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INACTIVATION OF THE LATERAL ORBITOFRONTAL CORTEX INCREASES DRINKING IN ETHANOL-DEPENDENT BUT NOT NON-DEPENDENT MICE

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Abstract

Long-term consumption of ethanol affects cortical areas that are important for learning and memory, cognition, and decision-making. Deficits in cortical function may contribute to alcohol-abuse disorders by impeding an individual's ability to control drinking. Previous studies from this laboratory show that acute ethanol reduces activity of lateral orbitofrontal cortex (LOFC) neurons while chronic exposure impairs LOFC-dependent reversal learning and induces changes in LOFC excitability. Despite these findings, the role of LOFC neurons in ethanol consumption is unknown. To address this issue, we examined ethanol drinking in adult C57Bl/6J mice that received an excitotoxic lesion or viral injection of the inhibitory DREADD (designer receptor exclusively activated by designer drug) into the LOFC. No differences in ethanol consumption were observed between sham and lesioned mice during access to increasing concentrations of ethanol (3–40%) every other day for 7 weeks. Adulterating the ethanol solution with saccharin (0.2%) or quinine (0.06 mM) enhanced or inhibited, respectively, consumption of the 40% ethanol solution similarly in both groups. Using a chronic intermittent ethanol (CIE) vapor exposure model that produces dependence, we found no difference in baseline drinking between sham and lesioned mice prior to vapor treatments. CIE enhanced drinking in both groups as compared to air-treated animals and CIE treated lesioned mice showed an additional increase in ethanol drinking as compared to CIE sham controls. This effect persisted during the first week when quinine was added to the ethanol solution but consumption decreased to control levels in CIE lesioned mice in the following 2 weeks. In viral injected mice, baseline drinking was not altered by expression of the inhibitory DREADD receptor and repeated cycles of CIE exposure enhanced drinking in DREADD and virus control groups. Consistent with the lesion study, treatment with clozapine-N-oxide (CNO) further enhanced consumption only in CIE exposed DREADD mice with no change in air-treated mice. These results suggest that the LOFC is not critical for the initiation and maintenance of ethanol drinking in non-dependent mice, but may regulate the escalated drinking observed during dependence.

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Keywords

Alcohol; frontal cortex; excitotoxic lesion; DREADD

11. INTRODUCTION

Alcohol is regularly consumed by a large (~70%) portion of the U.S. population and mortality due to alcohol-related causes is the third leading preventable cause of death in the United States (NSDUH, 2013). A large number of factors contribute to the risk for alcohol related problems including genetic factors, duration and intensity of drinking and co-occurrence of psychiatric disease and other illnesses. Elucidating how these and other factors contribute to an individual's risk for developing an alcohol use disorder is important for developing more effective treatments for alcohol abuse. Alcohol, like other addictive substances, activates midbrain reward-sensitive dopamine neurons that are designed to reinforce and guide responses to natural rewards such as food and social behavior (Hyman and Malenka, 2001). However, as drinking escalates, these reward pathways may become less important in driving alcohol intake as brain areas implicated in habitual and/or compulsive behaviors are engaged. These include striatal regions and those within prefrontal (PFC) and orbitofrontal (OFC) cortex that send and receive extensive projections to limbic and motor areas and that are critical for cognition, learning, and control over adverse and risky behaviors (Fuster, 2008). Although cortical regions including the OFC have long been suspected as being particularly impacted by alcohol in humans, until recently, few preclinical studies had examined the effects of acute and chronic ethanol exposure on this region.

In humans, the OFC is subdivided into several areas including Brodmann areas 10/11/13 and 47 (Fuster, 2008). In rodents, analogous regions have been described and include medial, ventrolateral and lateral subdivisions (Heidbreder and Groenewegen, 2003). The OFC receives inputs from all major sensory areas with especially robust inputs from olfactory and gustatory centers as well as those that signal emotional valence (e.g. amygdala). OFC neurons project widely to sub-cortical structures involved in reward evaluation and motor planning including ventral (nucleus accumbens, NAc) and dorsal striatum (DS) and ventral tegmental area (VTA) (Watabe-Uchida *et al*, 2012). Inactivating the OFC in mice disrupts goal-directed responding for food while leaving habit-based responses in the same animal intact (Gremel and Costa, 2013). Similarly, contralateral lesions of the lateral OFC and ventral dorsal lateral striatum prevented satiety-induced devaluation for food reward in mice (Gourley *et al*, 2013) and a similar effect was reported in monkeys following pharmacological inactivation of the lateral OFC (BA13) (West *et al*, 2011). These findings suggest that lateral OFC neurons are important in mediating reward-based behaviors through interactions with striatal and midbrain circuits. At the cellular level, results from ex vivo slice electrophysiology studies show that action potential spiking of LOFC neurons is

