Published in final edited form as:

Addict Biol. 2018 September ; 23(5): 1020–1031. doi:10.1111/adb.12551.

Functional role for suppression of the insular-striatal circuit in modulating interoceptive effects of alcohol

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Abstract

The insular cortex (IC) is a region proposed to modulate, in part, interoceptive states and motivated behavior. Interestingly, IC dysfunction and deficits in interoceptive processing are often found among individuals with substance-use disorders. Furthermore, the IC projects to the nucleus accumbens core (AcbC), a region known to modulate the discriminative stimulus/ interoceptive effects of alcohol and other drug-related behaviors. Therefore, the goal of the present work was to investigate the possible role of the IC→AcbC circuit in modulating the interoceptive effects of alcohol. Thus, we utilized a chemogenetic technique (hM4Di-Designer Receptors Activation by Designer Drugs) to silence neuronal activity in the IC of rats trained to discriminate alcohol (1 g/kg, IG) vs. water using an operant or Pavlovian alcohol discrimination procedure. Chemogenetic silencing of the IC or IC→AcbC neuronal projections resulted in potentiated sensitivity to the interoceptive effects of alcohol in both the operant and Pavlovian tasks. Together, these data provide critical evidence for the nature of the complex IC circuitry and specifically suppression of the insular-striatal circuit in modulating behavior under a drug stimulus control.

Keywords

accumbens; drug discrimination; insula

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Author Contributions

AAJ and JB were responsible for the study concept and design. AAJ, VEA, VM, and SP contributed to the acquisition of the animal data. ZAM conducted the electrophysiological experiments. AAJ and JB conducted data analysis and interpretation of findings. AAJ drafted the article. AAJ and JB provided critical revision of the article for important intellectual content. All authors critically reviewed content and approved the final version for publication.

Introduction

In human brain imaging studies, drugs and drug-related cues elicit activity within the insular cortex (IC; (Droutman et al., 2015; Jasinska et al., 2014; Naqvi and Bechara, 2010) and damage to the IC in humans (Droutman et al., 2015) or high frequency stimulation (Dinur-Klein et al., 2014) disrupts nicotine addiction. In the preclinical literature, pharmacological manipulation of the IC disrupts ongoing drug self-administration (Droutman et al., 2015; Kutlu et al., 2013; Pushparaj and Le Foll, 2015), relapse-like behavior (Cosme et al., 2015; Wu et al., 2014), and conditioned place preference (Cosme et al., 2015; Droutman et al., 2015; Li et al., 2013; Wu et al., 2014 but see Wu et al., 2016). Relatedly, the IC plays a major role in interoceptive processing while IC dysfunction along with dysregulation in body awareness is commonly found in individuals with substance-use disorders (Droutman et al., 2015; Paulus and Stewart, 2014). Therefore, in relation to addiction, the IC not only integrates information about interoceptive/internal cues (including drug states), but also their relevance to external cues, thus implicating a key role for the IC in driving drug-taking and -seeking behavior (Paulus and Stewart, 2014).

The IC receives somatosensory and viscerosensory information from the thalamus and somatosensory cortices in the granular IC (Craig, 2009). This information spreads vertically and is integrated through a columnar organization relayed to the agranular/anterior IC, a region highly interconnected with limbic structures (Craig, 2009; Gu et al., 2013; Maffei et al., 2012). As such the IC is positioned to be a central hub for interoceptive processing within the central nervous system (Gu et al., 2013; Naqvi and Bechara, 2010). Accordingly, a proposed function of the IC is to project relevant information on interoception to influence decision-making processes and drive motivated behavior through efferent limbic projections, which is highly relevant to drug-related behaviors (Naqvi and Bechara, 2010; Paulus and Stewart, 2014).

The suggested role of the IC in regulating drug-related interoceptive effects is based on findings from the clinical literature (Droutman et al., 2015; Paulus and Stewart, 2014). Interestingly, in the preclinical field, only recently has a functional role for the IC been identified in modulating the discriminative stimulus/interoceptive effects of a drug of abuse (Jaramillo et al., 2016). That is, pharmacological inactivation ($GABA_A + GABA_B$ agonist cocktail) of the IC in rats results in partial substitution for the discriminative stimulus effects of alcohol (i.e., produces some “alcohol-like” interoceptive effects). To extend that work, we sought to examine whether silencing the IC would potentiate the effects of alcohol and to determine the involvement of projections from the IC to the nucleus accumbens core (IC→AcbC; (Jaramillo et al., 2016; Wright and Groenewegen, 1996), a limbic region also implicated in modulating interoceptive sensitivity to alcohol (Besheer et al., 2003; Besheer et al., 2009; Hodge and Cox, 1998; Hodge et al., 2001; Jaramillo et al., 2016). In preclinical work, IC→AcbC projections have been shown to functionally regulate compulsive alcohol drinking (Seif et al., 2013) and imaging studies demonstrate strong functional connectivity between the IC and AcbC (Cauda et al., 2011) particularly in response to reward processing (Cho et al., 2013; Clithero et al., 2011). Therefore, understanding the neurobiological circuitry modulating the interoceptive effects of drugs and the potential role of the IC is important for the addiction field. Accordingly, the goal of this study is to further understand

the role of the IC and specifically the insular-striatal circuit (i.e., IC→AcbC) in modulating the discriminative effects of alcohol.

