TRACING OF ACCUMBENS AND AMYGDALA NEURONS REVEALS

DISTINCT CLUSTERS IN THE COMPULSIVE ALCOHOL-SEEKING CIRCUIT

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By

Hank Coshnear

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TRACING OF ACCUMBENS AND AMYGDALA NEURONS REVEALS
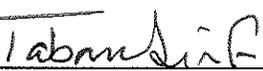
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Chapter 1: Introduction

Alcohol Use Disorders

Defined by the American Psychological Association's (APA) Diagnostic

and Statistical Manual of Mental Disorders V (DSM-V) as the presence of

symptoms including craving, tolerance, and withdrawal (2013), Alcohol Use

Disorders (AUDs)[1] have become a widespread and costly concern for the global

community. In 2013, 6.8 percent or 16.2 million adults in the United States were

heavy alcohol users, binge drinking (more than five drinks on the same occasion)

on five or more days in the past 30 days (SAMHSA, 2014). This alcohol use is

seen as a contributing factor to lost labor hours, medical visits, motor vehicle

accidents, and violent assaults, all of which have negative financial impacts on

society. The cost of excessive drinking to the U.S. economy in 2006 was

estimated at $223.5 billion, $170 billion of which was attributed to AUDs

(Bouchery et al., 2011). This cost represents nearly 2 percent of the 2006 United

States gross domestic product. Unfortunately, treatment for AUDs is not

particularly effective, as rates of relapse are high (O'Brien, 1997).

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[1] "Alcohol Use Disorder" is the updated DSM-V phrase for the colloquial term
"alcoholism." (APA, 2013) The DSM-V's "Alcohol Use Disorder" combines the two
disorders "alcohol abuse" and "alcohol dependence" used in previous edition of
the DSM.

Alcohol Use Disorders are diagnosed by the DSM-V (APA, 2013) upon the presence of at least 2 of the following criteria (at least 4 for moderate disorders and at least 6 for severe disorders):

1. Alcohol is consumed in larger amounts or for longer than intended.

2. There is a persistent desire or unsuccessful urge to cut down or control alcohol use.

3. A lot of time is spent obtaining alcohol, using alcohol, or recovering from the effects of alcohol.

4. Cravings, or strong desires or urges to use alcohol.

5. Recurrent alcohol use results in a failure to fulfill major role obligations at work, home or school.

6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.

7. Important social, occupational or recreational activities are given up because of substance use.

8. Recurrent alcohol use in situations in which it is physically hazardous.

9. Continuing to use, even when there exists a physical or psychological problem that could have been caused or made worse by the substance.

10. Tolerance, as defined by either of the following:

      a) A need for markedly increased amounts of alcohol to achieve intoxication or desired effect

b) A markedly diminished effect with continued use of the same amount of alcohol

11. Development of withdrawal symptoms, which can be relieved by using more alcohol.

The Substance Abuse and Mental Health Services Administration (SAMHSA) surveyed Americans in 2013 about their substance use. This survey, published in 2014 as the National Survey on Drug Use and Health (NSDUH), reflects the broadest and most current information about alcohol abuse and likely prevalence of AUDs in America. The NSDUH asked respondents aged 12 or older about their alcohol use in the past 30 days before the interview, defining binge drinking as drinking more than five drinks on one occasion and classifying heavy drinking as binge drinking on five or more of the last 30 days. 134 million Americans aged 18 or older were current drinkers of alcohol (SAMHSA, 2014). Out of those 134 million, 58.5 million were binge drinkers and 16.2 million were heavy drinkers (SAMHSA, 2014). Furthermore, "22.7 million individuals aged 12 or older needed treatment for an illicit drug or alcohol use problem" (SAMHSA, 2014). Because excessive alcohol use leads to tolerance and withdrawal buildup—important criteria in AUD diagnosis—and because of the rampancy of binge drinking and heavy drinking and the number of individuals needing treatment for AUDs and other Substance Use Disorders as evidenced by the NSDUH, there is reason to believe many Americans are suffering from AUDs.

Since AUDs are so widespread, they are deleterious to society on a large scale. Examples of the negative consequence of alcohol overuse include inability to work or get to work, increase in tendencies toward violent behavior, motor vehicle accidents, and hospital visits stemming from overdose or malnutrition. According to a study published in 2011 by Bouchery et al., excessive drinking cost the 2006 U.S. economy $223 billion, as well as 79,000 deaths and 2.3 million years of lost life. Furthermore, Bouchery et al. estimated that $129.1 billion (57.9%) of that cost was borne by government (including federal, state, and local agencies) and others in society, while only $92.2 billion (41.5%) was borne by the excessive drinkers and their families (2011). The annual financial cost of alcohol overuse is greater than the annual cost of cigarette smoking— $193 billion (Centers for Disease, Contol, and Prevention, 2008)—and the annual cost of physical inactivity—approximately $150 billion (Pratt et al., 2000).

Considering how ubiquitous and expensive excessive alcohol use has become, treatments demonstrate sub-optimal efficiency, not only in preventing the subject from drinking again (remission) but also in preventing the subject from returning to alcohol after a long period of time (relapse). In a 2006 longitudinal study by Moos and Moos, participants underwent treatment and attended Alcoholics Anonymous[2] sessions for 3 years and showed a 62.4% remission rate (p.216). In fact, even among the 62.4% that had shown remission for 3 years,

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[2] Founded in 1935, Alcoholics Anonymous is an international organization that conducts weekly local meetings with the stated purpose of helping alcoholics stay sober.

42.9% of them relapsed within the next 16 years (Moos & Moos, 2006, p.216).

The difficulty in achieving remission and preventing relapse that these data

suggest is a major obstacle in decreasing the rampancy of AUDs on a national

scale. Recent studies point to the compulsive nature of AUDs as the cause of this

difficulty.

Aversion-Resistant Alcohol Intake (ARAI) and Alcohol Addiction

Aversion-resistant alcohol intake (ARAI) in alcoholics is defined as an

impulsive and compulsive drive for ethanol that persists despite the awareness of

potential negative social, legal, and/or physical consequences. ARAI and its role

in a compulsive alcoholic's resistance to treatment have been well characterized

(Anton et al., 1996; Koob & Volkow, 2010; Naqvi et al., 2014; Sanchis-Segura &

Spinagel, 2006; Sinha, 2009).

ARAI is a key component of a "psychiatric-motivational framework" (Koob

et al., 2010, p.2) that conceptualizes alcohol addiction as both an impulse control

disorder and a compulsive disorder. Impulse control disorders are associated

with positive reinforcement: they are characterized by increasing anticipation

before committing an impulsive aspect and gratification upon completion.

Compulsive disorders are associated with negative reinforcement: they are

characterized by increasing anxiety and stress before committing a compulsive

repetitive behavior and relief from stress upon completion (APA, 2013). As the

subject transitions from occasional alcohol use to addiction, the subject's

motivation moves from impulsivity to compulsivity. This is also associated with a shift from positive reinforcement to negative reinforcement (Koob et al., 2010). In both of these stages, ARAI-related behavior can be seen as the subject's persistent prioritization of positive or negative reinforcement in the face of adverse consequence: impulsive alcohol intake is a prioritization of the immediate positive effects of alcohol over any conflicting interests, whereas compulsive alcohol intake is prioritization of the removal of withdrawal effects (seizures, delirium tremens, and shakiness) over any conflicting interests.

In alcohol addiction, the shift in motivational paradigm between impulsivity and compulsivity matches Solomon's opponent process theory of motivation (Solomon & Corbit, 1974). According to his theory, alcohol use causes a positive emotional state, which motivates the user to continue drinking and to have cravings for alcohol. Repeated exposure builds up tolerance, in this case manifested as decreasing affective valence. This decrease motivates further use and increased dosage. As use continues, negative emotional states (e.g. dysphoria, anxiety, and anhedonia) arise during withdrawal from alcohol. The user becomes motivated to increase use in order avoid these emotional states (Koob & Le Moal, 2005).

Aversion-resistant alcohol intake can also be conceived as an associative learning-induced adaptation or usurpation of the brain's incentive salience system. Alcohol is thought to usurp systems in the brain that judge salience

between incoming stimuli and direct animals to salient stimuli. Usurpation manifests in the addict's narrow focus on alcohol seeking despite the presence of other primary reinforcers (Koob et al., 2010). This theory helps explain the phenomenon of long-term relapse: adapted neural systems continue to assert incentive salience long after the drug has been consumed, which, combined with the right environmental cues and affective states, leads to relapse.