¹Abbreviations

LOFC-lateral orbitofrontal cortex; CIE-chronic intermittent ethanol; CNO-clozapine-N-oxide; DREADD-designer receptor exclusively activated by designed drug; GFP-green fluorescent protein; mPFC-medial prefrontal cortex; NAC-nucleus accumbens; NMDA-N-methyl-D-aspartate; VTA-ventral tegmental area;

inhibited by relevant concentrations of ethanol (U.S legal limit for intoxication is 0.08% or ~17 mM) via a glycine receptor dependent mechanism (Badanich *et al*, 2013b). Following repeated cycles of chronic intermittent ethanol (CIE) exposure that induces dependence (Lopez and Becker, 2005), LOFC neurons are hyperexcitable, show changes in markers of glutamatergic synaptic plasticity and are less sensitive to inhibition by acute ethanol (Nimitvilai *et al*, 2015). CIE exposed mice have also been shown to be impaired during the reversal phase of a naturalistic food foraging task shown to require the LOFC (Badanich *et al*, 2011; Bissonette *et al*, 2008).

While these findings clearly indicate that LOFC neurons are an important target for acute and chronic ethanol, little is known regarding whether LOFC neurons regulate voluntary ethanol consumption. In this study, we used two different mouse models of ethanol consumption and excitotoxic lesions and expression of inhibitory DREADD receptors to test if inactivation of the LOFC alters ethanol consumption. The results suggest that LOFC is not involved in initiating or maintaining baseline drinking in non-dependent mice but may serve to limit drinking during the development of dependence.

2. MATERIALS AND METHODS

2.1 Experimental Subjects

C57BL/6J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6–8 weeks of age and given at least one week to acclimate to the colony room before being used. Colony and testing rooms were maintained on a reverse light-dark cycle (lights off 9:00 AM). All procedures were carried out under protocols approved by the MUSC Institutional Animal Care and Use Committee and conform to NIH guidelines.

2.2 Surgeries

Neurotoxic lesions of the lateral OFC were generated using injections of N-methyl-D-aspartate (Sigma Aldrich, St Louis, MO) dissolved in 0.9% saline (Sigma Aldrich) at a concentration of 20 mg/mL (Bissonette *et al*, 2008). For sham lesions, an equivalent volume of 0.9% saline was injected. Coordinates for bilateral OFC lesions were: anterior-posterior, +2.6 mm from bregma; medial-lateral, ± 1.2 mm; dorsal-ventral, 2.2 from dura (Figure 1). At the injection site, 0.35 μ L of N-methyl-d-aspartate or saline was injected at a rate of 0.1 μ L/min, and the needle was left in place for 3 min. For viral expression studies, animals were injected at a rate of 0.1 μ L/min with 0.3 μ L of an AAV virus (UNC Viral Core) encoding the inhibitory (Syn-hM4Di-mCherry) DREADD receptor. Sham animals received an injection of a control AAV virus expressing green fluorescent protein (GFP). For both lesion and viral studies, mice were single housed after surgery and allowed 2 weeks to recover before beginning the drinking studies.

2.3 Histological Verification

At the end of the experiments mice that had received NMDA infusions were euthanized with a lethal dose of pentobarbital and perfused transcardially with 0.9 % saline solution followed by 4% formaldehyde. The brain was removed and fixed overnight in 4% formaldehyde followed by 30% sucrose-0.1M PBS for 48h. The brains were frozen and cut coronally in 30

µm slices using a Thermo Scientific Microm HM550 cryostat. To identify the lesion site, sections were processed for glial fibrillary acidic protein (GFAP; 1:500 dilution) and images of GFAP staining were collected using an Evos epifluorescent microscope (ThermoFisher, Rochester, NY). For viral injected animals, mice were euthanized by isoflurane anesthesia followed by rapid decapitation and brain slices (250 microns) were prepared using a Leica VT-1000S vibratome as previously described (den Hartog et al., 2013). Images of the fluorescent marker (e.g. mcherry, GFP) were collected using an Evos epifluorescent microscope and used to assess viral expression.

