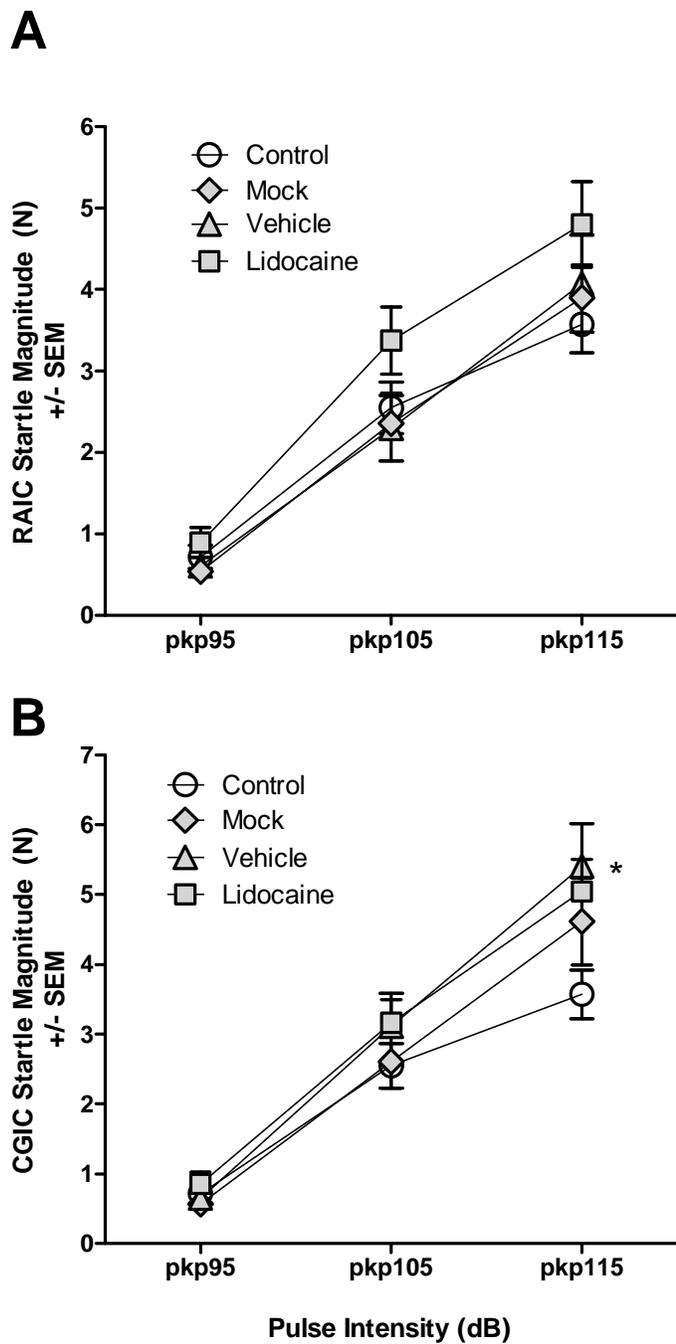


Figure 5-2. Effect of Infusion on Peak Startle Magnitude

Data show (a) no significant differences in any of the RAIC surgery or infusion groups, (b) significant effect of vehicle and lidocaine infusion into the CGIC on startle magnitude at pkp115dB compared to control animals (n=8-16/group). No effects were seen at lower dB. \* $p < 0.05$  vs. Control group.

## 5.5 Discussion

The use of acoustic startle to measure changes in sensory-motor processing as a result of aversive state (i.e. fear, anxiogenic drug treatment, withdrawal state) has been employed as a way to assess anxiety-like behavior in rodents. In experiment 2, lidocaine was shown to induce anxiety-like effects in the elevated plus maze when infused into either the rostral or caudal insular cortex. This effect may be attributed to a perturbation of the neural circuitry regulating emotional states (such as anxiety) or to a more general result of a negative effect of lack of interoceptive information after insular inactivation. In this acoustic startle experiment, we sought to confirm the anxiety-like effects of insula inactivation by approaching the question with a different measure of anxiety. Results indicated that there may be an effect of surgery and/or infusion on basal startle levels, as significant increases in startle magnitude were seen in CGIC surgery animals infused with either vehicle or lidocaine, compared to naïve non-surgery control animals (Figure 5-2b). While there were no statistically significant differences in the RAIC groups, there seems to be a trend for increased startle magnitude in all surgery groups (Mock, VEH, and Lido) compared to naïve non-surgery control animals (Figure 5-2a). This effect of surgery is in line with the data generated in the place conditioning paradigm, where surgery rats exhibited biases that were not present in non-surgery control rats and were consistent with increases in anxiety (tendency to prefer the darker compartment of the conditioning apparatus). While limited, the increase of startle magnitude in CGIC rats infused with vehicle or lidocaine is consistent with the increases in anxiety-like behavior seen in the elevated plus maze, and suggests that damage to the insula may be causing elevations in preconscious anxiety or behavioral responses of fear and uncertainty due to loss of interoceptive processing.

