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Effects of monoamines on the intrinsic excitability of lateral orbitofrontal cortex neurons in alcohol-dependent and non-dependent female mice

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Abstract

Changes in brain reward and control systems of frontal cortical areas including the orbitofrontal cortex (OFC) are associated with alcohol use disorders (AUD). The OFC is extensively innervated by monoamines, and drugs that target monoamine receptors have been used to treat a number of neuropsychiatric diseases, including AUDs. Recent findings from this laboratory demonstrate that D2, α 2-adrenergic and 5HT_{1A} receptors all decrease the intrinsic excitability of lateral OFC (lOFC) neurons in naïve male mice and that this effect is lost in mice exposed to repeated cycles of chronic intermittent ethanol (CIE) vapor. As biological sex differences may influence an individual's response to alcohol and contribute to the propensity to engage in addictive behaviors, we examined whether monoamines have similar effects on lOFC neurons in control and CIE exposed female mice. Dopamine, norepinephrine and serotonin all decreased spiking of lOFC neurons in naïve females via activation of G_{iα}-coupled D2, α 2-adrenergic and 5HT_{1A} receptors, respectively. Firing was also inhibited by the direct GIRK channel activator ML297, while blocking these channels with barium eliminated the inhibitory actions of monoamines. Following CIE treatment, evoked spiking of lOFC neurons from female mice was significantly enhanced and monoamines and ML297 no longer inhibited firing. Unlike in male mice, the enhanced firing of neurons from CIE exposed female mice was not associated with changes in the after-hyperpolarization and the small-conductance potassium channel blocker apamin had no effect on current-evoked tail currents from either control or CIE exposed female mice. These results suggest that while CIE exposure alters monoamine regulation of OFC neuron firing similarly in males and female mice, there are sex-dependent differences in processes that regulate the intrinsic excitability of these neurons.

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Disclosure

The authors declare no conflict of interest.

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Keywords

Chronic intermittent ethanol exposure; lateral orbitofrontal cortex; female mice; monoamine; G protein-coupled inwardly rectifying potassium channel; intrinsic excitability 1

1. Introduction

The orbitofrontal cortex (OFC) has been suggested to play an important role in integrating sensory and reward-related information in support of several aspects of learning such as choice behavior (Roesch and Olson, 2007), reversal learning (Bissonette *et al*, 2008; McAlonan and Brown, 2003) and the development of anticipation in response to either appetitive or aversive stimuli (Tremblay and Schultz, 1999). In addition, many studies have defined the OFC as critical for predicting outcomes that require one to incorporate new information not previously associated with the original learned association (Rudebeck and Murray, 2014; Stalnaker *et al*, 2015). Dysfunction of the OFC is associated with numerous neuropsychiatric diseases, including alcohol and drug abuse disorders (Fortier *et al*, 2008; Verdejo-Garcia *et al*, 2006). We reported previously that alcohol-dependent mice have impaired performance on reversal learning (Badanich *et al*, 2011), a behavior that requires an intact OFC (Bissonette *et al*, 2008). Deficits in reversal learning have also been observed in ethanol-exposed rats (Brown *et al*, 2007), primates (Jedema *et al*, 2011) and humans (Fortier *et al*, 2008; Verdejo-Garcia *et al*, 2006).

The OFC is extensively innervated by monoamines including dopamine (DA), norepinephrine (NE) and serotonin (5HT) (Agster *et al*, 2013; Homayoun and Moghaddam, 2008; Robbins and Arnsten, 2009). Alterations in the levels or function of monoamines and their receptors have been shown to underlie deficits in certain OFC-dependent behaviors (Clarke *et al*, 2005; Kheramin *et al*, 2004; Winstanley *et al*, 2006). A previous study in our laboratory demonstrated that, in brain slices obtained from naïve male mice, acute exposure to dopamine, norepinephrine and serotonin all decreased evoked firing of IOFC neurons (Nimitvilai *et al*, 2017). This occurred through stimulation of G protein-coupled inwardly rectifying potassium (GIRK; Kir3 family) channels coupled to D2, α 2-adrenergic and 5HT_{1A} receptors, respectively. Monoamine modulation of firing was lost in male mice exposed to repeated cycles of alcohol exposure that has been shown to produce dependence and escalations in voluntary ethanol consumption (den Hartog *et al*, 2016; Lopez and Becker, 2005). The results in male mice underscore the importance of monoamines as modulators of IOFC excitability and suggest that disruption of this process could contribute to various deficits associated with alcohol dependence. An important question that remains is whether these effects are selective for males as there are no reports on OFC neuron firing from control or alcohol-exposed female mice.

Although electrophysiological studies of neurons from female animals are few, a wealth of literature indicates that biological sex differences between males and females influences their behavioral responses to alcohol and contributes to the propensity to engage in addictive behaviors (Becker *et al*, 2017; Bobzean *et al*, 2014; Devaud *et al*, 2003). For example, females escalate alcohol use more rapidly (Becker *et al*, 2017) and develop addiction-like

behaviors faster than males (Anglin *et al*, 1987; Bobzean *et al*, 2014; Brady and Randall, 1999; Lancaster and Spiegel, 1992; Middaugh *et al*, 1999; Torres *et al*, 2014). In contrast, males exhibit greater withdrawal symptoms and have longer periods of abstinence than females (Devaud *et al*, 2003). In this study, we used whole-cell patch-clamp slice electrophysiology to examine the effects of monoamines on the intrinsic excitability of IOFC neurons in naïve female mice and those treated with repeated cycles of CIE exposure. The results demonstrate that dopamine, norepinephrine and serotonin each reduces the intrinsic excitability of IOFC neurons via activation of GIRK channels, and that this regulation is blunted in female mice following chronic exposure to alcohol.