2.4 Ethanol Drinking

Mice were individually housed for at least 2 weeks prior to initiating drinking and food was provided *ad libitum* at all times. Where appropriate, the placement of the drinking bottles was alternated for each session to control for side preferences and mice were weighed weekly. Sham cages had drinking tubes but no mice to account for accidental spillage or loss of fluid. Two drinking paradigms were used to assess the effects of OFC lesions on ethanol consumption.

2.4.1 Intermittent Access Drinking—OFC- and sham-lesioned mice were given intermittent access to one bottle of ethanol and one bottle of tap water during 24-h sessions. Mice received two water bottles on intervening days. Mice were serially offered 3%, 6%, 10%, 15% ethanol (v/v in water) on successive sessions and then 20% ethanol for 13 sessions (26 d). Ethanol was then increased to 40% (v/v) for 8 sessions (16 d). To test how tastants affect drinking, mice were then offered 40% ethanol sweetened with 0.02% saccharin for 8 sessions (16 d) followed by 40% ethanol mixed with 0.06 mM quinine for 5 sessions (10 d).

2.4.2 Two Bottle Choice Drinking—In the lesion study, separate groups of mice were used to examine the pattern of voluntary ethanol consumption before and after repeated cycles of chronic intermittent ethanol exposure that results in dependence and escalations in drinking (Lopez *et al*, 2005). To initiate drinking, mice were serially offered 3%, 6%, 10% and then 15% ethanol (v/v in water; 24-h sessions) on successive days and then underwent 4 weeks of baseline voluntary drinking (15% ethanol v/v). Baseline drinking used a 5-day 24 h access paradigm (weekdays) separated by 48 hrs of abstinence (weekends). Mice then were exposed to weekly cycles of chronic intermittent ethanol (CIE)- or air-chamber exposure as described in the next section. Each weekly CIE/air exposure period was followed by free-choice drinking for 5 test days and this pattern of CIE/drinking was repeated for a total of 4 cycles. During each 24-h drinking session, NMDA- and saline-lesioned mice were given access to one bottle of ethanol and one bottle of tap water. In the DREADD studies, drinking was monitored over a 3-hr period and all mice received daily i.p. injections of either saline (baseline and Tests 1, 2 and 5) or clozapine-N-oxide (CNO, Tests 3 and 4) 30 min prior the drinking sessions.

2.5 Chronic Intermittent Ethanol (CIE) Exposure

Chronic ethanol (or air) vapor exposure was delivered for 16 hours a day for 4 days in Plexiglas inhalation chambers as previously described (Nimitvilai *et al*, 2015). Chamber

ethanol concentrations were monitored daily, and airflow was adjusted to maintain ethanol concentrations within a range that yielded stable blood ethanol levels (150–200 mg/dl) throughout exposure. Prior to entry into the ethanol chambers, ethanol mice were injected (i.p.) with a loading dose of ethanol (1.6 g/kg; 8% w/v) and the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) in a volume of 20 ml/kg body weight. Air exposed mice were handled similarly, but received injections of saline and pyrazole before being placed in control chambers. The housing conditions were identical to those in the colony room. Blood samples were collected from sentinel mice exposed to the same alcohol vapor as test mice and blood ethanol concentrations were measured as described previously (Pava *et al*, 2012).

2.6 Slice Electrophysiology

Brain slices containing the lateral orbitofrontal cortex (IOFC) were prepared as previously described (Badanich *et al*, 2013a). For recordings, slices were perfused with 34°C aCSF (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1.3 MgCl₂, 2.0 CaCl₂, 0.4 ascorbate, 10 glucose, 25 NaHCO₃, adjusted to 290–310 mOsm; saturated with 95% O₂/5% CO₂, pH=7.4) at a flow rate of 2 ml/min. Recordings were localized to deep layers of the IOFC using an Olympus BX51W1 microscope (Center Valley, PA) equipped with infrared Dodt gradient contrast imaging (Luigs and Neumann, Ratingen, Germany). Thin-wall borosilicate glass electrodes (OD = 1.5 mm, ID = 1.17 mm) were pulled on a Sutter Instrument P97 Micropipette Puller (Novato, CA) and had tip resistances ranging from 2–5 MΩ. Patch pipettes filled with an internal solution (in mM; 120 K-Gluconate, 10 KCl, 10 HEPES, 2 MgCl₂, 1 EGTA, 2 NaATP, 0.3 NaGTP, adjusted to 294 mOsm, pH=7.4) were slowly lowered onto the layer V pyramidal neurons to obtain a seal (> 1 gigaohm) followed by breakthrough to gain whole-cell access. Recordings were carried out using an Axon MultiClamp 700B amplifier (Molecular Devices, Union City, CA) and Instrutech ITC-18 analog-digital converter (HEKA Instruments, Bellmore, NY) controlled by AxographX software (Axograph, Sydney, Australia) running on a Macintosh G4 computer (Apple, Cupertino, CA). Events were filtered at 4 kHz and digitized at a sampling rate of 10 kHz. To validate that expression of the inhibitory DREADD reduced the excitability of OFC neurons, current-evoked spike firing was monitored before and during perfusion of the slice chamber with CNO (5 μM).