Traditionally, drug discrimination methods have been used to investigate the discriminative stimulus/interoceptive effects of drugs (Solinas et al., 2006; Stoleran et al., 2011). Thus, the goal of the present work was to test the functional role of the IC and IC→AcbC circuit in modulating the interoceptive effects of alcohol in rats by implementing a chemogenetic strategy (i.e., hM4D Designer Receptors Exclusively Activated by Designer Drugs [DREADDs]). Moreover, we utilized two alcohol discrimination-training methods (i.e., operant and Pavlovian). Under both training conditions, behavior (i.e., lever selection in the operant method or goal-tracking in the Pavlovian method) is under the control of the alcohol (1 g/kg) drug state. Using two methods allows us to confirm functional involvement, and also to determine whether there is differential involvement of the IC→AcbC circuit related to the behavioral output (e.g., lever responding, goal-tracking). Based on our previous findings with the IC and the known functional importance of the AcbC in modulating sensitivity to alcohol (Besheer et al., 2009; Hodge and Cox, 1998; Jaramillo et al., 2016), we hypothesized that chemogenetic silencing of the IC and IC→AcbC projections would potentiate the interoceptive effects of alcohol, thus functionally demonstrating the role of the IC and insular-striatal circuit in modulating behavior under the stimulus control of alcohol.

Materials and Methods

Animals

Male Long-Evans rats (Harlan Sprague–Dawley, Indianapolis, IN) were individually housed in a vivarium maintained on a 12-h light/dark cycle (experiments conducted during the light cycle). Water was available ad libitum in the home cage and food intake was restricted to maintain body weight (325–340 g). Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Alcohol Discrimination Training and Testing Procedures

Rats were trained to discriminate alcohol (1 g/kg, IG) vs. water using operant (two-lever) or Pavlovian alcohol discrimination procedures as we describe in Jaramillo et al., 2016; Randall et al., 2016. Further details can be found in the Supplemental Materials.

Operant Alcohol Discrimination—During the alcohol and water training sessions (i.e., 10 min after alcohol or water IG, respectively), completion of a fixed ratio 10 (FR10) on the appropriate (i.e., correct) lever resulted in the delivery of sucrose reinforcer (i.e., 0.1 ml of 10% sucrose (w/v) solution; Fig. S2a). To confirm stimulus control, an initial cumulative alcohol dose-response curve (Table S1) was generated when the following accuracy criteria were met: the percentage of appropriate lever responses before the first reinforcer and during the entire session were >80% for at least 8 out of 10 consecutive days. For the remainder of the experiment, a rat was tested only if performance during 3 out of the previous 4 training sessions met the accuracy criteria. On the 2 min test sessions, FR10 on either lever resulted

in sucrose delivery and response accuracy (% alcohol-appropriate lever responses) was determined from the FR10 completed prior to reinforcer feedback (i.e., prior to sucrose delivery).

Pavlovian Alcohol Discrimination—During alcohol training sessions (10 min following alcohol, IG), each light presentation was followed by a sucrose presentation (i.e., 0.1 ml of 26% sucrose (w/v)). During water training sessions (10 min following water, IG), light presentations did not result in sucrose presentations (Fig. S2b). Head entries into the sucrose receptacle during the light presentation and the 15 sec preceding the light presentation were measured and a discrimination score was calculated. To confirm stimulus control, an initial cumulative alcohol dose-response curve (Table S1) was generated when the following accuracy criterion was met: the mean of the first discrimination score from the preceding two alcohol sessions had to be $\geq 150\%$ of the mean of the first discrimination score from the preceding two water sessions (Randall et al., 2016; adapted from Besheer et al., 2012; Palmatier et al., 2005). For the remainder of the experiment, a rat was tested only if performance during the previous 4 training sessions met the accuracy criteria. On the 2 min test sessions, there was a single 15-sec light presentation and thus the discrimination score reflects behavior prior to any sucrose delivery.

Viral Vectors

hM4D-DREADDs (AAV8-hSyn-DIO-hM4D(G_i)-mCherry; UNC Vector Core, NC; lot# 4980D & 4980H) or mCherry-Controls (AAV8-hSyn-DIO-mCherry; UNC Vector Core, NC; lot#4981CD), previously described by (Krashes et al., 2011; Roth, 2016), were combined with Cre recombinase (AAV8-CMV-Cre-GFP; Vector Biolabs, PA) in a ratio of 7:3 (v/v) and infused 2 μ l/side. This injection volume was selected based on a previous rat study that infused hM4D DREADDs into the IC (albeit at a volume of 3 μ l/side; (Mizoguchi et al., 2015) and on pilot studies in our lab. In pilot studies we found this volume was necessary to ensure effective DREADD expression and is likely related to our approach in which two AAV viruses (i.e., DREADD+Cre) need to be co-administered (Smith et al., 2016). Additionally, we utilized 33 gauge injectors, a 0.2 μ l/min flow rate, and left the injector in place for 10 min following the end of the 10 min infusion, as these are important strategies to further limit the spread of the viral injection (Smith et al., 2016).