2. Materials and Methods

2.1 Animals

Female C57/BL6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) (<https://www.jax.org/strain/013636>) at 9 weeks of age. They were group-housed (4/cage) and allowed to acclimatize to the colony room for at least one week in a temperature and humidity controlled AAALAC-approved facility. Animals were maintained on a 12-hour light/dark cycle with lights off at 09:00 am and had ad libitum access to food and water. Female mice were used without regard to the stage of the estrous cycle. All animals were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and all experimental methods were approved by the Medical University of South Carolina's Institutional Animal Care and Use committee.

2.2 Chronic intermittent ethanol exposure

After acclimatization, some mice were treated with repeated cycles of chronic intermittent ethanol (CIE) vapor exposure, a protocol that induces dependence in male (Lopez *et al*, 2005) and female mice (Jury *et al*, 2017), produces an impairment of OFC-dependent reversal learning (Badanich *et al*, 2011) and increases ethanol consumption (den Hartog *et al*, 2016). Briefly, mice were exposed to four cycles of either ethanol (CIE group) or air (control) in vapor inhalation chambers. Each cycle consisted of daily exposure to ethanol vapor for 16 hr followed by 8 hr of abstinence in the home cage. This was repeated each day for 4 consecutive days followed by 3 days of abstinence before beginning the next cycle of ethanol exposure. Ethanol (95%) was volatilized by passing air through a submerged air stone and the resulting vapor was mixed with fresh air and delivered to Plexiglas inhalation chambers (5 L/min) to maintain consistent ethanol concentrations between 17–21 mg/L air in the chamber. This yielded blood ethanol concentrations (BEC) in the range of 150–250 mg/dl. Prior to entry into the ethanol chambers, CIE mice were injected intraperitoneally (20 ml/kg body weight) with ethanol (1.6 g/kg; 8% w/v) and the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) to maintain stable blood ethanol levels. Air control mice were similarly handled but were injected with saline and pyrazole before being placed in air inhalation chambers. Chamber ethanol concentrations were monitored daily and air flow was adjusted to maintain concentrations within the specified range. In addition, blood samples were collected and determined from all animals to monitor BECs during the course of inhalation exposure. Average BECs during the 4 weeks of CIE exposure was 208.79 ± 15.88

mg/dl. Mice were sacrificed 3 and 7 days following the final vapor exposure (ethanol or air) and used for electrophysiological studies.

2.3 Preparation of brain slices

Brain slices containing the lateral orbitofrontal cortex (IOFC) were prepared for whole-cell patch-clamp electrophysiology experiments as previously described (Badanich *et al.*, 2013). Following brief anesthesia with isoflurane and rapid removal of the brain, the tissue was blocked coronally for the frontal cortex and the cerebellum and a portion of the dorsal mesencephalon were removed. The tissue block was mounted in a Leica VT1000S vibratome (Buffalo Grove, IL) containing ice-cold oxygenated (95% O₂, 5% CO₂) sucrose containing buffer and coronal sections (300 µm) were cut. Slices containing the IOFC were immediately placed in a holding chamber containing oxygenated artificial cerebral spinal fluid (aCSF) at 34°C for 30 min, and then kept at room temperature for at least 30 min before recordings. The composition of the cutting solution used was (in mM): 200 sucrose, 1.9 KCl, 1.2 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, 0.4 ascorbate, 10 glucose, 25 NaHCO₃, adjusted to 305–315 mOsm. The composition of the aCSF was (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. Both solutions were saturated with 95% O₂/5% CO₂ (pH=7.4).

2.4 Whole-cell patch-clamp electrophysiology

An individual slice was placed in the recording chamber and perfused with 34°C aCSF maintained 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 2.2–6.2 MΩ. Patch pipettes filled with an internal solution were slowly lowered onto the layer V pyramidal neurons to obtain a high-resistant seal (>1 GΩ) followed by breakthrough to gain whole-cell access.

To determine the effect of each monoamine on the intrinsic excitability of OFC neurons, current-clamp recordings were performed. Spike firing was induced by a series of current injections (0–220 pA in 20 pA increments, 750 msec duration for each step) through patch pipettes filled with a potassium gluconate internal solution (in mM; 120 KGluconate, 10 KCl, 10 HEPES, 2 MgCl₂, 1 EGTA, 2 NaATP, 0.3 NaGTP, adjusted to 294 mOsm, pH=7.4). Recordings were analyzed for the number of spikes in response to each current step in the absence and presence of monoamines and/or other drugs as described in the Results section. Resting membrane potential (RMP, mV) was obtained from the membrane potential just before initiation of current steps. After-hyperpolarization (AHP, mV) magnitude was calculated by subtracting the lowest potential achieved during the rebound hyperpolarization from the action potential threshold and reported values are the mean of the first three AHP magnitudes recorded.

To directly measure outward tail currents, IOFC neurons were voltage-clamped at –70 mV and depolarizing current steps (400 ms, from –20 to +30 mV with 10 mV between steps)

were applied before and during exposure to the SK channel inhibitor apamin (100 nM). SK currents were defined as the difference in peak magnitudes of tail currents obtained in the absence and the presence of apamin.

All whole-cell recordings were carried out using an Axon MultiClamp 700B amplifier (Molecular Devices, Union City, CA) interfaced with an Instrutech ITC-18 analog-digital converter (HEKA Instruments, Bellmore, NY). Data were acquired with AxographX software (Axograph, Sydney, Australia) running on a Macintosh G4 computer (Apple, Cupertino, CA). Events were filtered at 4kHz and digitized at a sampling rate of 10 kHz.