2.7 Data Analysis

Data were analyzed using a linear mixed model ANOVA with *post hoc* Bonferroni-corrected testing (SPSS software, version 23, IBM, Armonk, NY). Data were graphed using Prism software (version 6, Graphpad Software Inc., La Jolla, CA).

3. RESULTS

3.1 Intermittent Access Drinking

In the first study, the effect of an excitotoxic IOFC lesion on ethanol drinking was tested using an intermittent access paradigm in which animals were presented with escalating concentrations of ethanol along with water every other day. On off days, two bottles of water were available. As shown in Figure 1, consumption of ethanol by both groups of mice increased during each session as ethanol concentrations were ramped up from 3%–20%.

Consumption of 20% ethanol continued to increase until approximately day 19 and then was relatively stable. At day 35, the concentration of ethanol was increased to 40% and intermittent access was maintained until day 49. While there was a significant increase in drinking over the multiple sessions (mixed Anova, effect of concentration, $F_{5,231}=44.56$, $p<0.0001$), there was no effect of lesion on ethanol consumption (mixed Anova, effect of lesion $F_{1,79}=0.17$, $p<0.68$). As compared to the 20% solution, both sham ($F_{1,81}=12.29$, $p<0.001$) and lesioned ($F_{1,82}=15.96$, $p<0.0001$) mice consumed greater amounts of ethanol (g/kg; sham 3.1 ± 0.71 , $p<0.001$; lesion 2.36 ± 0.60 , $p<0.003$) when the 40% ethanol solution was available (Figure 1C). Water consumption was also monitored during each day of drinking and there were no significant differences observed between the two groups (data not shown). Ethanol preference (Figure 1C) was calculated by dividing the amount of ethanol consumed by the total amount of fluid (ethanol + water) taken each drinking day. Preference for ethanol increased as concentrations were ramped from 3–10% and then declined to approximately 50% for the 20% ethanol solution. As compared to the 20% EtOH solution, preference for 40% ethanol significantly declined in both groups (Preference; sham -13.25 ± 2.50 , $p<0.0001$; lesion -17.29 ± 2.14 , $p<0.0001$). However, as with consumption, no lesion-dependent effects on ethanol preference were noted across the range of ethanol concentrations tested. When averaged over the drinking sessions, preference for 20% ethanol was significantly higher than that of 40% for both groups (sham $F_{1,87.47}=82.75$, $p<0.0001$; lesion $F_{1,88.45}=39.13$, $p<0.0001$).

To gauge whether OFC lesions affects ethanol consumption or preference in the presence of sweet or bitter tastants, saccharin (0.2%) was added to the 40% ethanol solution for 8 sessions beginning at day 51 followed by ethanol (40%) plus quinine (0.06 mM) at day 67 (Figure 2A). Analysis of these data revealed a significant effect of tastants on consumption ($F_{2,146.58}=27.24$, $p<0.0001$) and preference ($F_{2,140.39}=29.43$, $p<0.0001$) for the 40% ethanol solution. Pairwise comparisons showed that saccharin enhanced consumption of 40% ethanol as compared to 40% alone to a similar extent in both groups (Figure 2C; g/kg; sham 3.61 ± 0.88 g/kg, $p<0.0001$; lesion 2.37 ± 0.74 g/kg, $p<0.0001$). The increase in drinking of the saccharin/ethanol mixture was mirrored by a similar increase in preference in both groups (Preference; sham 8.02 ± 2.26 , $p<0.002$; lesion 7.92 ± 1.92 , $p<0.0001$) with no effect of genotype. When quinine was added, both drinking and preference were reduced to levels similar to 40% EtOH in both groups with no effect of lesion. Overall, these results show that lesions of the IOFC have little effect on voluntary ethanol consumption in a model of non-dependent drinking.