ARAI is linked to both impulsive and compulsive aspects of excessive alcohol use. It is also correlated with a neuroadaptive change in incentive salience. Thus, in order to understand ARAI, it is important to understand the neural systems that are usurped by alcohol in AUDs and the neural bases for increasing motivation to seek out and consume the drug. The first part in gaining this understanding is learning how to effectively model ARAI and AUDs in a preclinical manner.

Modeling AUDs and ARAI: self-administration and 2-bottle choice paradigms

A crucial part of understanding the neural basis of alcohol use disorders and aversion-resistant alcohol intake is creating effective preclinical models for alcohol addiction in animals. While it is not possible to directly measure affective states or awareness of adverse consequences in non-human models, it is possible to observe animal behavior and infer a certain degree of motivation. By allowing animals to self-administer alcohol (in the form of 20% ethanol) or giving

animals a choice between alcohol and water, researchers can observe criteria 1, 4, 10, and 11 of the DSM-V's criteria for AUDs (escalation, extinguishment/reinstating, tolerance, and withdrawal). Comparison of animal drinking rates to human drinking rates (via blood alcohol levels) can show similarity of abuse.

Extended access to alcohol during self-administration studies allowed researchers to measure many of these criteria. First, rats given extended access to alcohol showed escalation in intake over the course of several days (Koob, 2009). Furthermore, rats were shown to increase self-administration during acute and protracted withdrawal and when made dependent on alcohol, obtained blood levels consistent with those exhibited by moderate or heavy alcohol abusers (Gilpin & Koob, 2008). Finally, alcohol dependent rats showed increased alcohol-induced reinstatement after extinction (Deroche-Gamonet et al., 2004). These data suggest that the murine self-administration model of alcohol is compatible with many of the motivational and behavioral components of human alcohol use disorders.

One effective and instructive form of self-administration is the 2-bottle choice paradigm. In this paradigm, animals are habituated to 20% ethanol over the course of 12 weeks. After the 12-week training period, animals are provided a choice between 20% ethanol and water (two-bottle choice, 2BC) for 20 minutes every 24 hours, and consumption is measured by bottle weight before and after

the 20 minute drinking sessions (Hopf et al., 2010). The benefits of this paradigm include the ability to analyze conditioned place preference—where the animal spends more time on the side where the ethanol bottle is located—or preference for alcohol—where the animal consumes alcohol more than water. The 2BC system also allows the animal to drink water if it is thirsty and not be forced to drink the ethanol.

The most relevant advantage of the 2BC paradigm for ARAI research is the ability to introduce quinine into the ethanol in order to create an aversive stimulus (Hopf et al., 2010; Hopf & Lesscher, 2014; Loi et al., 2010; Wolffgramm et al., 2000; Wolffgramm & Heyne, 1991). Quinine is very bitter and when mixed with the ethanol (.1g quinine/L ethanol) it causes rats not habituated to alcohol to drastically reduce consumption (Hopf et al., 2010). However, when rats are exposed to alcohol using 2BC and an intermittent access paradigm,[3] they develop a quinine-resistant motivation for alcohol (Hopf et al., 2010; Seif et al., 2013). Moreover, data suggest that this resistance to quinine aversion is motivated by alcohol specifically: when rats were given sucrose or water adulterated with quinine, they displayed a decrease in intake (Hopf et al., 2010).

Similarly, Seif et al. (2013) used a footshock model to demonstrate ARAI using a physical aversive stimulus. After 2.5 months of 2BC training and 7-8

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[3] Intermittent Access to Alcohol (IAA): Animals are given access to 20% ethanol 3 of the 7 days per week, with at least 1 day in between the 24-hour alcohol access sessions (Hopf et al., 2010). This paradigm shows greater overall alcohol intake than the continuous access paradigm (Simms et al., 2008).

weeks of operant training, rats were introduced to daily intermittent footshock, where 1 in 8 FR3[4] lever presses paired with a light footshock (0.2mA, 0.5s) (Seif et al., 2013, p.8). After the first shock, all rats showed reduced responding. However, Seif et al. found that after the 5th or 6th footshock session, about half of the rats that were shock-resistant—that is, they did not significantly reduce intake or number of lever presses in the presence of the intermittent footshock stimulus (2013, p.3).

The 2BC self-administration model combined with quinine matches well with human aversion-to-physical-discomfort resistance: alcoholics, once dependent, will drink "non-beverage alcohol despite the bad taste and the presence of toxic compounds" (Hopf & Lesscher, 2014, p.4) such as mouthwash, eau de cologne, and rubbing alcohol. Furthermore, alcohol abuse can often cause "gastrointestinal problems such as diarrhea and intestinal bleeding" (Hopf & Lesscher, 2014, p.4). These noxious effects clearly show that alcohol intake continues despite physical harm or negative consequences. Moreover, the quinine and ARAI paradigm models multiple aspects of alcohol addiction, including greater motivation for the drug, greater reinstatement, and greater responding without drug delivery (Belin et al., 2011; Deroche-Gamonet et al., 2004; Hopf et al., 2010; Hopf & Lesscher, 2014; Lesscher et al., 2010; Seif et al.,

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[4] 1 in 8 fixed ratio 3 (FR3) schedule means positive reinforcement (20% ethanol) was delivered after every three lever presses, and 1 in every 8 reinforcers was paired with footshock. Essentially, 1 in every 24 lever presses produced footshock.

2013). Rats showing quinine-resistant intake have also shown disrupted patterns of intake and increased preference for higher concentrations of alcohol (Spanagel & Holter, 1999; Vengeliene et al., 2013; Wolffgramm et al., 2000). Taken together, the data from these experiments support the quinine-resistant intake model as a useful model for human AUDs and for studying human ARAI.

Because of some criticism (Hopf et al., 2010) regarding the quinine-resistant intake model (e.g., level of aversiveness of quinine, timing of aversion with respect to intake), the footshock-resistant intake model is an important addition and alternative to the quinine-resistant intake model (Hopf et al., 2010). Seif et al. demonstrated (2013) that the footshock model effectively parallels the resistance to reduction found in the quinine model. Also, Seif et al. found that an important cortical-nucleus accumbens input mediates reduction in both quinine and footshock, with inhibition of this input leading to reduction in intake. Interestingly, this reduction in intake only occurred in the presence of aversive stimuli (2013). This correlates with previous work showing that cortical inputs are preferentially involved in aversion-resistant intake (Naqvi & Bechara, 2010; Tiffany, 1990). Regarding the criticism of timing—human alcoholics don't generally incur adverse consequences (job loss, incarceration, pathological disorder) until hours or days after the alcohol intake, while rats taste quinine with each sip—the 1 in 8 FR3 footshock model has been argued to create the "anticipation of footshock" (Seif et al., 2013; Vanderschuren & Everitt, 2004) so

that this anticipation is sufficient as a deterrent to intake. This model, then, imitates the anticipation of future consequences in humans. This argument is supported by the fact that the rats in the 1 in 8 FR3 paradigm that had the relevant cortical-accumbens inputs "often received no shocks per session" (Hopf & Lesscher, 2014, p.6). In other words, when mental conflict was drastically lessened, the rats' anticipation of footshock was sufficient to eliminate response.

It is interesting to note that in the rat ARAI experiments, only a subpopulation of animals show ARAI (Hopf et al., 2010; Hopf & Lesscher, 2014; Naqvi & Bechara, 2010; Seif et al., 2013; Vanderschuren & Everitt, 2004; Vengeliene et al., 2009; Wolffgramm et al., 2000); this matches the observation that only a subpopulation of human alcohol or cocaine users develop addiction. It may be true that all humans and rats can develop ARAI, but a subpopulation of subjects may not respond to the particular intensity of aversiveness or of the consequences in each case. The Hopf et al. 2010 and Seif et al. 2013 experiments illuminate part of this contention, as nearly all of the rats showed resistance at 30mg/L of quinine but only some did at 100mg/L. Additionally, degree of impulsivity in each subject may contribute to the degree of aversion resistance, as studies in cocaine showed rats that had high responses also had exhibited high basal impulsivity and novelty seeking (Belin et al., 2011; Belin et al., 2012; Belin et al., 2008).

Behaviorally speaking, quinine-resistant and footshock-resistant alcohol intake has been well characterized. However, the neurological bases for this behavior are not completely understood.