We note that there were no major methodological differences between this study in females and our previous reports in male mice (Nimitvilai *et al*, 2016, 2017).

2.5 Drugs

Dopamine hydrochloride was purchased from Alfa Aesar (Ward Hill, MA). Serotonin hydrochloride, α -methyl-5HT, quinpirole hydrochloride, SCH23390, WAY100635, yohimbine, naftopidil hydrochloride, nomifensine and apamin were purchased from Tocris Biosciences (Minneapolis, MN). Sulpiride and ML297 were purchased from Abcam (Cambridge, MA). Norepinephrine, barium and all reagents used to prepare aCSF, sucrose-containing and internal pipette solution were purchased from Sigma (St. Louis, MO).

2.6 Statistical Analysis

Experimental data are expressed as mean $\pm$ SEM and were analyzed with Prism software (GraphPad Software, San Diego, CA) using standard methods (one-way and two-way repeated measures ANOVA, paired and unpaired two-tailed *t*-test). Comparisons were considered significantly different when $p < 0.05$.

3. Results

To study the effects of monoamines on the intrinsic excitability of IOFC neurons from female mice, a series of current injections from 0 to 220 pA were used to evoke firing. In the absence of any modulator, the number of spikes at the 220 pA current step ranged from 5 to 21, with a mean basal firing rate of 10.60 ± 0.53 (N=42). Other electrophysiological characteristics of IOFC neurons obtained from naïve female mice are summarized in Table 1. There was no obvious difference in spike number of IOFC neurons of naïve female mice when compared to that previously reported for male mice (11.11 ± 0.54 , N=66) (Nimitvilai *et al*, 2017). Likewise, no differences in any electrophysiological characteristic of IOFC neurons between naïve female and male mice (refer to Table 1 in Nimitvilai *et al*, 2016).

3.1 Monoamines decrease the intrinsic excitability of lateral OFC neurons in naïve female mice through $G_{i/o}$ -coupled receptors

3.1.1 Dopamine—Under current-clamp mode, action potential (AP) spiking was significantly reduced by dopamine (50 μ M; Figure 1A; two-way repeated measures ANOVA, $F_{(1,11)} = 6.134$, $*p = 0.03$; N=12) and this effect reversed upon washout. To determine which subtype of DA receptor mediated inhibition of firing, spiking was

monitored in the presence of receptor selective compounds. Alone, the D1 receptor antagonist SCH23390 (10 μ M) had no effect on AP spiking (two-way repeated measures ANOVA, $F_{(2,12)} = 9.383$, $q=0.79$, $p>0.05$; $N=7$), while in the presence of SCH23390, DA (50 μ M) still produced a significant reduction in spike firing of IOFC neurons ($q=3.293$, $*p<0.05$; Figure 1B). In contrast, DA (50 μ M) failed to decrease spiking in the presence of the D2 receptor antagonist sulpiride (10 μ M) (two-way repeated measures ANOVA, $F_{(2,26)} = 0.1226$, $q=0.467$, $p>0.05$; $N=14$; Figure 1C). Sulpiride by itself did not alter neuronal firing ($q=0.091$, $p>0.05$; Figure 1C). Selective activation of D2 receptors by the D2 receptor agonist quinpirole (5 μ M) also significantly reduced spike firing of IOFC neurons (Figure 1D; two-way repeated measures ANOVA, $F_{(1,10)} = 17.95$, $**p=0.0017$; $N=10$). Thus, DA-induced inhibition of firing of IOFC neurons in naïve female mice requires activation of D2 receptors.

3.1.2 Norepinephrine—Norepinephrine (NE, 50 μ M) also produced a reversible reduction in AP spiking of IOFC neurons in naïve female mice (Figure 2A; two-way repeated measures ANOVA, $F_{(1,7)} = 6.223$, $*p=0.0413$; $N=8$). Application of the $\alpha 2$ adrenergic receptor antagonist yohimbine (100 nM), which by itself did not alter cell firing, prevented NE inhibition of spiking (Figure 2B; two-way repeated measures ANOVA, $F_{(2,12)} = 0.3736$, $q=0.2486$, $p=0.6960$; $N=7$). In contrast, NE still induced a decrease in AP firing when applied in the presence of the $\alpha 1$ adrenergic receptor antagonist naftopidil (10 μ M) (Figure 2C; two-way repeated measures ANOVA, $F_{(2,20)} = 5.12$, $q=3.186$, $**p<0.01$; $N=11$). Alone, naftopidil had no significant effect on neuron excitability ($q=1.332$, $p>0.05$). Together, these data indicate the involvement of $\alpha 2$ -adrenergic receptors in NE-mediated inhibition of IOFC neuron spiking.

3.1.3 Serotonin—Similar to that observed for DA and NE, acute application of serotonin (5HT, 50 μ M) significantly and reversibly decreased AP spiking of IOFC neurons in naïve female mice (Figure 2D; two-way repeated measures ANOVA, $F_{(1,7)} = 7.698$, $*p=0.0275$; $N=8$). The inhibitory effect of 5HT was blunted by the 5HT_{1A} receptor antagonist WAY100635 (100 nM) that by itself did not alter firing ($q=2.368$, $p>0.05$), as the number of spikes in the presence of WAY100635 and 5HT was not different from that obtained in WAY100635 alone (Figure 2E; two-way repeated measures ANOVA, $F_{(2,12)} = 9.688$, $q=2.03$, $p>0.05$; $N=7$). In contrast, no change in IOFC firing was observed in the presence of the 5HT₂ agonist α -methyl-5HT (50 μ M; Figure 2F; two-way repeated measures ANOVA, $F_{(1,7)} = 1.004$, $p=0.3496$; $N=8$). These results suggest that in female mice, serotonin decreases IOFC neuron excitability through activation of 5HT_{1A} receptors.