3.2 CIE Drinking

In the second study, the effects of OFC lesions on ethanol drinking were examined using a vapor exposure paradigm model that produces dependence and escalations in drinking (Lopez *et al*, 2005). Following surgery and recovery, animals were given 24 h access to 15% ethanol for five days a week followed by a 48 h abstinence period. This was continued for a total of 4 weeks to establish a stable baseline of drinking prior to CIE treatment and there were no differences in drinking between sham and OFC-lesioned mice during any of the baseline periods (data not shown). Following the fourth weekly baseline drinking period, animals were exposed to daily episodes of either ethanol or air vapor for four consecutive

days with each episode consisting of 16 h of ethanol or air exposure followed by 8 h in the homecage. At the end of the fourth day of exposure, animals were returned to the homecage for 72 h and then began the next week of drinking. This pattern of interleaved episodes of CIE exposure and drinking was repeated for a total of 4 cycles. Blood ethanol concentrations of animals undergoing CIE treatment averaged 166.4 mg/dl (± 7.42) over the 4 cycles.

There were no significant effects of the lesion or CIE treatment on body weight over the course of the study (data not shown). However, as reported previously (Lopez *et al*, 2005), both sham and lesioned mice exposed to CIE treatment showed a similar increase in drinking as compared to air controls (Figure 3A; mixed Anova $F_{1,313}=137.5$, $p<0.0001$). Following the second and third CIE exposures, OFC-lesioned mice showed an additional increase in ethanol consumption as compared to non-lesioned CIE treated mice (Test 2, $F_{1,414}=6.11$, $p<0.014$; Test 3, $F_{1,419}=4.18$, $p<0.04$). This change was not significantly correlated with lesion size (r values; Lesion-Air=0.28, $p=0.46$; Lesion-CIE=-.12, $p=0.75$). After the 4th and final cycle of CIE exposure, 0.06 mM quinine was added to the ethanol solution and drinking was assessed for an additional three weeks (Test 4, 5, 6). During Test 4, the addition of quinine reduced drinking in all groups but CIE treated OFC lesioned mice still showed enhanced consumption as compared to sham-lesioned CIE treated mice ($F_{1,414}=5.77$, $p<0.017$). During Test 5 and 6, both groups of CIE treated mice continued to show greater drinking than air-exposed mice but there was no longer any effect of lesion on ethanol consumption. To assess whether OFC lesions affected the magnitude of the quinine-mediated reduction in drinking, the percent change in drinking from Test 3 to Test 4 was analyzed for each group (Figure 3D). Quinine produced a significant reduction in drinking in the sham-air ($t_8=7.81$, $p<0.0001$; paired t-test), lesion-air ($t_8=5.20$, $p<0.0008$; paired t-test) and sham-CIE ($t_8=3.06$, $p<0.02$; paired t-test) groups while the difference in the lesion-CIE mice showed a trend towards a significant reduction ($t_9=2.15$, $p<0.06$). Similar to its effect on ethanol consumption, CIE treatment had significant effects on water consumption with CIE exposed mice showing an overall decrease in water drinking as compared to air controls (Figure 3B; $F_{1,234}=12.25$, $p<0.001$). CIE treated groups also showed a significant increase in preference for ethanol (Figure 3C; $F_{1,210}=52.99$, $p<0.0001$) as compared to the air controls. However, analysis of the CIE treated groups showed no statistically significant effect of lesion on preference or water consumption despite a trend for increased water drinking especially during Test 4.

3.3 CIE and DREADDs

To test whether temporary inactivation of the lateral OFC would also enhance drinking in CIE exposed mice, separate groups of mice received injections of AAV viruses encoding the inhibitory DREADD receptor hM4Di or GFP into the lateral OFC (Figure 4B). After recovery from surgery and establishment of baseline drinking, mice underwent weekly cycles of CIE or air treatment followed by weekly episodes of drinking as described above. In these studies, mice had access to ethanol or water over a 3 h period rather than 24 h in order to better match the predicted half-life of CNO (Guettier *et al*, 2009). To validate that activation of the inhibitory DREADD reduces LOFC neuron excitability, slice electrophysiology was used to monitor current-evoked spiking in individual neurons before and during application of CNO (Figure 4A, C). Current-evoked spike firing in DREADD-

expressing OFC neurons was significantly reduced following application of CNO (5 μ M) to the slice. For the 200 pA current step, LOFC neurons generated 18.5% of the number of action potentials obtained before CNO treatment while during the 300 pA injection, the number of action potentials reached an average of 38% of the pre-drug control value (Figure 4C).