Experimental Procedures

Experiment 1: Validation of DREADDs—Naïve rats (n=10) received bilateral microinjection of hM4D-DREADDs in the IC (AP +3.2, ML $\pm$ 4.0, DV -6.0 from skull; Fig. 1h–i). Seven weeks later, allowing time for expression of DREADDs, brain tissue was collected to confirm hM4D-mCherry expression in the IC (Fig. 1a–b and 1f–g) or to validate functional activity by *ex vivo* slice electrophysiological recordings in the IC (Fig. 1c–e), following bath application of clozapine-N-oxide (10 μ M CNO; Fig. 1k–l). Additionally, to confirm neuronal expression, tissue was analyzed for immunofluorescence co-localization of hM4D-DREADDs and NeuN or GFAP (i.e., neuronal and glial markers, respectively) in the IC (Fig. 1f–g).

Experiment 2: Examination of the functional role of the IC and IC→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure

A. IC silencing: Discrimination-trained rats received bilateral infusion of hM4D-DREADDs into the IC (n=11). Following 1 week of recovery, training continued until stable discrimination was established (>6 weeks). To determine a functional role of the IC in modulating the discriminative stimulus effects of alcohol, rats received CNO (0, 1 mg/kg, intraperitoneal [IP]) and 45 min later an alcohol dose (0.1, 0.3, 1.0 g/kg, IG), after which rats were placed in the chambers for a test session (Fig. 2a). Another group of discrimination-trained rats that did not receive surgery were used as CNO-injected behavioral controls (n=7).

B. IC→AcbC silencing: To determine the role of IC→AcbC projections, another group of discrimination-trained rats were infused with hM4D-DREADDs (n=6) or mCherry-Controls (n=6) in the IC and implanted with bilateral AcbC cannulae. Following acquisition of stable discrimination behavior (>6 weeks), rats received intra-AcbC infusion of CNO (0 or 9 μM/ side) 5 min prior to alcohol (0.3, 0.5, 1.0 g/kg, IG) and then underwent a test session (Fig. 3a).

Experiment 3. Examination of the functional role of the IC on the alcohol drug state utilizing a Pavlovian alcohol discrimination procedure

A. IC silencing: Rats trained in the Pavlovian alcohol discrimination procedure received bilateral infusion of hM4D-DREADDs (n=18) or mCherry-Controls (n=6) into the IC. Following recovery and continued training (>6 weeks), testing began. Rats received CNO (0, 1 mg/kg, IP) and 45 min later an alcohol dose (0, 0.1, 1.0 g/kg, IG) after which rats were placed in the chambers for a test session (Fig. 4a).

B. IC→AcbC silencing: Following systemic CNO testing, the same hM4D-DREADD and mCherry-Control rats were implanted with bilateral AcbC cannulae. Two rats were included in the hM4D group that had not undergone the systemic CNO testing. Rats received pretreatment of CNO (0 or 0.3 μM/side) 5 min prior to alcohol (0, 0.1, 1.0 g/kg, IG) and then underwent a test session (Fig. 5a).

Tissue Preparation, Immunohistochemistry Procedure, and Analysis

Tissue collection, immunohistochemistry (IHC) staining and verification procedures were similar to those described in (Jaramillo et al., 2016). All procedural details are described in the Supplementary Materials and Methods. Only rats with accurate injections and placements were included in the analyses and data presentation.

Drugs

Alcohol (95% w/v) was diluted in distilled water to a concentration of 20% (v/v) and administered IG, with volumes varied by weight to obtain the desired dose. For systemic administration CNO (1.0 mg/kg), injected at a volume of 1 ml/kg (NIDA Drug Supply Program, NC or Enzo Life Sciences, NY), was dissolved in 1% dimethyl sulfoxide in water (v/v) or 0.9% saline (w/v), respectively, or in aCSF for intracranial administration (0.3 or 9

μM). The CNO doses were chosen based on previous work (Krashes et al., 2011; Roth, 2016; Stachniak et al., 2014) and pilot studies from our lab.