3.2 Role of monoamine transporters on monoamine-induced inhibition of lateral OFC neurons in naïve female mice

We reported previously that low concentrations of DA (1 and 100 nM) that normally have no effect on IOFC neuron firing in naïve male mice, significantly reduce evoked spiking when a monoamine transporter antagonist is present (Nimitvilai et al, 2017). To assess whether active uptake of monoamines in slices obtained from female mice affects monoamine inhibition of firing, low concentrations (1 and 100 nM) of each monoamine were tested in the absence or presence of the nonspecific monoamine transporter inhibitor nomifensine. In

the absence of nomifensine, 1 and 100 nM DA did not alter neuronal firing (Figure 3A; two-way repeated measures ANOVA, main effect of DA, $F_{(2,20)} = 2.107$, $p=0.1478$; $N=11$). Likewise, 1 and 100 nM NE (Figure 3B; two-way repeated measures ANOVA, main effect of NE, $F_{(2,10)} = 1.181$, $p=0.3463$; $N=6$) or 5HT (Figure 3C; two-way repeated measures ANOVA, main effect of 5HT, $F_{(2,10)} = 1.151$, $p=0.3549$; $N=6$) had no effect on spiking. However, in the presence of nomifensine (10 μM) that itself had no effect (two-way repeated measures ANOVA, $F_{(1,32)} = 0.0137$, $p=0.9076$), low concentrations of DA (two-way repeated measures ANOVA, $F_{(3,27)} = 14.78$, post hoc 1 nM DA $q=3.971$, $**p<0.001$; 100 nM DA $q=5.152$, $***p<0.0001$; Figure 3D), NE ($F_{(3,27)} = 10.5$, post hoc 1 nM NE $q=2.843$, $*p<0.001$; 100 nM NE, $q=5.217$, $***p<0.0001$; Figure 3E) or 5HT (two-way repeated measures ANOVA, $F_{(3,21)} = 11.98$, post hoc 1 nM 5HT, $q=3.597$, $**p<0.001$; 100 nM 5HT $q=4.447$, $***p<0.0001$; Figure 3F) all inhibited spike firing. These data suggest that monoamines are actively regulated by monoamine transporters in cortical slices prepared from female mice.

3.3 Role of GIRK channels in the inhibitory effects of monoamine

Agonist binding to $G_{i\alpha}$ -coupled receptors such as D2, α_2 -adrenergic or 5HT_{1A} results in $\beta\gamma$ subunit activation of inhibitory G protein-coupled inwardly rectifying potassium (GIRK) channels. In naïve male mice, blocking GIRK channels with barium attenuates monoamine inhibition of IOFC excitability, and the GIRK channel activator ML297 by itself reduces AP spiking (Nimitvilai *et al*, 2017). To examine whether a similar process is involved in the monoamine inhibition of IOFC spiking in female mice, we tested the effects of different GIRK channel modulators on IOFC firing. The GIRK activator ML297 (10 μM) produced a significant reduction in AP spiking of IOFC neurons in naïve females (Figure 4A; two-way repeated measures ANOVA, $F_{(1,15)} = 35.99$, $***p<0.0001$; $N=16$) and blocking GIRK channels with a low concentration of barium (100 μM) caused a small but significant increase in neuronal firing (Figure 4B; two-way repeated measures ANOVA, $F_{(1,20)} = 33.07$, $***p<0.0001$; $N=19$). In the presence of barium, neither DA (Figure 4C; two-way repeated measures ANOVA, $F_{(2,10)} = 8.951$, $q=0.1142$ $p>0.05$; $N=6$), NE (Figure 4D; $F_{(2,10)} = 8.765$, $q=1.135$ $p>0.05$; $N=6$) nor 5HT (Figure 4E; $F_{(2,12)} = 7.471$, $q=0.8876$ $p>0.05$; $N=7$) had any significant effect on firing. Together, these results suggest that, in female mice, DA, NE and 5HT inhibit IOFC excitability through $G_{i\alpha}$ -coupled activation of GIRK channels.

3.4 Withdrawal from CIE increases excitability of lateral OFC neurons in female mice

Withdrawal from chronic intermittent ethanol (CIE) exposure robustly increases intrinsic excitability of IOFC neurons in male mice, and this increase is associated with a reduction in the amplitude of the after-hyperpolarization (AHP) and a functional downregulation of small conductance potassium (SK) channels (Nimitvilai *et al*, 2016). Here, we examined whether CIE also alters the excitability of IOFC neurons in female mice. Four weekly cycles of CIE exposure followed by withdrawal produced an enhancement of AP spiking as compared to air controls (two-way ANOVA: main effect of CIE, $F_{(2,128)} = 16.83$, $***p<0.0001$; Figure 5A). Post-hoc comparisons revealed significant differences in spiking for 3-day ($q=5.773$, $p<0.0001$; $N=45$) and 7-day ($q=2.252$, $P<0.05$; $N=15$) withdrawal groups. There were no differences in the magnitude of the AHP among air (14.37 ± 0.33 mV), 3D-WD CIE (14.28 ± 0.27 mV) and 7D-WD CIE (13.33 ± 0.96 mV) groups (One-way ANOVA, $F_{(2,211)} =$

0.7815, $p > 0.05$; Figure 5B). There were also no significant differences in the resting membrane potential, input resistance, or height, threshold, width and rise time of action potentials between naïve, air and CIE groups (all one-way ANOVAs, $p > 0.05$.)