As shown previously, exposing mice to repeated cycles of CIE exposure enhanced ethanol consumption as compared to that in air controls (Figure 4D; mixed Anova $F_{1,179}=86.51$, $p<0.0001$). Ethanol consumption was further enhanced in DREADD-CIE mice following CNO (10 mg/kg; i.p.) administration ($F_{1,251}=6.46$, $p<0.012$; DREADD-CIE mice vs. AAV-CIE controls; $F_{1,335}=5.25$, $p<0.023$ DREADD-CIE mice vs. Test 3 DREADD-CIE mice) but this effect was only observed during Test 4. This effect was not significantly associated with degree of viral expression (r values; DREADD-Air=0.16, $p=0.79$; DREADD-CIE=-.38, $p=0.31$). CNO had no effect on ethanol consumption in DREADD or AAV injected mice in the air control groups whether given before Test 3 or 4. Drinking was monitored for an additional week (Test 5) with all animals receiving saline injections prior to each daily drinking session. Under these conditions, drinking in both CIE exposed groups declined (Figure 4D), although DREADD-CIE mice continued to show a significant increase in consumption as compared to AAV-CIE mice ($F_{1,251}=4.9$, $p<0.028$). Water consumption during the limited 3 h access period was essentially negligible (mls, mean $\pm$ SEM; AAV-Air 0.08 ± 0.01 ; AAV-CIE 0.11 ± 0.02 ; DREADD-Air 0.11 ± 0.02 ; DREADD-CIE 0.10 ± 0.03) and thus preference ratios were not determined.

4. DISCUSSION

The major findings of this study indicate that inhibition of the LOFC produces selective effects on voluntary ethanol consumption that manifest during the development of ethanol dependence. Thus, mice with an excitotoxic lesion or viral delivery of an inhibitory DREADD to the LOFC showed similar consumption of ethanol under baseline conditions but drank more following repeated cycles of ethanol vapor exposure that itself enhances drinking. These changes emerged only after multiple cycles of CIE and eventually dissipated following withdrawal from chronic ethanol exposure. These results suggest that the LOFC's role in regulating ethanol consumption is both dynamic and state-dependent and may be especially impacted in long-term alcohol dependent subjects.

Although there is a large literature with respect to manipulations that affect voluntary alcohol consumption in rodents, relatively few of these have examined how disruption of cortical areas affects drinking and none appear to have specifically targeted the lateral OFC. In an early study, sham and lesioned animals had access to 10% ethanol as the only liquid for a nine week period (Hollway *et al*, 1979). No differences in intake were observed between controls and those in which an area of cortex above the dorsal hippocampus of adult rats was removed by aspiration while rats with a dorsal hippocampal lesion showed increased consumption. Deckel *et al*. (Deckel *et al*, 1996) used aspiration lesions of the rat dorsal prefrontal cortex and examined consumption of alcohol with a sucrose fading technique. They reported no effect of lesion on intake of a 20% sucrose-only solution but lesioned rats drank more than controls when offered 5% ethanol containing 15% or 20%

sucrose. When sucrose was reduced to 3% (with 5% ethanol), lesioned rats drank less than controls while no differences were noted at a higher ethanol concentration (10% ethanol plus 5% sucrose). It is not clear whether in the Deckel et al. study, the aspiration lesions spared the orbitofrontal cortex but the authors state that they took care to avoid more lateral regions of prefrontal cortex that encompass gustatory cortex suggesting that this was the case. No difference in consumption of 6% ethanol was reported for female rats with ibotenic acid lesions of the medial prefrontal cortex (Hansen *et al.*, 1995) although water intake was significantly enhanced in lesioned animals by the third post-operative week. In the Hansen study, the lesion was centered on the medial PFC (including prelimbic and infralimbic areas) and also encompassed ventromedial aspects of the orbitofrontal cortex but did not extend into more lateral areas. The latter two studies used limited access drinking protocols that do not typically induce dependence but model patterns of consumption associated with low to moderate intake in humans. In a recent study using an elegant genetic approach, neuronal ensembles in the mPFC previously activated during cue-induced responding for ethanol were ablated prior to reinstatement testing (Pfarr *et al.*, 2015). Animals with neuronal cell death restricted to the infralimbic portion of the mPFC showed increased operant responding for ethanol while no change was noted in prelimbic targeted animals.