Data Analysis

For Experiment 1, *t*-tests, were used to analyze the effects of CNO on modulation of synaptic transmission. For Experiment 2 (i.e., operant drug discrimination), a three-way analysis of variance (ANOVA) with CNO treatment and alcohol dose as within subject factors, and DREADD group as a between subject factor, was used to analyze response accuracy and response rate. Response accuracy was expressed as the percentage of alcohol-appropriate lever responses prior to delivery of the first reinforcer. If during the test session an animal did not complete an FR10, data from that animal were not included in the response accuracy analysis, but were included in the response rate analysis. Partial substitution for the alcohol training dose (1 g/kg) was defined as >40% and <80% alcohol-appropriate responses. Full substitution for the alcohol training dose (1 g/kg) of alcohol was defined as 80% alcohol-appropriate responding (Jaramillo et al., 2016; Solinas et al., 2006). Response rate (responses/min) was analyzed for the entire session and served as an index of motor activity. For Experiment 3 (i.e., Pavlovian discrimination), three-way ANOVA (CNO treatment and alcohol dose as within subject factors, and DREADD group as a between subject factor) was used to analyze discrimination score and locomotor rate was analyzed for the entire session and served as an index of motor activity. The discrimination score was calculated by subtracting the number of head entries that occurred in the 15 s before light onset (i.e., pre-CS) from the head entries that occurred during the 15-s light CS (Besheer et al., 2012; Palmatier et al., 2005; Randall et al., 2016). Full substitution for the alcohol training dose (1 g/kg) was determined when the discrimination score did not differ from the vehicle+alcohol training dose condition (Randall et al., 2016). Tukey *post hoc* analyses were used to explore significant main effects and interactions. Data represented as means ± S.E.M. and significance was declared at *p* 0.05. Figures were assembled using Photoshop (Adobe, CA).

Results

Experiment 1: Validation of DREADDs

Representative hM4D-mCherry expression is shown in IC (Fig. 1a–b, f–g) and in the AcbC (Fig. S1), 7 weeks following bilateral intra-IC viral vector infusion (hSyn-DIO-hM4D(Gi)-mCherry+Cre). To test the efficacy of the hM4D-DREADDs, using whole-cell current ramp recordings (Fig. 1c–e), resting membrane potential (RMP; representative trace in Fig. 1d–e) and rheobase were measured before and after 10 μM CNO bath application (n=8 cells from 6 rats). Following CNO, a significant hyperpolarization (pre-CNO: -62.7 ± 1.6 mV [Fig. 1d]; post-CNO -66.3 ± 2.1 mV [Fig. 1e]; delta: -3.5 ± 1.3 mV, $t(7)=2.84$, $p<0.03$, [Fig. 1c]), and a significant change in rheobase (pre-CNO: 119.4 ± 45.4 nA; post-CNO: 215.1 ± 72.0 nA; delta: 104.7 ± 36.0 nA; $t(7)=2.70$, p 0.03) were found. Thus, these findings together with previous studies (Stachniak et al., 2014) confirm the functional activity of the hM4D-DREADDs in the IC and the ability to functionally manipulate synaptic activity. Additionally hM4D-mCherry expression was co-localized to NeuN, a neuronal cell marker

(Fig. 1f), and not to the glia marker, GFAP (Fig. 1g). Thus, confirming DREADD expression within neurons and not glia due to the hSyn neuron-specific promotor (Kugler et al., 2003).

Experiment 2: Examination of the functional role of the IC and IC→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure

A. IC silencing—In the hM4D group, 3 rats died or had to be sacrificed due to complications following an injection, 1 was excluded due to behavioral attrition (i.e., failure to meet testing criteria), and 1 had an inefficient hM4D-DREADD infusion (i.e., no hM4D-mCherry expression likely due to a clogged injector). These rats are not included in any analyses or figures. Thus, the data presented are based on hM4D-DREADD (n=6) and CNO-Controls (n=7) groups. Representative hM4D-mCherry expression and individual bilateral hM4D-mCherry expression are represented in Figure 2b and 2c, respectively.

The three-way ANOVA of alcohol-appropriate responses showed a significant main effect of alcohol dose [$F(2,22)=116.95$, $p<0.000$; Fig. 2d], with an increase in alcohol-appropriate responding at the training dose (1 g/kg) relative to the lower alcohol doses, demonstrating appropriate discriminative stimulus control. There was also a significant main effect of CNO treatment [$F(1,11)=8.71$, $p=0.01$; Fig. 2d], with increased alcohol-appropriate responding following CNO ($p<0.05$). Indeed, in the hM4D group following CNO treatment, partial substitution (>40%) for the alcohol training dose was observed at the lowest alcohol dose (0.1 g/kg) tested. There was no main effect of DREADD group. Additionally, three-way ANOVA on response rate demonstrated no significant effects (Fig. 2e), indicating similar response rates between the two groups, and that alcohol and CNO treatment did not affect response rate.

Given that we observed partial substitution for the alcohol training dose (1 g/kg) at the lowest alcohol dose tested (0.1 g/kg) in the hM4D group, in a follow-up assessment, we tested CNO prior to water (IG) to determine whether we would observe partial substitution. A paired t-test on alcohol-appropriate responses following water (IG) revealed no significant effect ($p<0.3$) with vehicle treatment (7.2 ± 1.5) compared to CNO treatment (29.5 ± 46.3) in the hM4D group. Thus the water (IG) condition was not tested in the CNO-Control group or in the subsequent analysis.