We then tested whether the lack of change in AHP amplitude after CIE treatment was a result of unaltered SK channel function. Depolarizing voltage steps (400 ms, from -20 to $+30$ mV with 10 mV between steps) were applied to IOFC neurons of air or CIE groups to activate an outward tail current that is mediated in part by SK channels. There was a significant main effect of CIE treatment on total tail current amplitude (two-way ANOVA; $F_{(2,282)} = 8.634$, $p = 0.0002$) with amplitudes from both the 3-day ($q = 5.723$, $p < 0.001$; $N = 19$) and 7-day ($q = 3.974$, $p < 0.05$; $N = 15$) CIE groups being significantly lower from the air control ($N = 17$; -apamin traces in Figure 5C–E). The SK channel inhibitor apamin (100 nM) had no effect on tail currents from air control animals (two-way ANOVA; $F_{(1,16)} = 3.264$, $p = 0.0897$, Figure 5C) or those withdrawn from CIE treatment (3-day WD, two-way ANOVA; $F_{(1,17)} = 3.634$, $p = 0.0737$, Figure 5D; 7-day WD, two-way ANOVA; $F_{(1,14)} = 3.196$, $p = 0.0955$, Figure 5E). As a further test of SK channel function, we measured spike firing of IOFC neurons in control female mice in the absence and presence of apamin. As shown in Figure 5F, apamin had no effect on IOFC firing as compared to control conditions in the absence of apamin (two-way ANOVA; $F_{(1,12)} = 0.467$, $p = 0.5074$; $N = 13$). Together, these results suggest that, unlike CIE male mice, the increase in intrinsic excitability of IOFC neurons in CIE females was not accompanied by a decrease in AHP amplitude or a change in SK channel function. These findings also suggest that apamin-sensitive SK channels do not actively regulate spiking of IOFC neurons in female mice.

3.5 Withdrawal from CIE blunts the inhibitory effects of monoamines and the GIRK channel activator in female mice

While CIE exposure and withdrawal enhanced the intrinsic excitability of IOFC neurons from female mice, this treatment has also been shown to attenuate the inhibitory effects of DA, NE, 5HT and the GIRK activator ML297 in male mice (Nimitvilai *et al.*, 2017). Here, we examined whether CIE also interferes with the inhibitory effects of monoamines and the GIRK activator on spiking of IOFC neurons from female mice. Figures 6A–D show the number of spikes at the 220 pA current injection step in the presence of DA, NE, 5HT or ML297 with values normalized to pre-drug baseline. As previously observed (Figures 1, 2 and 4), AP spiking recorded in air-treated female mice was significantly reduced by DA (Figure 6A; two-tailed paired t -test, $t(13) = 3.877$, $**p = 0.0019$), NE (Figure 6B; two-tailed paired t -test, $t(11) = 3.772$, $**p = 0.0031$), 5HT (Figure 6C; two-tailed paired t -test, $t(11) = 2.466$, $*p = 0.0313$) or ML297 (Figure 6D; two-tailed paired t -test, $t(16) = 6.648$, $***p < 0.0001$). In contrast, spiking of IOFC neurons from CIE exposed female mice was unaffected by DA (two-tailed paired t -test, $t(7) = 1.174$, $p = 0.2786$), NE (two-tailed paired t -test, $t(8) = 1.614$, $p = 0.1453$), 5HT (two-tailed paired t -test, $t(7) = 0.811$, $p = 0.4441$) or ML297 (two-tailed paired t -test, $t(13) = 0.8796$, $p = 0.3950$). Together, these findings suggest that $G_{i\alpha}$ -coupled monoamine receptors reduce the excitability of IOFC neurons in naïve female mice via activation of GIRK channels, and that this effect is blunted by CIE exposure.

4. DISCUSSION

The major findings of the present study demonstrate that acute application of DA, NE and 5HT all reduce current evoked firing of IOFC neurons from naïve female mice, and that this action is mediated primarily via $G_{i\alpha}$ -coupled D2, α_2 and 5HT_{1A} receptors, respectively. In addition, monoamine inhibition of firing was dependent on GIRK channels as a low concentration of barium occluded monoamine-induced inhibition of spiking and the GIRK channel activator ML297 itself reduced neuronal firing. After exposure to chronic ethanol treatment, the intrinsic excitability of IOFC neurons from female mice was enhanced, and monoamines and ML297 no longer decreased spike firing. Together with similar findings reported previously for IOFC neurons in male mice (Nimitvilai *et al*, 2017), these results suggest that monoamines are an important modulator of the intrinsic excitability of IOFC neurons and suggest that loss of this action following chronic ethanol exposure could contribute to the impairment of OFC-related behaviors observed in alcohol-dependent individuals.

In this study, micromolar concentrations of DA, NE and 5HT were required to alter firing of IOFC neurons in female mice. This is similar to that reported for IOFC neurons of male mice (Nimitvilai *et al*, 2017) and for effects on neurons in other cortical areas (Di Pietro and Seamans, 2011; Gonzalez-Islas and Hablitz, 2001; Nimitvilai *et al*, 2012; Trantham-Davidson *et al*, 2014; Wallace *et al*, 2014). The need for such high concentrations of monoamines in these ex vivo slice studies is perhaps surprising given that the affinity of receptors for monoamines is typically in the nanomolar to low micromolar range with those coupled to G_i/o proteins showing relatively higher affinity than others (Alexander *et al*, 2017). The relatively thick (~300 μ m) slices used in this and other electrophysiology studies may limit diffusion of monoamines and thus contribute to the need for higher concentrations. It is also likely that active uptake reduces extracellular levels of monoamines since DA, NE or 5HT at concentrations as low as 1 and 100 nM significantly decreased spike firing when the nonspecific monoamine transporter inhibitor nomifensine was co-applied. Interestingly, evoked firing was not affected when nomifensine or selective monoamine receptor antagonists were applied alone, consistent with previous findings in male mice (Nimitvilai *et al*, 2017). These results suggest that there is little tonic regulation of firing by monoamines in cortical slices under standard recording conditions, but that transporters actively regulate monoamines added exogenously to the slice.