In the present study, NMDA-mediated lesions of LOFC had no effect on voluntary baseline drinking with either an intermittent or every day drinking paradigm. Thus, mice tested in the intermittent access (IA) paradigm consumed ~8–10 g/kg of ethanol per 24 h consumption while those in the every day model consumed slightly less (~6–7 g/kg per 24 h). In both protocols, there was no effect of LOFC lesion on alcohol drinking despite the wide range of ethanol concentrations tested (3–40%) and lesioned and sham animals showed similar changes in consumption in the presence of tastants that enhanced or reduced ethanol intake. These findings suggest that LOFC is not involved in the initial acquisition or maintenance of ethanol consumption with protocols that yield low to moderate levels of consumption.

Lesioned animals in the current study that were exposed to repeated cycles of ethanol vapor exposure that reliably produces dependence (Griffin *et al.*, 2009) showed significant, albeit modest (~20%) increases in consumption as compared to sham controls. Interestingly, this effect emerged only after the second cycle of CIE exposure and was recapitulated in DREADD expressing animals following the second CNO administration period. These findings suggest that a form of learning may occur during each CIE/drinking cycle that influences consumption in subsequent sessions. Lesions or DREADD-induced inactivation of the LOFC presumably disrupt this learning leading to an inability to restrict ethanol drinking. Although speculative, this could reflect a role of the LOFC and related areas (e.g. dorsal striatum, nucleus accumbens, amygdala) in establishing a balance between goal-directed and habitual responding for alcohol that guides future consumption. Loss or impairment of this learning signal may then result in an overshoot during the next drinking cycle leading to higher rates of consumption. Studies in the literature using food reinforcement show that lesions or inactivation of the lateral OFC disrupt the ability of the animal to adjust responding following devaluation of the reward indicating a critical role of the OFC in goal-directed behaviors (Gremel *et al.*, 2013). This appears to involve OFC projections to striatal areas as mice with unilateral asymmetric lesions of LOFC and ventrolateral striatum are insensitive to treatments that degrade the relationship between

responding and delivery of the reward (Gourley *et al*, 2013). Following repeated cycles of CIE exposure and withdrawal, striatal-based habit-like learning is enhanced (DePoy *et al*, 2013) as is aversion-resistant drinking (e.g. footshock, quinine) suggesting that alterations in corticostriatal circuits such as those including the LOFC may develop during alcohol dependence and contribute to escalations in ethanol intake and loss of control over drinking (Hopf and Lesscher, 2014). The reduced quinine sensitivity of OFC-lesioned CIE exposed mice observed in the present study may reflect these types of alterations.

In contrast to LOFC, output from the insular cortex and mPFC may drive habitual/compulsive drinking as inhibiting these inputs in the nucleus accumbens reduces intake in quinine resistant rats (Seif *et al*, 2013). These findings suggest that medial and lateral areas of PFC/OFC play different roles in regulating drinking and undergo different adaptations in response to ethanol. Data from emerging studies of mPFC/OFC electrophysiology and function support this idea with region-dependent differences observed for ethanol sensitivity of NMDA EPSCs (Badanich *et al*, 2013a; Tu *et al*, 2007; Weitlauf and Woodward, 2008); action potential firing (Badanich *et al*, 2013a; Tu *et al*, 2007), CIE-induced changes in AMPA/NMDA ratio and GluN2B subunit expression (Kroener *et al*, 2012b; Nimitvilai *et al*, 2015), alterations in spine dynamics (Holmes *et al*, 2012; Kim *et al*, 2014; Kroener *et al*, 2012a; McGuier *et al*, 2015) and alcohol seeking behavior (Pfarr *et al*, 2015). Thus, imbalances in the function or output of mPFC/OFC may be a critical factor underlying the gradual loss of control over drinking in alcohol-addicted individuals.

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Past Month among Persons Aged 18 or Older, by Demographic Characteristics: Percentages, 2012 and 2013. 2013

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Figure 1.

Effects of an excitotoxic lesion of the LOFC on ethanol consumption by mice on the intermittent access paradigm. (A) Schematic showing anatomical location of LOFC (left panel, shaded area, figure from Allen Brain Atlas) and examples of GFAP immunoreactivity from saline and NMDA injected animals. Positive GFAP staining is white. Right panel are atlas figures with shading indicating average extent of lesion (numbers are distance (mm) from bregma). (B) No effect of lesion on ethanol consumption (mean $\pm$ SEM, g/kg) during intermittent access to ethanol containing solutions. Animals were given 24 h access to increasing concentrations of ethanol every other day with water available every day. (C) No

effect of lesion on ethanol preference for different concentrations of ethanol. (D) Sham and lesioned mice consumed more ethanol during access to the 40% ethanol solution as compared to the 20% ethanol solution (* $p < 0.05$; **** $p < 0.0001$). (E) Preference for the 40% ethanol solution was reduced as compared to that of the 20% solution (**** $p < 0.0001$). Number of animals per group: Sham (8); Lesion (11).