B. IC→AcbC silencing—Two rats were sacrificed due to illness (1 in hM4D and 1 in mCherry-Control group), and 2 rats in the mCherry-Control group died due to complications following an IG injection. These rats are not included in any analyses or figures. Thus, the data presented are based on hM4D-DREADD (n=5) and mCherry-Control (n=3) groups. Representative hM4D-mCherry and mCherry-Control expression are represented in Figure 3b and 3c, respectively. Representative AcbC injector tip placements (Fig. 3d) for hM4D-mCherry (red) and mCherry-Control (blue) groups are depicted Figure 3e and 3f, respectively.

Three-way ANOVA of alcohol-appropriate responding showed a significant main effect of alcohol dose [$F(2,12)=24.32$, $p<0.001$; Fig. 3g] with an increase in alcohol-appropriate responding as the alcohol dose increased, demonstrating appropriate discriminative stimulus control. There was no main effect of CNO treatment or DREADD group. However, there

was a significant three-way interaction (CNO treatment X DREADD group X alcohol dose; $F(2,12)=6.23$, $p=0.01$). In the hM4D group, there was a significant increase in alcohol-appropriate responses following intra-AcbC CNO relative to vehicle at the 0.3 and 0.5 g/kg doses ($p<0.05$). In contrast, there were no CNO-induced differences in the mCherry-Control group at any of the alcohol doses. Indeed, in the hM4D group partial substitution ($>40\%$) for the alcohol training dose was observed at 0.3 and 0.5 (g/kg) following intra-AcbC CNO. Additionally, three-way ANOVA of response rate demonstrated no significant main effects or interactions (Fig. 3h).

Experiment 3. Examination of the functional role of the IC on the alcohol drug state utilizing a Pavlovian alcohol discrimination procedure

A. IC silencing—In the hM4D group, 2 rats died due to complications following an IG injection, and 8 had inefficient hM4D-DREADD expression (i.e., unilateral or no hM4D-mCherry expression). These rats are not included in any analyses or figures. Thus, the data presented are based on hM4D-DREADD ($n=6$) and mCherry-Controls ($n=6$) groups. Representative hM4D-mCherry expression and individual bilateral hM4D-mCherry expression are represented in Figure 4b and 4c, respectively.

The three-way ANOVA of discrimination scores showed a significant main effect of alcohol dose [$F(2,20)=19.98$, $p=0.001$; Fig. 4d], with elevated discrimination scores as the alcohol dose increased, demonstrating appropriate alcohol stimulus control. There was also a significant main effect of CNO treatment [$F(1,10)=7.59$, $p=0.02$; Fig. 4d], and a significant CNO treatment by DREADD group interaction [$F(1,10)=6.89$, $p=0.03$; Fig. 4d], with elevated discrimination scores following CNO treatment in the hM4D group. That is, in the hM4D group, IC silencing produced alcohol-like effects at 0 (water) relative to vehicle treatment ($p=0.05$). Indeed, following intra-AcbC CNO, full substitution for the alcohol training dose following water was observed, in the hM4D group. Additionally, three-way ANOVA of locomotor rate revealed a main effect of alcohol dose [$F(2,20)=6.78$, $p=0.006$; Fig. 4e], with a general increase in locomotor rate at the alcohol training dose relative to the lower alcohol doses (0 and 0.1 g/kg; $p<0.05$). As this is an index of overall motor activity in the chamber during the session, this increase in locomotor rate at the training dose may represent a general increase in anticipatory behavior. There was no main effect of CNO treatment or DREADD group on locomotor rate.

B. IC→AcbC silencing—After completion of systemic CNO testing the same rats were utilized for intra-AcbC CNO testing, with the addition of 2 rats that did not undergo systemic CNO testing. In the hM4D group, 2 rats were sacrificed due to illness and behavioral attrition (i.e., failure to meet testing criteria). In the mCherry-Control group 1 rat was sacrificed due to illness. These rats are not included in any analyses and are not shown in Figure 5. Thus, the data presented are based on hM4D-DREADD ($n=6$) and mCherry-Control ($n=5$) groups. Representative AcbC injector tip placements (Fig. 5b) for hM4D-mCherry (red) and mCherry-Control (blue) groups are depicted Figure 5c and 5d, respectively.

The three-way ANOVA of discrimination scores showed a significant main effect of alcohol dose [$F(2,18)=43.05$, $p = 0.001$; Fig. 5e] with elevated discrimination scores as the alcohol dose increased, demonstrating appropriate alcohol stimulus control. There was no main effect of CNO treatment or DREADD group. However, there was a significant alcohol dose by DREADD group interaction [$F(2,18)=6.92$, $p = 0.006$; Fig. 5e], with elevated discrimination scores with increasing alcohol dose in the hM4D group. That is, in the hM4D group, IC silencing potentiated sensitivity to 0.1 (g/kg) alcohol relative to vehicle treatment ($p = 0.05$). Indeed, in the hM4D group, following CNO, full substitution for the alcohol training dose at water and the lowest alcohol dose (0.1 g/kg) was observed. Additionally, three-way ANOVA of locomotor rate revealed a main effect of alcohol dose [$F(2,18)=3.93$, $p<0.04$; Fig. 5f], consistent with our previous assessment (Fig. 4e). There was no effect of CNO treatment or DREADD group.