Neurocircuitry of addiction: neurological bases for compulsive alcohol intake

The regions of the brain associated with addiction and ARAI are those typically associated with awareness (the insula and prefrontal cortex), reward (the striatum, including the nucleus accumbens), and fear/arousal (the central amygdala) (See Figure 1). The insula and medial prefrontal cortex (mPFC) are implicated in craving and relapse, and dysfunction in these areas can reduce intake (Koob et al., 2010; Naqvi & Bechara, 2010; Tiffany et al., 2000). The striatum, including the nucleus accumbens (NAc), has been studied exhaustively regarding its connection to reward, habitual drug use, and expression of habits (Belin et al., 2008; Hopf & Lesscher, 2014; Koob et al., 2010; Seif et al., 2013; Tiffany, 1990; Vanderschuren & Everitt, 2004). The central amygdala (CeA) has been studied in detail with respect to changes in neuronal activation (Childress et al., 1999; Schneider et al., 2001), overall amygdala volume (Makris et al., 2008), and signal peptide concentration (Pandey et al., 2008; Roberto et al., 2004; Roy & Pandey, 2002) in substance abusers. In recent studies, these regions have been shown to interact in order to produce aversion-resistant alcohol intake; however, the circuitry and molecular interactions between these areas have not

been fully explored. The following sections will discuss what is known about each brain area in regards to how it regulates compulsive alcohol intake.
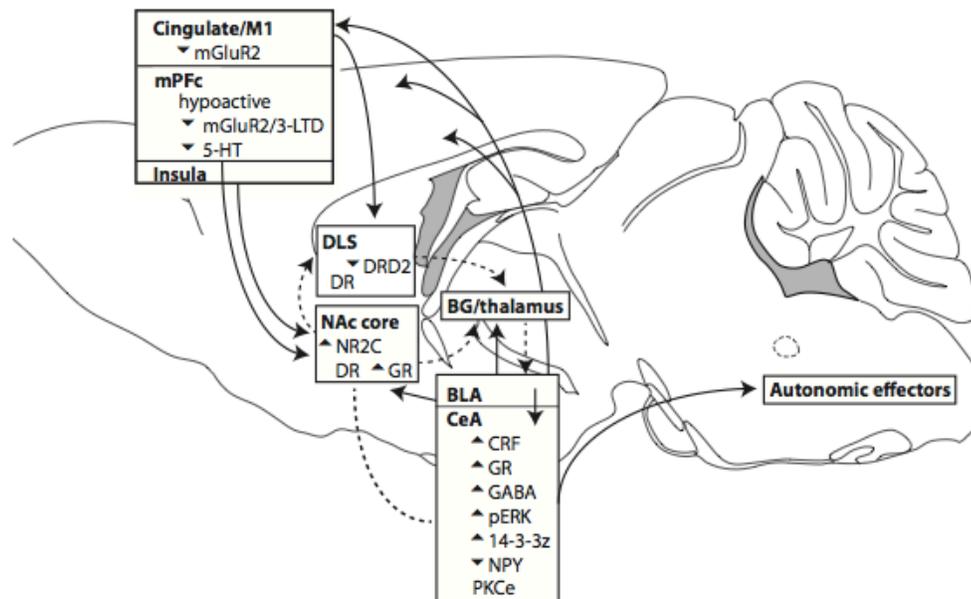
Figure 1: Sagittal section through a rat brain, showing brain circuits thought to take part in ARAI, including molecules and neural adaptations involved (Childress et al., 1999; Everitt & Robbins, 2005; Koob, 2009; Naqvi & Bechara, 2010). This circuit is activated during conflict of desire (wanting ethanol vs. aversion). It functions as a top-down circuit, with cortical areas processing conflict. Cortical areas (Cingulate cortex, mPFC, Insula) and striatum (DLS, NAc core) directly mediate compulsivity, and CeA has been shown through knockout studies to promote compulsive alcohol drinking (Naqvi & Bechara, 2010). The authors of these studies have theorized that the CeA may enact physical and/or hormonal sensations that cause feedback leading the subject to act (Naqvi & Bechara, 2010). BG: Basal ganglia, DLS: dorsolateral striatum, BLA: basolateral amygdala, DR: dopamine receptor, GR: glucocorticoid receptor. Borrowed with permission from (Hopf & Lesscher, 2014) .

Cortical areas and compulsive alcohol intake

Many researchers studied the role of cortical areas in compulsive alcohol intake. These researchers found that prefrontal activity correlates with craving and relapse and that cortical dysfunction decreases behavioral control (Koob et al., 2010; Naqvi & Bechara, 2010; Tiffany et al., 2000). More recently, Seif et al. 2013 found new data on the cortical neurons likely responsible for this mediation of behavior. Seif et al. found (2013) that optogenetic inhibition of insular or mPFC inputs to the nucleus accumbens core reduces both quinine- and footshock-resistant intake. By contrast, animals optogenetically inhibited but allowed to drink without aversive stimuli showed normal intake, suggesting that these cortical inputs were not active in those circumstances (Seif et al., 2013). This research found—via *in vitro* electrophysiology—that alcohol-drinking rats underwent adaptations in NMDA receptors in these cortical-accumbens core inputs independently of the introduction of aversion. Specifically, these NMDA receptors were hyperpolarized, meaning that they required a smaller stimulus to fire. Given that these adaptations occurred in alcohol-drinking rats and that when the relevant neurons were shut off, the rats showed aversion, it seems likely that insula-NAc or mPFC-NAc neuronal firing is part of a compulsive drinking circuit.

One of the most important implications of this research is that brief interventions, shown to be the most effective form of treatment for AUDs (Moyer & Finney, 2004), could be used to aid the subject in associating conflict with

drinking. Brief interventions typically involve short counseling sessions that aim at reducing drinking to moderate levels. Brief interventions use motivational interviewing to provide personalized feedback on the patient's AUD and persuade them to reduce their alcohol intake (Moyer & Finney, 2004). The development of drugs targeted specifically to cortical-accumbens core NMDA receptors in combination with these brief interventions could reduce a subject's compulsive drive for alcohol more effectively than any current psychological or pharmacological treatment.

Striatum and compulsive alcohol intake

The striatum, especially the NAc core, has also been extensively studied with respect to compulsive alcohol intake. Activity in the NAc is associated with a feeling of reward in all classes of abused drugs (Everitt & Robbins, 2005; Hopf & Lesscher, 2014; Koob et al., 2010; Pierce & Kumaresan, 2006), and dopamine levels in the NAc rise in response to all of these drugs (Di Chiara & Imperato, 1988). As dopamine acts as a signal for reward, it is thought that the striatum is responsible for the reinforcing aspect of drug use. Furthermore, inputs to the NAc core have been shown to regulate ARAI (Seif et al., 2013). In addition, dopamine receptors within the NAc sustain footshock-resistant cocaine seeking (Hopf & Lesscher, 2014, p.8; Saunders et al., 2013).

Although the NAc is active during any reinforcing event, the dorsal striatum (DLS) appears to be recruited during the development of compulsive

drug seeking (Everitt et al., 2008; Koob et al., 2010, p.5). When the NAc core

was lesioned on one side of the brain and combined with dopamine receptor

blockade in the contralateral dorsal striatum, although no effect was seen right

after acquisition, a second-order schedule resulted in a large decrease in cocaine

seeking. (Everitt et al., 2008; Koob et al., 2010, p.6). Furthermore, inactivation of

the DLS has been shown to increase sensitivity to devaluation in rats undergoing

a habitual self-administration paradigm (Corbit et al., 2012). In other words,

inactivation of the DLS caused habitual alcohol-seeking rats to reduce intake

before which they had been shown to be unable. This shows that the DLS plays

a major part in the transition to habitual or compulsive drug use, such as when

alcohol use becomes an AUD.

Central amygdala and compulsive alcohol intake

Evidence suggests a role for the central amygdala (CeA) in compulsive

alcohol intake. Habitual users of alcohol and cocaine show decreased amygdala

volume (Makris et al., 2008), which is correlated with a greater propensity for

craving and relapse (Wrase et al., 2008).

Excessive alcohol use also affects the levels of many neurotransmitters

and signaling factors in the CeA. Escalation of alcohol intake is paralleled by

increased levels of corticotropin releasing factor (CRF) and phosphorylated ERK

and by decreased levels of neuropeptide Y (NPY), brain-derived neurotrophic

factor (BDNF), and phosphorylated CREB (Merlo Pich et al., 1995; Pandey et al., 2008; Roy & Pandey, 2002; Sanna et al., 2002). Also, by increasing NPY levels or by blocking CRF receptors in the CeA, escalation of intake is eliminated (Funk et al., 2006; Gilpin & Koob, 2008). This suggests that the CeA plays many roles in responding to escalation and developing addictive behavior.