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Figure 2.

Effect of an excitotoxic lesion to the LOFC on the ability of sweet and bitter tastants to alter ethanol drinking. (A) No effect of lesion on consumption of 40% ethanol adulterated with either saccharin (0.2%) or quinine (0.06 mM). (B) No effect of lesion on preference for 40% ethanol adulterated with either saccharin (0.2%) or quinine (0.06 mM). (C) Sham and lesioned mice consumed greater amounts of 40% ethanol in the presence of saccharine as compared to ethanol alone or ethanol plus quinine (oneway Anova, **, $p < 0.01$; ***, $p < 0.001$). (D) Sham and lesioned mice showed greater preference for 40% ethanol in the presence of saccharine as compared to ethanol alone or ethanol plus quinine (oneway Anova, **, $p < 0.01$; ***, $p < 0.001$). Number of animals per group: Sham (8); Lesion (11).

Figure 3.

Excitotoxic lesion to the LOFC enhances ethanol consumption in dependent but not non-dependent mice. Groups of mice were exposed to repeated cycles of chronic intermittent exposure (CIE) to ethanol vapor or air followed by weekly drinking sessions. (A) CIE exposure enhances ethanol drinking as compared to air-exposed mice (###; main effect of CIE on ethanol consumption, mixed Anova, $p < 0.0001$). Lesioned mice exposed to two or more cycles of CIE exposure (Tests 2–4) consume more ethanol than non-lesioned CIE treated mice ($*p < 0.05$; $**p < 0.01$). Data represent mean ($\pm$ SEM) daily ethanol consumption (g/kg) for each group during baseline, after each CIE exposure (Test 1–4) and during post-

CIE periods when quinine was added to the ethanol solution. (B) No effect of LOFC lesion on water consumption during baseline drinking or following CIE exposures. Symbol (###, main effect of CIE on water consumption, mixed Anova, $p < 0.01$). (C) No effect of LOFC lesion on ethanol preference during baseline drinking or following CIE exposures. Symbol (###, main effect of CIE on ethanol preference, mixed Anova, $p < 0.0001$). (D) Lesioned CIE treated mice show reduced sensitivity to quinine adulteration of ethanol solution. Data represent mean ($\pm$ SEM) percent inhibition of drinking by quinine during Test 4 relative to Test 3. Ethanol consumption data from panel A. Symbols (**, $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$; paired t-test). (E) Atlas figures with shading indicate average extent of lesioned area (numbers indicate distance (mm) from bregma). Number of animals per group: Sham Air (9), Lesion Air (9), Sham CIE (9), Lesion CIE (10).

Figure 4.

Effect of expression of the inhibitory hM4Di DREADD in LOFC on spike firing and ethanol consumption. (A) Representative traces showing action potentials from an LOFC neuron during sequential current injections before (baseline) and during application of CNO (5 μ M). (B) Anatomical location of injection site for AAV virus in LOFC (shaded area in left panel, figure from Allen Brain Atlas) and fluorescent/bright field images (right panel) of hM4Di-mCherry expression two weeks after viral injection. Atlas figures with shading indicate average extent of lesioned area (numbers indicate distance (mm) from bregma). (C) Effect of CNO on action potential number. Columns show number of spikes during injection of current during the last 3 minutes of CNO exposure expressed as a percent of the baseline (mean $\pm$ SEM). Symbol (*): value significantly different from control (N=7, $p < 0.001$, one-sample t-test). (D) Effect of the inhibitory hM4Di DREADD on ethanol consumption in CIE exposed mice. Following establishment of baseline drinking (15% ethanol v/v; 3 hr limited access), groups of virus injected mice (AAV; DREADD) were exposed to repeated cycles of

air or ethanol vapor (CIE) interleaved with weekly assessments of drinking (Test 1–5). During Tests 3 and 4, all mice received an injection of CNO (10 mg/kg) 30 min prior to the introduction of the ethanol bottle. Symbols (###: main effect of CIE treatment on ethanol consumption, Mixed Anova, $p < 0.0001$; *: Test 4; DREADD-CIE consumption significantly greater than AAV-CIE group, Mixed Anova, $p < 0.01$), Test 5; DREADD-CIE consumption significantly greater than AAV-CIE group, Mixed Anova, $p < 0.05$). Number of animals per group: AAV Air (8), DREADD Air (6), AAV CIE (9), DREADD CIE (10).