Discussion

The present findings demonstrate that suppression of neuronal activity in the IC and specifically its projections to the AcbC are important for the expression of the interoceptive effects of alcohol. By chemogenetically silencing the IC, in rats trained on the two-lever operant alcohol discrimination task or the Pavlovian discrimination task, we show increased sensitivity to the interoceptive effects of alcohol. Chemogenetic silencing of IC→AcbC projections further extend these findings and support our hypothesis of a role for suppression of the insular-striatal circuit in modulating the discriminative stimulus effects of alcohol.

Insular-Striatal Interoceptive Circuitry

Drug discrimination procedures have been used to identify several receptor systems that modulate the discriminative stimulus effects of alcohol (GABA_A, NMDA, serotonin, opioid, mGluRs (Besheer et al., 2009; Grant, 1999; Kostowski and Bienkowski, 1999). Therefore, while the present study has identified that suppression of a specific circuit, in part, is sufficient for the expression of the interoceptive effects of alcohol, it will be important for future work to identify the receptor mechanism(s) within this circuit. Based on the extensive alcohol discrimination literature, pharmacological manipulations that inhibit the CNS (e.g., GABA agonists, NMDA antagonists) tend to produce “alcohol-like” effects (Grant, 1999; Kostowski and Bienkowski, 1999) and these effects likely involve inhibition in the AcbC (Besheer et al., 2009; Hodge and Cox, 1998; Jaramillo et al., 2016). Interestingly, the present data demonstrates a stronger behavioral effect following IC→AcbC silencing which demonstrates a behavioral-specific role for the IC→AcbC projections that may be overshadowed following general silencing of all IC outgoing projections. Furthermore, given the presumed glutamatergic projections from the IC→AcbC (Leong et al., 2016; Seif et al., 2013), we posit that chemogenetic silencing of these projections following activation of the G_{i/o} DREADDs likely results in inhibition of the AcbC. Consistent with this suggestion are previous findings showing that inhibition of the AcbC with GABA agonists produces “alcohol-like” effects (Besheer et al., 2003; Hodge and Cox, 1998; Hodge et al., 2001). Interestingly, Seif et al., (2013) demonstrated increased NMDAR function in the IC→AcbC circuit attributed to compulsive drinking, demonstrating the circuit as a site of alcohol action after extensive alcohol history. Therefore, it will be relevant for future discrimination studies

to investigate the role of IC→AcbC in modulating the interoceptive effects of higher alcohol doses (i.e., >1.0 g/kg), as it is possible that the circuit may be recruited to a greater (or lesser) degree at higher alcohol doses and following a history of exposure to higher alcohol doses. Together, these data suggest that IC projections to the AcbC may provide a tonic level of glutamatergic excitation and the expression of the interoceptive effects of alcohol is a consequence of decreased glutamatergic tone in the AcbC (i.e., insular input). However, future experiments would need to directly test this hypothesis along with other IC outgoing projections (e.g., IC projections to amygdala, mPFC). Additionally, although strategies were taken to localize chemogenetic manipulations to the IC, the potential for the functional contribution of neighboring regions needs to be considered.

Although increased sensitivity to alcohol following silencing of the IC and IC→AcbC projections was observed under both discrimination training conditions, full substitution for alcohol was only observed in the Pavlovian-trained group. That is, under the Pavlovian discrimination training conditions, silencing of the IC and IC→AcbC projections potentiated the effects of the low alcohol dose (0.1 g/kg) resulting in full substitution for the 1 g/kg training dose an effect that was also observed in the absence of alcohol (i.e., following water, IG). In contrast, under the operant discrimination training conditions, silencing the IC and IC→AcbC projections resulted in partial substitution for the alcohol training at the 0.1 g/kg dose, and 0.3 and 0.5 g/kg alcohol doses, respectively. Together, these data suggest that the Pavlovian discrimination procedure may be a more sensitive tool for detection of low drug doses than the operant procedures, which is consistent with previous suggestions (Besheer et al., 2012; Palmatier et al., 2005; Randall et al., 2016), and is likely related to the different response costs and distinct behavioral outputs (i.e., lever response, goal-tracking) of the procedures (see Besheer et al., 2012; Palmatier et al., 2005). Moreover, by definition the two discrimination-training procedures are inherently different and thus, alcohol and CNO doses were selected in accordance with each procedure. Thus, parallel examination of the discrimination behavior in both procedures allows for a point of comparison and identification of potential overlap in mechanism/neural circuitry. To this end, these data suggest overlap, but also, the possibility of the recruitment of different IC projections and/or neuromodulator systems by the different procedures.