The insula/mPFC, NAc, and CeA all are part of a theoretical compulsive drinking circuit—brain modules that once regulated appraisal and approach behavior with respect to strong reinforcers and have been hijacked in drinking animals to cause escalation of intake and resistance to aversion.

Cortical projection experiments: How is the ARAI circuit organized?

From the evidence presented, different aspects of compulsive alcohol drinking (including ARAI) seem to be associated with different areas of the reward/arousal pathway. It has been shown that inputs from the aINS/mPFC to the NAc regulate ARAI (Seif et al., 2013). The insula has also been shown to project to many of the other implicated areas in this pathway, including the CeA and locus coeruleus and adjacent parabrachial neurons (LCPB) (Jasmin et al., 2004). These projections are thought to mediate compulsive drinking, but very little data has been collected about these projections. Characterization of the neuronal projections of the aINS and determination of neuronal segregation of these projections will aid in understanding AUDs and ARAI. Compulsive behavior has long been an obstacle in treating AUDs; the ability to target a drug directly to

a projection or set of projections responsible for this behavior could lead to novel and innovate treatments for these disorders.

The experiment undertaken here used dual injections of fluorescently labeled beads to trace where aINS projections originated in the insula; fluorescence from two separate projections was combined and examined for colocalization (Dunn et al., 2011). If fluorescence from both beads were observed in the same neuron, evidence would have pointed to one population of neurons that regulates the whole compulsive drinking circuit. If neuronal colocalization were not observed, evidence would have pointed to separate clusters of insular neurons that each regulates a different aspect of compulsive drinking. Although this research has been done previously, injections were performed in only one brain region per animal; thus, variance between subjects was very likely and previous results might not accurately represent the variation.

A major advantage of this experiment was that the results were interesting and informative regardless of the outcome: All of the regions investigated (aINS, LCPB, NAc) were known to regulate alcohol consumption as well as stress and aversion responses. If data had shown that neuronal subpopulations are segregated, we could investigate how each subpopulation affects behavior separately and how antagonism of each subpopulation might contribute to treatment of compulsive ethanol consumption. If data had shown that neuronal subpopulations are integrated and the same neuronal cluster projected to

different targets, we could investigate how these neurons coordinate activity in multiple downstream sites. Also, as individual aINS neurons could have projected to multiple downstream targets, we could study whether only one or all of these projections are necessary for compulsive drinking.

Chapter 2: Materials and Methods

Unless specified, all chemicals and reagents were from Sigma (St. Louis, MO).

Male Long-Evans rats were used in this experiment. Procedures for the maintenance and use of experimental animals conformed to the regulations of the UCSF Committee on Animal Research and were carried out in accordance with the guidelines of the NIH regulations on animal use and care (Publication 85-23, Revised 1996).

Intracerebral injections of fluorescent bead retrograde tracers

Animals were anesthetized with a mixture of 5% isoflurane and 95% oxygen. They were placed in a stereotaxic frame, the skull was exposed via a midline incision, and a burr hole was drilled at the appropriate stereotaxic coordinates. A 33-gauge microinjector needle was fixed to a 2-μl Hamilton syringe, filled with tracer, and lowered to the target. 0.3-0.5 μl of Lumafluor retrobeads (Durham, NC), conjugated to either Alexa Fluor 488 or 594 were

slowly injected into each injection site. Each animal received a total of 4 retrograde tracer injections (two in each hemisphere). Two sites specifically targeted for injection are given with their stereotaxic coordinates and angles of injection in Table 1, and the combinations of these sites constituting the three evaluated injection pairs are shown in Table 2. The present studies focused on comparing aINS neurons that project to the NAc versus LC.

Perfusion and sectioning

3-4 weeks after surgery, rats were deeply anesthetized with 5% isoflurane and 95% oxygen, injected with pentobarbital (100 mg/kg, IP), and then killed by vascular perfusion, first with a brief rinse of PBS, followed for 5 min with 4% paraformaldehyde. The brains were immediately removed, stored overnight in fresh fixative, and then transferred to PB (pH 7.4) containing 25% sucrose for 48 hours. The brains were coated in OCT (Tissue-Tek, USA) to prevent ice crystals from damaging tissue and were subsequently frozen at $-20°C$. Coronal sections were cut on a sliding microtome at 50 μm and stored at $4°C$ in sodium PBS.

Microscopy

Sections were mounted on glass slides in rostrocaudal sequence and air-dried. Sections were coverslipped using Vectashield with DAPI (Vector Laboratories, Burlingame, CA) for nuclear staining. The sections were visualized with a Life Technologies (Pleasanton, CA) EVOS FL equipped for brightfield and fluorescent microscopy.

Retrogradely labeled neurons in the aIns exhibiting Alexa Fluor 488, Alexa Fluor 594, and both fluorophores were visualized in every section as green, red, and yellow, respectively. Widefield images were captured using the GFP and Texas Red filter cubes[5] and red and green channels were merged to show colocalization using FIJI. Images were taken at 2x magnification to show overall structure and confirm placement of injections and at 10x magnification to show individual fluorescent beads.

Fluorescence controls

Control injection sites where both retrograde tracers were injected into the same target area (e.g. NAc-NAc) were chosen in order to allow estimation of false negatives (i.e. the relative incidence of green-only and red-only labeled aINS neurons when both tracers were injected into the same target area) (See Table 2). Control images of fluorescence in the NAc and LCPB injection sites were captured using the widefield microscope. Images were qualitatively analyzed for successful injections where fluorescence was markedly greater in the expected channel (the channel respective to the bead injected) than in the alternate channel (the channel used by the bead injected into the alternate injection site).

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[5] For filter cube spectral data, see
https://www.thermofisher.com/content/dam/LifeTech/global/technical-reference-library/newsletters-journals/bioprobes/pdfs/bp70/bp70-evos-light-cube-poster.pdf

Table 1. Stereotaxic coordinates[6] for injection sites.

Injection Site	AP	ML	DV	Angle
NAc core	**+2.2**	**+/- 2.7**	**-7.48**	**8°**
LCPB	**-13.93**	**+/- 1.3**	**-8.66**	**30°**

Table 2. Injection site combinations.

Case	NAc		LC
NAc-NAc	**488 + 594**		
LC-LC			**488 + 594**
NAc-LC	**488**		**594**

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[6] In mm relative to bregma, using flat-skull techniques.
AP, Anterioposterior; ML, mediolateral; DV, dorsoventral.

Chapter 3: Results

Homotypic injections (NAc-NAc and LC-LC)

Positive controls for this experiment used homotypic injections: bilateral injections into a single target area using both retrograde tracers. Correct placement and fluorescence from both injections was confirmed at 2X magnification both at the nucleus accumbens injection site (n=2) and at the LCPB site (n=3) (See Figures 2 & 3). The merged channels images showed complete overlap between the signal from the Alexa Fluor 488 beads and the Alexa Fluor 594 beads.

Slides of anterior insular projections of NAc and LCPB neurons targeted by these homotypic injections were imaged at 10x magnification to visualize individual cells and compare bead signal between channels. The retrograde labeled insular neurons highlighted by fluorescent beads were consistent between channels (See Figure 4)(n=5). Both the 488 and the 594 beads appeared to localize to the same cell bodies in the insular slices. This was consistent with expectations for the homotypic injections and with the results from imaging the injection sites.