In the present study, current-evoked AP spiking of IOFC neurons was significantly enhanced in female mice following a 3-day or 7-day withdrawal from repeated cycles of chronic ethanol vapor exposure. In our previous report, the increase in spike firing of IOFC neurons from CIE treated male mice was accompanied by a significant reduction in AHP amplitude and apamin-sensitive, small conductance potassium (SK) channel currents that contribute to the AHP (Nimitvilai *et al*, 2016). Despite a similar increase in firing, there were no changes in AHP amplitude and unlike in male mice, the SK channel blocker apamin had little effect on tail currents in neurons from either control or CIE treated female mice. Consistent with this finding was the lack of effect of apamin on current-evoked spiking. Together, these findings suggest that apamin-sensitive potassium currents are not a major contributor to the AHP or the regulation of firing in IOFC neurons from female mice. Ethanol-induced

changes in AHP and SK function have been reported in areas such as the nucleus accumbens following withdrawal from chronic ethanol exposure (Hopf *et al*, 2010; Padula *et al*, 2015). However, these studies were performed using only male animals leaving open the question of whether neurons in other regions of the female mouse brain also lack apamin-sensitive currents. Despite the lack of change in the AHP and the absence of apamin-sensitive currents, CIE exposed female mice did show reduced amplitude in current-evoked tail currents. The channels underlying these currents are not yet known but could represent a novel site of action for ethanol in female mice that contributes to the enhanced firing following CIE treatment. Unlike changes in apamin-sensitive AHP and outward tail currents that in IOFC neurons appear to be gender-specific, CIE exposed male and female mice both show resistance to the GIRK channel activator ML297 indicating some commonality of action. While the results of the present study have identified at least two differences in the effects of CIE exposure on OFC neuronal excitability between male and female mice, it is noted that reversal learning deficits have been reported in both genders with an alcohol exposure history (An and Zhang, 2015; Badanich *et al*, 2011; O'Leary-Moore *et al*, 2006) and in mice that display enhanced OFC firing due to reduced function of fast-spiking interneurons (Bissonette *et al*, 2015). These results suggest that regardless of the underlying mechanisms, disruption of optimal firing of OFC neurons by long-term exposure to ethanol may contribute to deficits in cognitive performance often reported in alcohol dependent individuals.

The increase in firing observed in CIE treated female mice in the present study was accompanied by a loss of monoamine sensitivity similar to that previously reported in male mice. The mechanism underlying the inability of monoamines to inhibit firing is currently unknown but could reflect changes in transporter function, monoamine receptor expression or changes in G-protein coupling to GIRK channels. We note that in male mice, CIE exposure did not alter the expression of Gi/o proteins or several GIRK channel subunits including GIRK1 that is activated by ML297 (Nimitvilai *et al*, 2017). Interestingly, other studies report that chronic ethanol exposure induces an increase in monoamine uptake rate in several brain areas of rodents and monkeys (Budygin *et al*, 2003; Budygin *et al*, 2007; Carroll *et al*, 2006; Sahr *et al*, 2004; Shibasaki *et al*, 2010). In addition, long-term exposure to alcohol has been shown to increase the expression of 5HT1A and 5HT1B receptors in the dorsal raphe nucleus and globus pallidus (Nevo *et al*, 1995), while decreases have been reported for 5HT1A receptors in the hippocampus and anterior/posterior cortices (Nevo *et al*, 1995), 5HT2A receptors in the cortex (Pandey *et al*, 1992) and D2 receptors in the striatum and nucleus accumbens (Feltmann *et al*, 2017; Volkow *et al*, 1996). While changes in transporter function and receptor expression could contribute to the blunted actions of monoamines on OFC firing, this implies a coordinated and receptor selective dysregulation of three different neurotransmitter systems and would not explain the lack of effect of ML297 on IOFC firing in CIE treated female (this study) and male mice (Nimitvilai *et al*, 2017). Future studies are needed to determine if this phenomenon is restricted to OFC neurons and whether CIE exposure results in changes in G protein/GIRK channel trafficking that could help explain the findings.

An important caveat regarding the results of the present study is that female mice were used without regard to their estrous cycle. We chose this approach since there were no prior

studies of OFC electrophysiology in female mice and a full characterization of monoamine action on OFC neuron firing across the multiple stages of the estrous cycle would require large numbers of animals. We note that our slice experiments were conducted using mice of slightly different ages and were performed during various days of the week that would be expected to capture different phases of the estrous cycle in individual mice. If estrous cycle affects monoaminergic signaling, this could have contributed to some of the variability observed for the effects of monoamines on neuron spiking. In support of this are results suggesting that ovarian hormones can affect monoamine release and monoamine receptors differently throughout the estrous cycle. For example, using quantitative microdialysis, basal extracellular levels of DA in the striatum (Xiao and Becker, 1994) and prefrontal cortex (Dazzi *et al*, 2007) were reported to be greater during estrus than diestrus in female rats, and the increased release of DA was associated with enhancement of D1 and/or downregulation of D2 receptors (Becker, 1990). Similarly, ovarian hormones have also been shown to influence serotonergic and noradrenergic systems in several brain areas, including hippocampus, raphe nuclei, hypothalamus and prefrontal cortex (Duchesne *et al*, 2009; Lu and Bethea, 2002; Parada *et al*, 1991; Pecins-Thompson and Bethea, 1999). Future studies are needed to address whether monoamine inhibition of IOFC neuronal excitability varies across the estrous cycle.