These results require careful interpretation, however, as all RAIC and CGIC animals were tested in the elevated plus maze prior to testing in startle (meaning they received either vehicle or lidocaine infusions, or some combination thereof). Knowing that multiple infusions of both vehicle and lidocaine blunted the anxiety-like effects in the plus maze, it is possible that this prior exposure negated any significant effects on startle. As a follow-up, naïve surgery rats were being tested under the same startle conditions, but the experiments were not completed at the time of this thesis.

## 6 Conclusions

The Insula has been identified as an integral part of the interoceptive circuit and has been implicated in regulating changes in hedonic processing of interoceptive information. The Insula is responsible for integrating a wide array of interoceptive signals (including sensual touch, thirst, pain, itch, hunger, temperature, and metabolic states) with homeostatic information in order to produce a relevant representation of the body and suggest behavior appropriate to the current situation (Craig 2002). While implicated in a vast number of functions, its role in drug craving and addiction became particularly interesting after evidence emerged showing that insula inactivation could disrupt drug seeking and conditioning in both humans and rats (Contreras et al. 2007; Naqvi et al. 2007; Hollander et al. 2008; Forget et al. 2010).

In this thesis, reversible inactivation of both the caudal granular insular cortex (CGIC) and rostral agranular insular cortex (RAIC) was used to examine the role of the insula and the interoceptive circuit on behavior. Interestingly, albeit not initially anticipated, insular manipulation resulted in alterations to behavior consistent with an increased anxiety-like state in the 1) place conditioning, 2) elevated plus maze, and 3) acoustic startle paradigms. In the conditioned place aversion test (Experiment 1), RAIC and CGIC surgery animals showed an intrinsic bias for the dots compartment which was not present in non-surgery rats. Testing in the elevated plus maze (Experiment 2) revealed a significant anxiety-like effect following insula inactivation on the behavioral measures of percent time, percent distance and number of entries in the open arms of the maze. Lastly, a trend for elevated startle magnitude (Experiment 3) was seen in RAIC and CGIC rats, although only rats with CGIC surgery receiving vehicle or lidocaine infusions showed significantly higher startle responses. The data suggest that lack of insula functioning produces a negative affective state where the rat is left to

react with fear and uncertainty to novel situations since an appropriate behavioral response is unclear. The interoceptive circuit, therefore, is important in the coordination of internal and external stimuli and necessary for suggesting behaviors. Although not yet confirmed, it also supports a role for the insula in mediating negative emotional states such as anxiety and drug addiction. These anxiety-like effects have not been reported before in insula inactivation studies, but are consistent with what is known from insula lesions in humans.

In humans, the clinical effects of insula damage have been evaluated (Ibanez et al. 2010), however, no descriptions of increased “anxiety”, per se, have been reported. Studies of tumor and stroke damage occurring in the insula show effects on autonomic processing (including cardiovascular effects), taste and gustatory deficits, impaired auditory and somatosensory processing (including alterations to bodily awareness), reduced pain response, increased neglect, and impaired emotional state recognition (Ibanez et al. 2010). Most relevant to this thesis are the effects on mood and willed action resulting from an interruption of insula-frontal lobe connectivity (Manes et al. 1999;Ibanez et al. 2010). Clarke, et al. (2008) and Weller, et al. (2009) also showed that patients with insular lesions exhibited altered risk-taking behavior and more cautious decision making when compared to normal controls (Clark et al. 2008;Weller et al. 2009;Jones et al. 2010). This is attributed to a lack of appropriate affective response due to impaired processing of interoceptive information (Jones et al. 2010). Based on the review of human lesions, Ibanez, et al (2010) propose a model where the insula controls the “basal subjective states that mediate action preparation through temporal coordination between interoceptive and exteroceptive process” (Ibanez et al. 2010). Basically, insula damage may cause changes to behavior consistent with a lack of integration of external and internal information required for decision-making

processes. With no input from interoceptive circuits, decisions requiring assessment of physiological or environmental state become unclear. This uncertainty may be the “anxiety-like” state detected in these current studies; a preference for darker/enclosed spaces, decreased approach behavior (increased avoidance), and increased startle magnitude all suggest that inactivation of the insula begets a negative emotional state where the animal is unable to process information about its surroundings and reacts with uncertainty and fear.

As mentioned before, conclusions from these studies are limited. Follow-up experiments will be necessary to determine exactly what is mediating these effects and to suggest appropriate experimental conditions moving forward. Inactivation using an agent other than lidocaine (such as Muscimol or TTX) would need to be used to confirm that the anxiety-like effect is specific to the lack of insula function in general as opposed to a specific effect of lidocaine (i.e. blockage of fibers of passage). Additional control infusion sites will be required to verify the effects are due to the novel inactivation of the insula and not a result of diffusion into nearby regions. Repeated infusions of smaller volumes of lidocaine are also needed to limit the extent of tissue damage resulting from numerous injections into the same region (as shown in experiment 2, these may be important considerations). Additionally, it may be necessary to target the insula from a different direction to avoid damage to the somatosensory cortices, which (as a component of the interoceptive circuit) may be compounding effects (as seen in experiment 1 with place conditioning).

Overall, the results of this thesis indicate that the insula plays an important role in the interoceptive pathway responsible for coordinating the integration of external and internal stimuli as a means to generate behavior responses appropriate to the current state of the body. Understanding the role of the insula in drug addiction, anxiety, and

other related indications may provide an important approach for treatment of these disorders in the future.

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