As the images were taken in widefield, it is possible that signal appeared to colocalized but beads actually labeled two cells on top of each other, which could not be visualized without a confocal z-stack. However, given the amount of

yellow signal and the number of cells labeled with yellow makes this possibility

very unlikely.
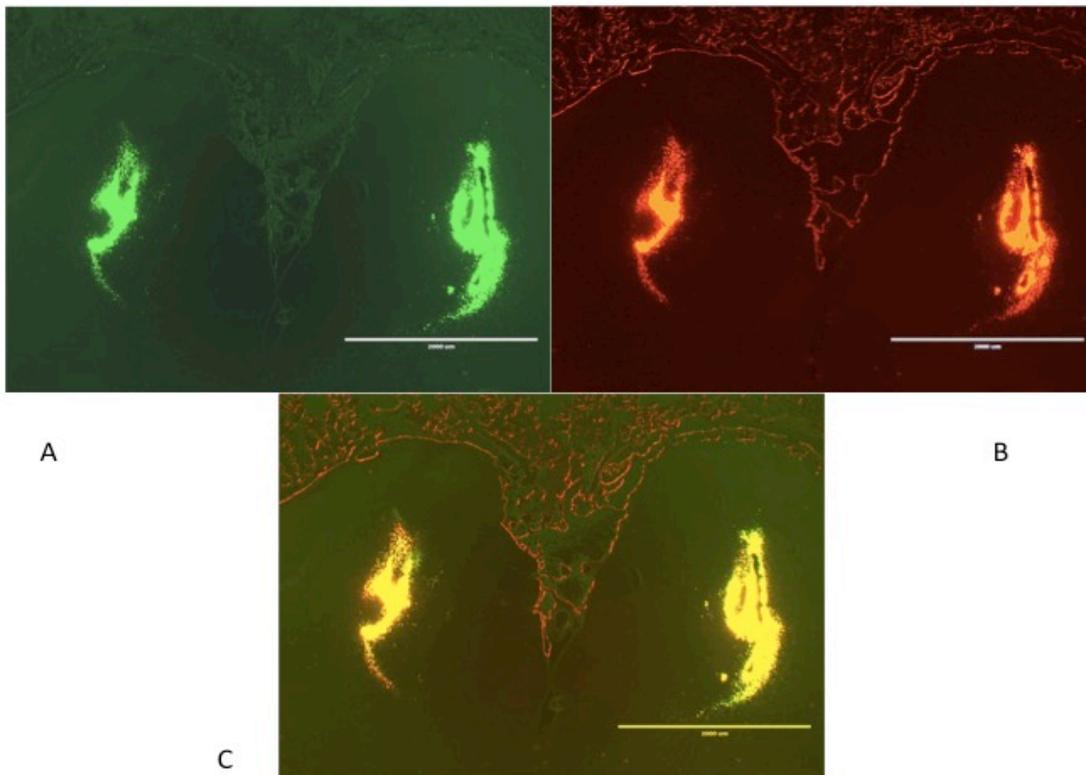
Figure 2. Results of homotypic bilateral retrograde tracer injection into the nucleus accumbens (NAc) (n=2). 2x magnification. A: Alexa Fluor 488 injection site. B: Alexa Fluor 594 injection site. C: Merged channels. Yellow signal is colocalized signal from both channels. Scale bars indicate 2000 um.
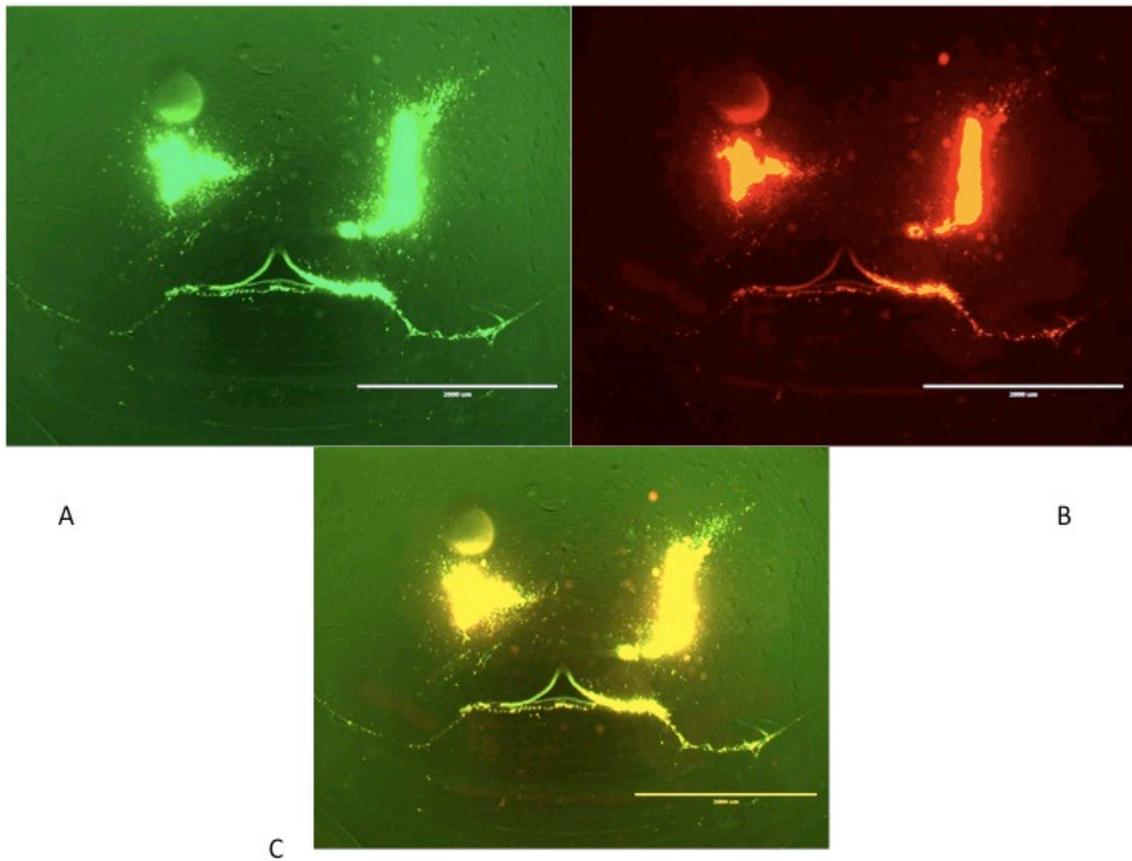
Figure 3. Results of homotypic bilateral retrograde tracer injection into the LCPB (n=3). 2x magnification. A: Alexa Fluor 488 channel B: Alexa Fluor 594 channel C: Merged channels. Yellow signal is colocalized signal from both channels. Scale bars indicate 2000 um.
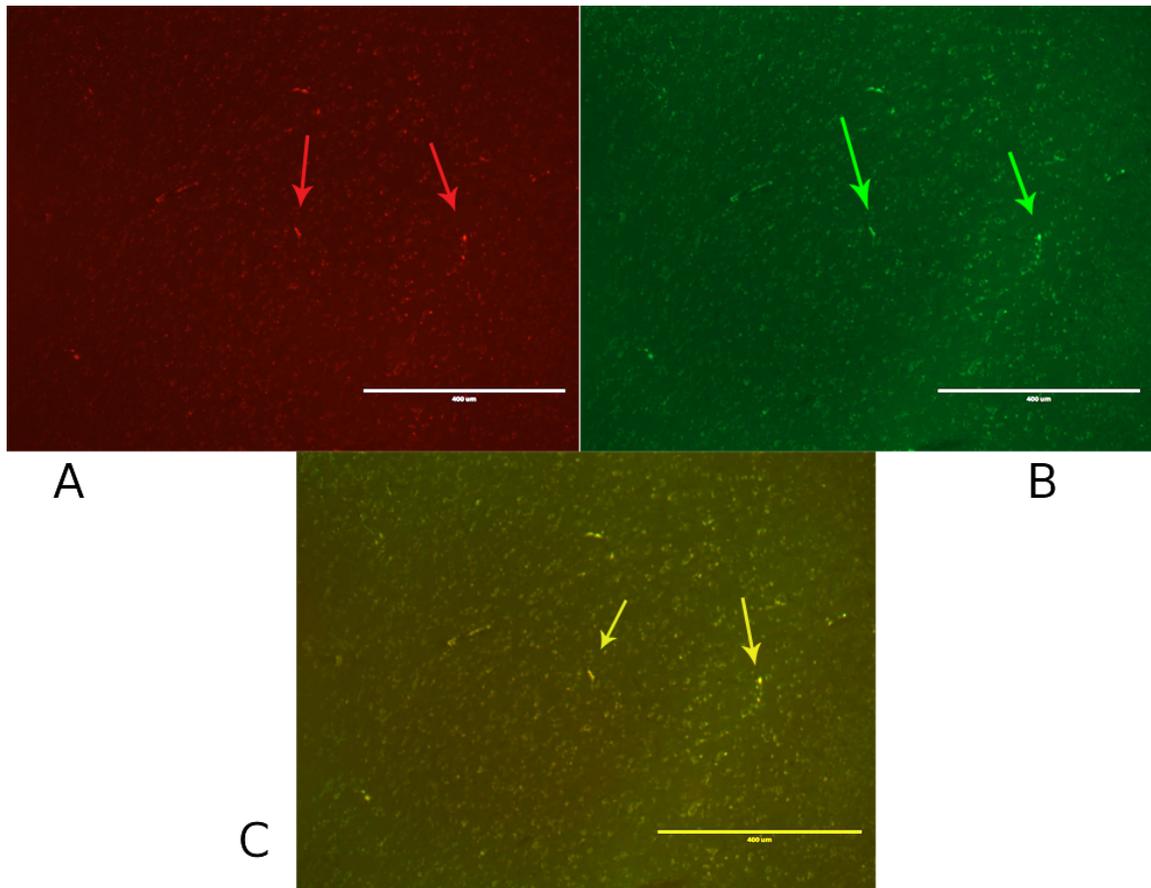
Figure 4. Highlighted neurons in the anterior insula resulting from homotypic bilateral retrograde tracer injections (n=5). 10x magnification. A: Alexa Fluor 488 channel B: Alexa Fluor 594 channel C: Merged channels. Yellow puncta are colocalized beads. Red, green, and yellow arrows indicate examples of double labeling. Scale bars indicate 400 um.