Finally, imbalance or dysfunction of monoaminergic systems has been linked to functional abnormalities in the OFC and deficits in behavioral flexibility that require an intact OFC (Clarke *et al*, 2004; Lee *et al*, 2007; Masaki *et al*, 2006; Pardey *et al*, 2013; Ridley *et al*, 1981; Walker *et al*, 2006; Wischhof *et al*, 2011; Zeeb *et al*, 2010). In addition, monoamine neurotransmitters have been associated in alcoholism and other substance abuse disorders (Noble, 2000). The findings of the current study combined with our previous report (Nimitvilai *et al*, 2017) suggest that monoamines play a role in modulating IOFC neuronal excitability in both male and female mice and that changes in monoamine action following CIE exposure may contribute to deficits in cognitive functions mediated by each monoamine. The profound loss of monoamine inhibition following CIE treatment also suggests that pharmacological treatments focused on specific receptor subtypes may not be effective in restoring the balance of monoamine signaling in alcohol-dependent individuals. The lack of change in the AHP amplitude in female mice following CIE exposure also highlights a growing awareness that there are likely diverse actions of alcohol between males and females, and that the same treatment might not generate equivalent outcomes between genders.

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Highlights

- DA, NE and 5HT all reduced evoked firing of OFC neurons in female mice
- This action required GIRK channels and D2R, α 2R and 5HT1_A receptors
- Chronic intermittent ethanol (CIE) exposure increased firing of OFC neurons
- After CIE treatment, DA, NE and 5HT no longer blocked firing of OFC neurons

Figure 1.

Dopamine decreases current-induced spiking of IOFC neurons from female mice via D2 receptors. (A) Representative traces show decreased action potential (AP) spiking in the presence of 50 μ M DA as compared with control. The graph shows the effect of dopamine on AP spiking (mean $\pm$ SEM) induced by a series of current injections (40–220 pA) in IOFC neurons. In comparison with control, DA produced a significant decrease in basal firing rates of IOFC neurons (two-way repeated measures ANOVA: main effect of DA, $F_{(1,11)} = 6.134$, $*p=0.03$; $N=12$). (B) The D1 receptor blocker SCH23390 (10 μ M) itself had no effect on DA-induced inhibition ($q=0.79$, $p>0.05$). A significant reduction of spiking was observed when a combination of DA and SCH23390 was applied (two-way ANOVA, $F_{(2,12)} = 9.38$, $q=3.293$, $*p=0.0035$; $N=7$). (C) Sulpiride (10 μ M) by itself did not alter AP spiking ($q=0.0908$, $p>0.05$) but prevented the inhibition of firing by DA (two-way ANOVA, $F_{(2,26)} = 0.1226$, $q=0.467$, $p>0.05$; $N=14$). (D) The D2 receptor agonist quinpirole (5 μ M) significantly decreased AP spiking as compared with control (two-way ANOVA, $F_{(1,10)} = 17.95$, $**p=0.0017$; $N=10$).

Figure 2.

Acute application of norepinephrine or serotonin decreases current-induced spiking of IOFC neurons from female mice via α_2 -adrenergic or 5-HT_{1A} receptors, respectively. Representative traces show decreased action potential (AP) spiking in the presence of 50 μ M NE or 5-HT as compared with control. (A) Effect of NE on AP spiking (mean $\pm$ SEM) induced by a series of current injections (40–220 pA) in IOFC neurons. NE (50 μ M) produced a significant decrease in current-evoked AP spiking (two-way ANOVA, $F_{(1,7)} = 6.223$, $*p=0.04$; $N=8$). (B) The α_2 -adrenergic receptor blocker yohimbine (100 nM) by itself did not alter cell firing (two-way ANOVA, $F_{(2,12)} = 0.37$, $q=0.5927$, $p>0.05$; $N=7$), but prevented NE-induced inhibition of spiking (no difference in spiking between yohimbine alone and yohimbine + NE; $q=0.2486$, $p>0.05$). (C) The α_1 -adrenergic receptor blocker naftopidil (10 μ M) alone did not alter spike firing (two-way ANOVA, $F_{(2,20)} = 5.12$, $q=1.332$, $p>0.05$; $N=11$) or prevent NE inhibition of spike firing (naftopidil alone versus naftopidil + NE; $q=3.186$, $**p<0.01$). (D) Effect of 5-HT on AP spiking (mean $\pm$ SEM) induced by a series of current injections (40–220 pA). 5-HT (50 μ M) produced a significant decrease in current-evoked spiking (two-way repeated measures ANOVA, $F_{(1,7)} = 7.698$, $*p=0.0275$; $N=8$). (E) The 5-HT_{1A} receptor antagonist WAY100635 (100 nM) alone had no effect on AP firing of IOFC neurons (control versus WAY100635, $q=2.368$, $p>0.05$) but

attenuated spike inhibition by 50 μ M 5-HT (no difference in number of spikes between WAY100635 alone WAY100365 + 5-HT; two-way ANOVA, $F_{(2,12)} = 9.688$, $q=2.03$, $p>0.05$; $N=7$). (F) The 5-HT₂ receptor agonist α -methyl-5HT (50 μ M) did not affect AP spiking (two-way ANOVA, $F_{(1,7)} = 1.004$, $p=0.3496$; $N=8$).

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Figure 3.