Implications and Considerations

Previous work has demonstrated involvement of the IC in taste processing, albeit gustatory cortex (granular; Maffei et al., 2012), consummatory feeding, and the processing of anticipatory food cues (Kusumoto-Yoshida et al., 2015). Additionally, the IC has been implicated in altered decision-making in methamphetamine-treated rats (Mizoguchi et al., 2015), and reduced IC activity is observed during cognitive control tasks in drug-dependent populations (Paulus and Stewart, 2014). Consideration of these appetitive and cognitive processes is important for the interpretation of the outcome of the present studies. Silencing the IC or IC→AcbC projections did not alter response rates (operant discrimination groups) or locomotor rate (Pavlovian discrimination group), confirming that changes in discrimination performance were not due to nonspecific changes in motor output, or motivation to respond for the sucrose reinforcer. This latter point also suggests that there was likely no change in sucrose palatability. However, it is also important to note that for the

tests in both discrimination procedures, the primary dependent measure of alcohol sensitivity (alcohol-appropriate responses or discrimination score) is determined prior to sucrose delivery, which makes an explanation based on altered taste or consummatory behavior less likely. Next, consideration of the known role of the IC in processing external cues associated with food or drugs (Cosme et al., 2015; Li et al., 2013; Wu et al., 2014) but see (Kusumoto-Yoshida et al., 2015; Wu et al., 2016) is important. For example, optogenetic silencing of the IC decreases cue (tone and light)-triggered food-seeking (i.e., goal-tracking) behavior (Kusumoto-Yoshida et al., 2015). Therefore, the behavioral findings (i.e., after chemogenetic silencing of the IC and the IC→AcbC projections) of a specific increase in alcohol-appropriate responding and lack of change in response rate (in the operant task), and an increase in goal-tracking following the light cue with no change in locomotor rate (in the Pavlovian task), are likely not related to deficits in cue processing and are specific to the drug state. Next, it is possible that IC or IC→AcbC silencing impaired decision-making processes. Indeed, in the two-lever operant discrimination task, 50% responding on either lever may suggest such impairment. However, discrimination performance following chemogenetic silencing was either below or above this level. Additionally, in the Pavlovian procedure, discrimination scores were at levels comparable to the training condition, again making an argument of impaired decision making less tenable.

An important feature of the present work is the incorporation of the mCherry-Control groups. That is, the lack of effect of CNO on alcohol discrimination behavior in these control groups throughout the studies allows us to conclude that the CNO doses used (systemically and intra-AcbC) do not have “alcohol-like” effects and do not potentiate the effects of alcohol. These doses also did not affect general response rate or locomotor behavior. These are important findings as CNO, a major metabolite of the anti-psychotic drug clozapine, may have biological effects (perhaps due to retroconversion to clozapine or N-desmethylozapine) which are likely to depend on animal, strain, and CNO dose (Chang et al., 1998; Jann et al., 1994; MacLaren et al., 2016). Additionally, clozapine can serve as a discriminative stimulus in drug discrimination experiments (Goudie et al., 1998; Prus et al., 2016) and has been shown to decrease alcohol-stimulated activity (Thrasher et al., 1999), an effect absent in our CNO-manipulations (Fig. 4d and 5f). Thus, the inclusion of these CNO-only control groups and the absence of behavioral effects within the context of this study are highly relevant. However, given the long-term nature of these behavioral experiments, animals were lost due to behavioral attrition, complications with injections, or inaccurate viral injections/cannulae placements and this is most pertinent to the mCherry-Controls of Experiment 2B. Therefore, while the lack of CNO effect in this control group should be interpreted with caution given the small sample size, it is important to note that there was also no behavioral effect of CNO in any of other three control groups. Additionally, of relevance to all the experimental groups, is that it is important to consider that behavior is trained to be under alcohol stimulus control, and rats in all groups had to meet criteria prior to each test, which not only ensures stable behavior but also supports that changes in that behavior are related to the chemogenetic manipulations.

Overall Summary

Together these data identify a role for the IC→AcbC circuit in modulating behavior dependent on an alcohol drug state, which to date has been unstudied. Here, we demonstrate the ability to change behavior that is under the control of an alcohol-interoceptive state through chemogenetic silencing of IC→AcbC circuit. That is, silencing of this circuit potentiates and produces “alcohol-like” effects. This is highly relevant given that interoceptive drug cues can impact drug-taking, -seeking, and relapse-like behaviors (Paulus and Stewart, 2014; Stoleran, 1992; Verdejo-Garcia et al., 2012). Thus, it is possible that the decrease in alcohol self-administration shown by previous studies following IC manipulations (Pushparaj and Le Foll, 2015; Seif et al., 2013) may be related to the manipulations producing “alcohol-like” effects. Together with the previous data, these findings inform us of the complex IC structure while providing evidence of the critical nature of IC circuitry in underlying an alcohol drug state, which may have implications drug taking and seeking behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Thomas Kash for helpful discussions regarding the utilization of DREADDs, Dr. Michael Chua for assisting in the training and use of fluorescent microscopy, and the NIDA Drug Supply Program for providing the CNO. This work was supported, in part, by grant AA019682 to JB from the National Institute on Alcohol Abuse and Alcoholism and by the Bowles Center for Alcohol Studies at the University of North Carolina at Chapel Hill, and grant AA011605. AAJ was supported, in part, by an NSF Graduate Research Fellowship DGE-1144081 and then by F31AA024973 while working on data collection and the writing of this manuscript. VM was supported by T32 NS007431. The authors declare no conflict of interest.