Heterotypic injections (NAc – LC)

Heterotypic injections, or injections where beads were inserted in two different brain regions, were analyzed for heterogeneity (n=4). Injection sites for each condition (NAc 488 – LC 594 or NAc 594 – LC 488) were imaged at 2X magnification to confirm placement and to serve as negative controls: images were taken in both Alexa Fluor 488 and Alexa Fluor 594 channels at each site. Correct placement was confirmed at all injection sites. All injection site images were positive for signal in the channel corresponding with the color of the injected fluorescent beads and negative for signal in the channel not corresponding to the color of the injected fluorescent beads (See Figures 5 & 6).

Slides of insular tissue from these heterotypic injected brains were imaged at 10X magnification to reveal cell body fluorescence in neuronal projections from the NAc and LCPB. Distinct, discrete populations of cell bodies in the anterior insula (AIns) were seen while comparing signal from each fluorescence channel (See Figure 7) (n=4). There was no qualitative colocalization seen between the Alexa Fluor 488 and Alexa Fluor 594 beads. These results were consistent between samples and consistent with the results from the injection site controls.

Particular instances of lack of colocalization, or exclusion, were enlarged and bleed through ratio was calculated to account for the bleed through seen in both channels (See Figure 8 and Table 3). The mean brightness of these regions of interest, or ROIs, was used to create bleed through ratios that quantify how much signal in each channel is due to bleed through as opposed to true signal from the fluorescent beads. There was some bleed-through from the 488 beads into the 594 channel in cases where only 488 beads were injected into a particular site, but the level of fluorescence was 55% lower than that of the expected channel (See Table 3). This bleed-through can happen when the fluorescence filters on the microscope slightly overlap, but filter cube data from the manufacturer shows no overlap[7].

As most cells typically contain some basal autofluorescence, autofluorescence was analyzed by calculated the mean brightness of an ROI in Figure 7 with signal and comparing it to an ROI in Figure 7 without any expected signal (See Figure 9 and Table 4). As the ratios of autofluorescence in each sampled pair match the bleed through ratios found in the same image (Table 3), autofluorescence may account for the apparent bleed-through in both channels.

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[7] These data can be found on Life Technologies's website:
https://www.thermofisher.com/content/dam/LifeTech/global/technical-reference-library/newsletters-journals/bioprobes/pdfs/bp70/bp70-evos-light-cube-poster.pdf
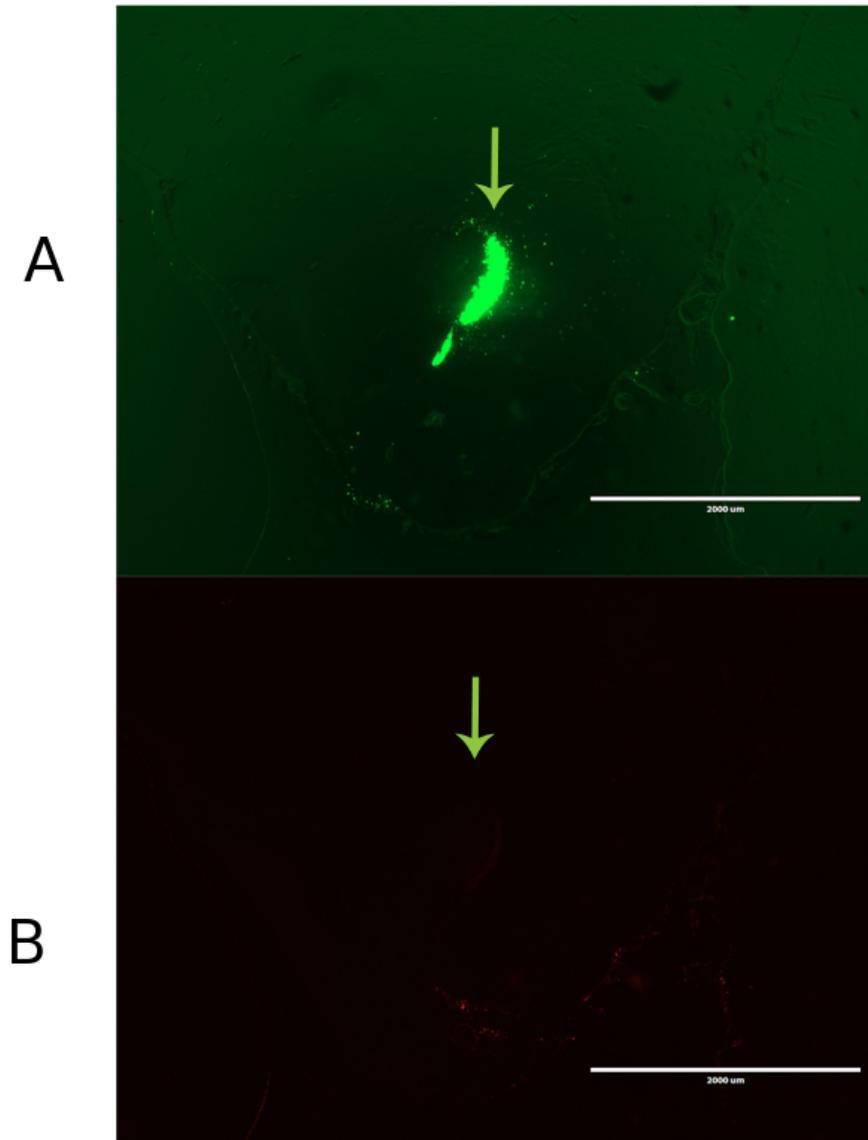
Figure 5. Results of 2x scan on heterotypic injection site (NAc). Injection beads were labeled with Alexa Fluor 488. A: 2x scan with 488 filter. B: 2x scan with 594 filter. Scale bars indicate 2000 um. Green arrows indicate where signal was seen in the 488 channel and where signal could be expected in the 594 channel.
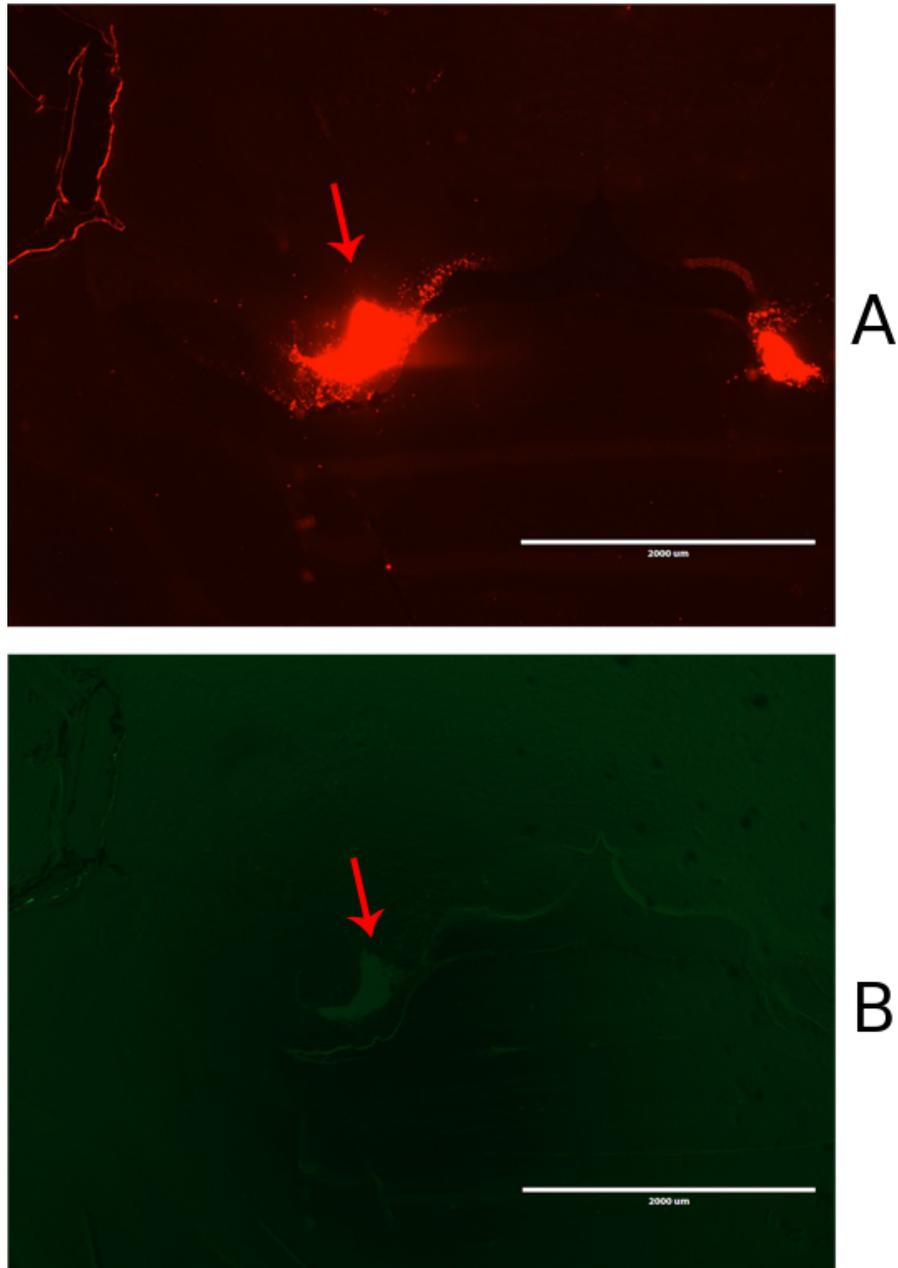
Figure 6. 2x scan of heterotypic injection site (LCPB). Beads were labeled with Alexa Fluor 594. A: 2x scan with 594 channel. B: 2x scan with 488 channel. Scale bars indicate 2000 um. Red arrows indicate where signal was seen in the 594 channel and where signal was expected in the 488 channel.
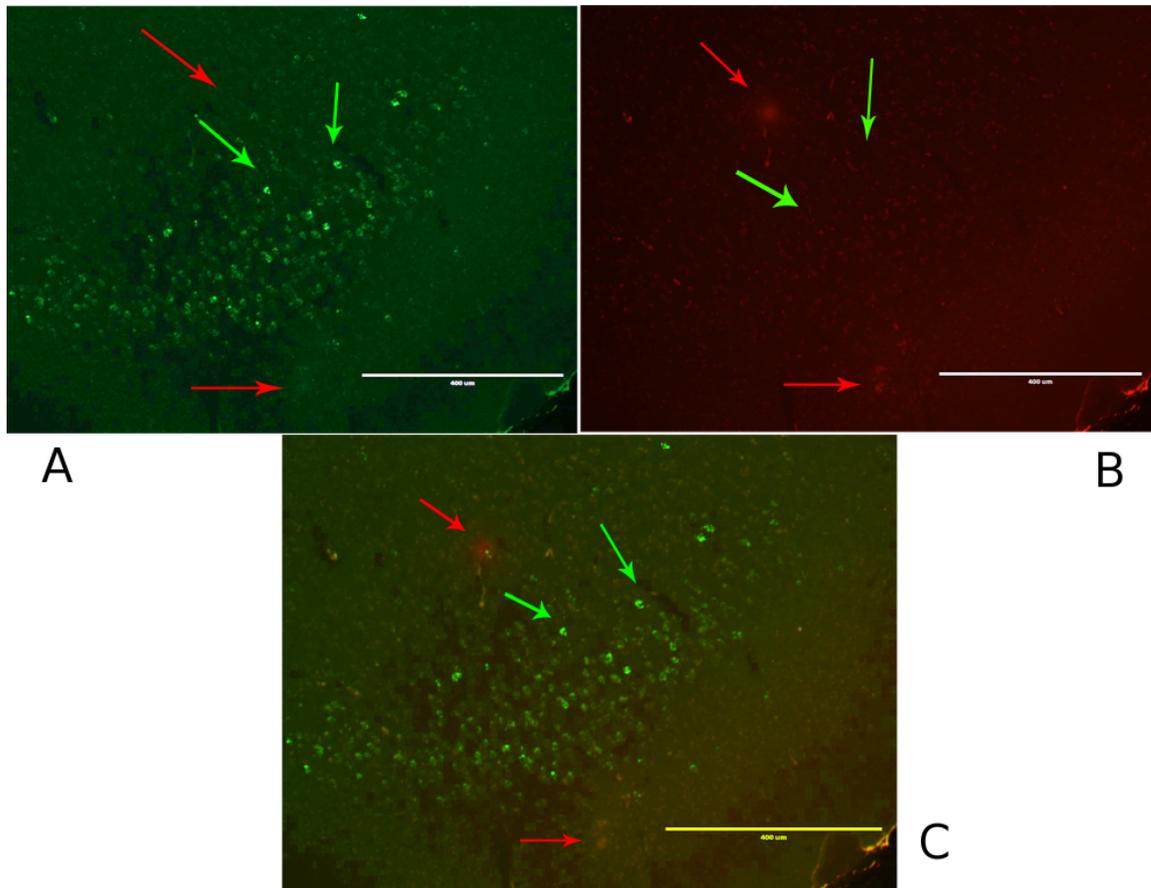
Figure 7. 10x scan of anterior insula tissue. Fluorescent beads are shown as puncta. A: 10x scan on 488 channel. B: 10x scan on 594 channel. C: merged channels. Red and green arrows indicate examples of single labeling. Scale bars indicate 400 um.
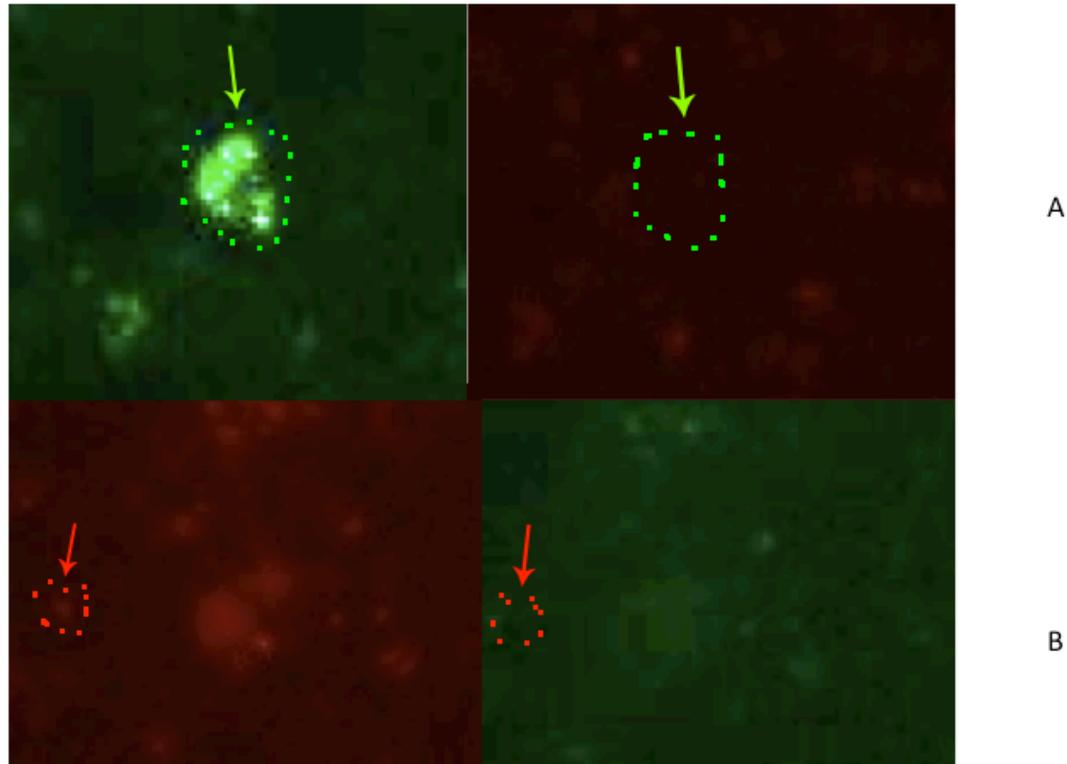
Figure 8. 600% enlarged ROIs from Figure 7. A: Showing presence of signal in 488 channel and lack of signal in 594 channel. B: Showing presence of signal in 594 channel and lack of signal in 488 channel. Green and red arrows and dotted lines indicate ROIs.