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Figure 1. hM4D-DREADD validation for chemogenetic silencing of IC

(a) Representative m-Cherry immunofluorescence in IC (2X, 2 mm scale bar) and (b) IC neurons (10X, 100 μ m scale bar) following stereotaxic injection of AAV-hSyn-DIO-hM4D_i-mCherry+Cre into IC. (c) Decreased membrane potential in IC neurons, (d) demonstrated by representative traces of neuronal firing and (e) neuronal silencing following bath application of clozapine-n-oxide (CNO; 10 μ M). (f) Co-localization of mCherry with neuronal marker NeuN demonstrates dense co-localization (marked by white circles), and (g) no co-localization with the glial marker GFAP (40X, 100 μ m scale bar).

Figure 2. Chemogenetic silencing of IC increases sensitivity to the interoceptive effects of alcohol in an operant alcohol discrimination task

(a) Schematic diagram of test session. (b) Representative intra-IC hM4D-mCherry expression (2X, 1 mm scale bar) (c) with schematic demonstrating individual bilateral expression in discrimination-trained rats. (d) Silencing of IC, by CNO, increased the percentage of alcohol-appropriate responses, with partial substitution (>40%) for the alcohol training dose observed at the lowest alcohol dose (0.1 g/kg) in the hM4D group. (e) CNO did not affect response rate (responses/min). Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. *Significant main effect of CNO treatment (three-way ANOVA, $p < 0.05$). Values on graphs represent mean $\pm$ S.E.M.

Figure 3. Chemogenetic silencing of IC→AcbC projections increases sensitivity to low alcohol doses in an operant alcohol discrimination task

(a) Schematic diagram of test. (b) Representative intra-IC hM4D-mCherry expression and (c) intra-IC mCherry-Control expression (2X, 1 mm scale bar) with schematics demonstrating individual bilateral expression in discrimination-trained rats, respectively. (d) Representative bilateral AcbC injector tip placements from individual discrimination-trained rats in hM4D (depicted as red circles) or Control (depicted as blue circles) groups. (e–f) Corresponding photomicrographs (4X, 200 μm scale bar) showing an injector tip (arrow) for

each group, respectively. **(g)** Infusion of CNO into AcbC increased percentage of alcohol-appropriate responses following 0.3 and 0.5 g/kg alcohol in the hM4D group. **(h)** Response rate (responses/min) was unaffected by CNO or alcohol dose. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. *Significant difference from Vehicle in hM4D group ($p<0.05$). Values on graphs represent mean $\pm$ S.E.M.

Figure 4. Chemogenetic silencing of IC substitutes for the interoceptive effects of alcohol in a Pavlovian alcohol discrimination task

(a) Schematic diagram of test. (b) Representative intra-IC hM4D-mCherry expression and (c) intra-IC mCherry-Control expression (2X, 1 mm scale bar) with schematics demonstrating individual bilateral expression in discrimination-trained rats, respectively. (d) Silencing of IC, by CNO, following water increased the discrimination score (head entries into the liquid receptacle during the 15-s light CS minus head entries 15 s before light onset) in the hM4D group. (e) Locomotor rate (beam breaks/min) was increased with alcohol dose. *Significant difference from Vehicle in hM4D group ($p < 0.05$), #Significant main effect of alcohol (three-way ANOVA, $p < 0.05$). Values on graphs represent mean $\pm$ S.E.M.

Figure 5. Chemogenetic silencing of IC→AcbC projections substitutes for the interoceptive stimulus effects of alcohol in a Pavlovian alcohol discrimination task

(a) Schematic diagram of test. (b) Representative bilateral AcbC injector tip placements from individual discrimination-trained rats in hM4D (depicted as red circles) or Control (depicted as blue circles) groups. (c–d) Corresponding photomicrographs (4X, 200 μm scale bar) showing an injector tip (arrow) for each group, respectively. (e) Infusion of CNO into AcbC following 0.1 g/kg alcohol, increased the discrimination score (head entries into the liquid receptacle during the 15-s light CS minus head entries 15 s before light onset) in the hM4D group. (f) Locomotor rate (beam breaks/min) was increased with alcohol dose.

*Significant difference from Vehicle in hM4D group, #Significant main effect of alcohol (three-way ANOVA, $p < 0.05$). Values on graphs represent mean $\pm$ S.E.M.