Table 3. Mean Intensities and Bleed Through Ratios			
	488 channel mean	**594 channel mean**	**Bleed through Ratio**
488 ROI	64.343	15.173	23.5%
594 ROI	15.778	33.667	46.9%
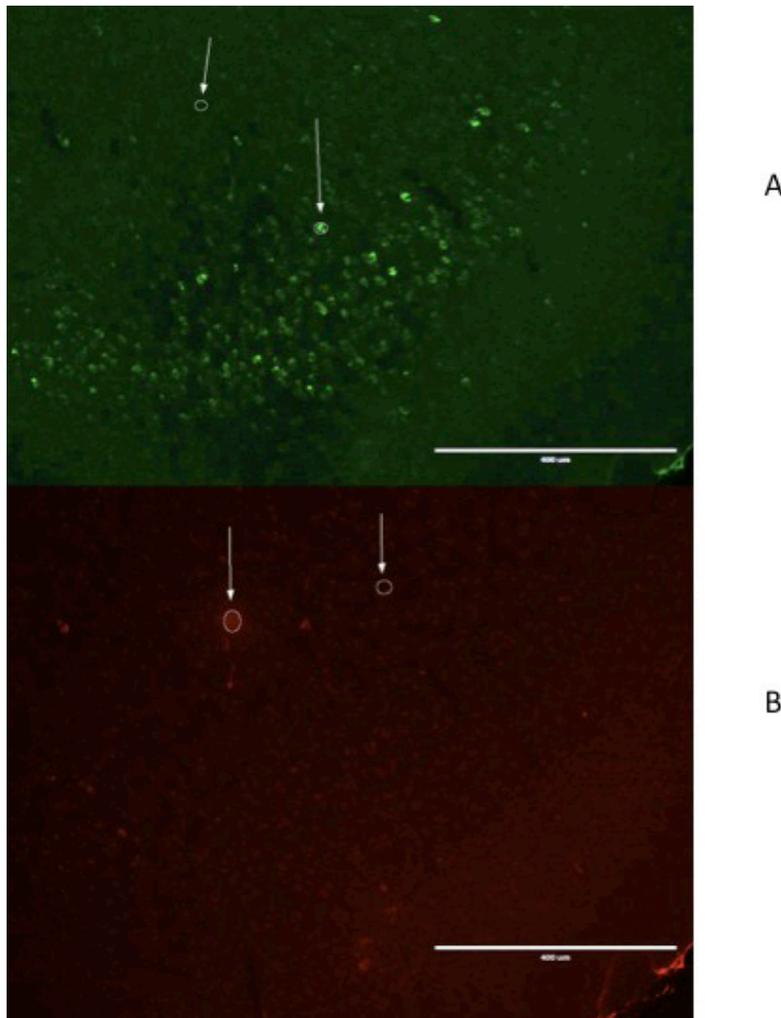
Figure 9. Autofluorescence ROIs from Figure 7. A: 488 channel autofluorescence ROIs. B: 594 channel autofluorescence ROIs. Scale bar indicates 400 um. White arrow pairs indicate ROIs used to calculate autofluorescence ratios.

Table 4. Mean intensities and Autofluorescence			
	Area with signal mean	No signal mean	Autofluorescence Ratio
488 channel ROI	77.686	17.066	22.0%
594 channel ROI	36.302	14.966	41.2%

Chapter 4: Discussion

Given the results of this experiment, we can conclude that our original hypothesis of segregated neuronal subpopulations in the ARAI circuit was confirmed. We found distinct subpopulations of insular neurons that projected to the nucleus accumbens and locus coeruleus, leading us to believe that there are separate brain subsystems that collectively produce aversion-resistant alcohol intake (ARAI). This ARAI can then be thought of as a gestalt of its component functions, each of which can be investigated on its own to examine its contributions to alcohol-seeking behavior.

The insular fibers that project to the NAc, whose NMDA receptors were previously shown to regulate ARAI (Seif et al., 2013), have even more relevance in alcohol abuse research as this experiment shows that this Ins-NAc connection can be targeted without disrupting other insular functions. If the population of neurons that project to the NAc had been shown to project to the LC as well, silencing these neurons could have unintended side effects. However, since these projections originate from different insular neurons, the Ins-NAc connection can be disrupted benignly, decreasing only the insular contribution to the ARAI circuit. This has enormous potential for drugs that can specifically target against alcohol abuse.

One caveat for this experiment is that colocalization for widefield images is difficult to quantify (Dunn et al., 2011). An attempt was made to prove colocalization in the homotypic injections (Figure 4) by the sheer number of yellow puncta, and an attempt was made to prove exclusion in the heterotypic injections (Figures 7 & 8, Table 3) by the relative bleed through in each channel. However, these data do not completely show quantitative colocalization, as a Pearson's Colocalization Constant (PCC) would need to be found for each image; this would only be possible if the slides had been imaged using a confocal microscope, as the PCC can only be calculated using three-dimensional voxels (Dunn et al., 2011).

Future studies

Although our research qualitatively confirms our hypothesis, more work could be performed to optimize the results: a future study could use Imaris software (Bitplane, St. Paul, MN) to compile a three-dimensional model of the injection sites from the individual brain slices. This model would be useful for identifying the volume of each injection and for determining how many off-target neurons uptook fluorescent beads during injections. Imaris software could also be used to create a 3D model of the insular slices, which would be useful for more precisely identifying the insular location of each neuronal body that projects to the NAc and to the LCPB. Finally, a future study could image slides using a confocal microscope. This could be used to quantitate the results of the

heterotypic injections: calculating the PCC for each image would yield statistical colocalization data, which could quantitatively confirm that none of the heterotypic injections resulted in colocalized beads.

Our research, which confirms the presence of discrete modules within the ARAI circuit, could have implications for drug abuse therapy in general and for other behavioral circuits. If the aINS-NAc connection can be specifically silenced to mediate abuse in alcohol-seeking behavior, perhaps a future experiment could attempt to regulate nicotine or cocaine cravings, for which previous studies have implicated similar mechanisms in the nucleus accumbens (Childress et al., 1999; Everitt et al., 2008; Koob, 2009; Saunders et al., 2013). Similarly, researchers could target other compulsive behaviors such as lying or gambling, as the reward pathway for these behaviors also involves the nucleus accumbens (Cardinal et al., 2005). Ultimately, this research would have translational medicine potential for studies attempting to silence ARAI in humans; although an intracranial NAc injection as drug therapy would be too invasive for alcohol-seeking humans, perhaps another mechanism can be found to directly target those AIns-NAc fibers.

As this experiment did not investigate projections from the insula to the central amygdala (CeA), another limbic region associated with ARAI (Pandey et al., 2008; Roberto et al., 2004), a future bead tracing study could determine whether this connection is similarly segregated. This study could use heterotypic

injections in the NAc or LC versus the CeA as experimental conditions. Alternatively, researchers could investigate the dorsolateral striatum (DLS), as it has also been shown to take part in ARAI (See Figure 1, Hopf and Lesscher, 2014).

In conclusion, this research demonstrates that the neurons in the nucleus accumbens, locus coeruleus, and anterior insula that coordinate to mediate alcohol abuse behavior such as ARAI are organized in segregated clusters that can each be individually targeted for potential drug therapy. Homotypic injections resulted in complete colocalization of injected fluorescent beads, while heterotypic injections resulted in complete non-colocalization of the beads. Therefore, an injection in the NAc will not disrupt AIns-LC function, and vice versa. As the AIns-NAc neurons have been shown to regulate aversion-resistant alcohol intake, this connection is ripe for drug therapy studies and has great potential for novel therapeutic interventions for compulsive alcohol seeking in humans.

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