Low concentrations of monoamines decrease current-induced spiking of IOFC neurons from female mice when monoamine transporters are suppressed. Graphs show effect of each monoamine on AP spiking (mean $\pm$ SEM) of IOFC neurons induced by a series of current injections (40–220 pA) in the absence (A–C) or presence (D–F) of nomifensine. Without nomifensine, low concentrations (1 and 100 nM) of DA (A; two-way ANOVA: main effect of DA, $F_{(2,20)} = 2.107$, $p = 0.1478$), NE (B; two-way ANOVA: main effect of NE, $F_{(2,10)} = 1.181$, $p = 0.3464$) or 5HT (C; two-way ANOVA: main effect of 5HT, $F_{(2,10)} = 1.151$, $p = 0.3549$) had no effect on AP spiking of IOFC neurons. In the presence of 10 μ M nomifensine, DA (D; 1 nM, two-way ANOVA, $F_{(3,27)} = 14.78$, $q = 3.971$, $^{**}p < 0.001$; 100 nM ($q = 5.152$, $^{***}p < 0.0001$)), NE (E; 1 nM, two-way ANOVA, $F_{(3,27)} = 10.5$, $q = 2.843$, $^{*}p < 0.05$; 100 nM $q = 5.271$, $^{***}p < 0.0001$) and 5HT (F; 1 nM, two-way ANOVA, $F_{(3,21)} = 11.98$, $q = 3.597$, $^{**}p < 0.01$; 100 nM $q = 4.447$, $^{***}p < 0.001$) inhibited spike firing. Nomifensine itself did not affect spiking ($q = 0.1431$, $p > 0.05$).

Figure 4.

Effects of GIRK channel modulators on current-induced spiking of IOFC neurons from naïve female mice. Graphs show the effect of each drug on AP spiking (mean $\pm$ SEM) induced by a series of current injections (40–220 pA) in IOFC neurons. (A) The GIRK activator ML297 (10 μ M) produced a significant decrease in current-evoked spiking of IOFC neurons (two-way repeated measures ANOVA, $F_{(1,15)} = 35.99$, $***p < 0.0001$; $N=16$). (B) The GIRK channel inhibitor barium (100 μ M) caused a small but significant increase in spiking (two-way repeated measures ANOVA, $F_{(1,20)} = 33.07$, $***p < 0.0001$; $N=19$). In the presence of barium, the monoamines (50 μ M) no longer inhibited spike firing (C; DA, two-way repeated measures ANOVA, $F_{(2,10)} = 8.951$, $q=0.1142$, $p > 0.05$; $N=6$; D; NE, two-way repeated measures ANOVA, $F_{(2,10)} = 0.0063$, $q=1.135$, $p > 0.05$; $N=6$; E; 5HT, two-way repeated measures ANOVA, $F_{(2,12)} = 7.471$, $q=0.8876$, $p > 0.05$; $N=7$).

Figure 5.

CIE exposure of female mice enhances the intrinsic excitability of IOFC neurons without altering the amplitudes of after-hyperpolarization and outward tail currents. (A) Representative traces show increased action potential (AP) spiking in mice withdrawn for three days (3D-WD) and seven days (7D-WD) following CIE treatment as compared with air control. Graph shows number of spikes (mean $\pm$ SEM) of IOFC neurons from air and CIE treated female mice plotted against a series of current injections (40–220 pA). In comparison with air control (N=72), basal firing rates of IOFC neurons were significantly enhanced in 3D-WD (N=45) and 7D-WD (N=15) CIE groups (two-way ANOVA: main effect of CIE $F_{(2,128)} = 16.83$, $***p < 0.0001$; *post-hoc* comparison: air vs 3D-WD CIE, $q = 5.773$, $p < 0.0001$ and air vs 7D-WD CIE, $q = 2.252$, $p < 0.05$). (B) CIE had no significant effect on AHP amplitude as compared to air controls (one-way ANOVA, $F_{(2,211)} = 0.7815$, $p = 0.4591$; *post-hoc* comparison: $q = 0.2241$ for air vs 3D-WD CIE and $q = 1.243$ for air vs 7D-WD CIE). (C–E) Representative traces show total tail current amplitudes in the absence (black) and presence (gray) of the SK channel blocker apamin (100 nM) in air control, 3D-WD and 7D-WD CIE groups, respectively. Graphs show comparison of the peak outward tail current amplitudes (mean $\pm$ SEM) in response to depolarizing voltage steps (400 ms, from -20 to $+30$ mV with 10 mV between steps) in the absence and presence of apamin.

The SK channel blocker apamin did not significantly alter the tail current amplitude of IOFC neurons in air controls (C; two-way ANOVA; $F_{(1,16)} = 3.264$, $p=0.0897$; $N=17$), 3D-WD CIE (D; two-way ANOVA; $F_{(1,17)} = 3.634$, $p=0.0737$; $N=19$) and 7D-WD CIE (E; two-way ANOVA; $F_{(1,14)} = 3.196$, $p=0.0955$; $N=15$) groups. (F) Graph shows number of spikes (mean $\pm$ SEM) of IOFC neurons from control female mice in the absence and presence of apamin. Apamin did not affect IOFC firing as compared to baseline recordings without apamin (two-way ANOVA; $F_{(1,12)} = 0.467$, $p=0.5074$; $N=13$).

Figure 6.

CIE exposure blunts the inhibitory effects of monoamines. Summary graphs show number of spikes at the highest current injection (220 pA) expressed as percent change from control baseline (without drug) in response to each treatment condition. (A) DA significantly inhibited AP spiking of IOFC neurons in air-treated mice (two-tailed paired t -test, $t(13) = 3.877$, $**p=0.0019$) but not those in the 3D-WD group (two-tail paired t -test, $t(7) = 1.17$, $p=0.2786$). (B) NE significantly reduced evoked AP spiking in air control mice (two-tailed paired t -test, $t(11) = 3.772$, $**p=0.0031$), but not those in the 3D-WD group (two-tailed paired t -test, $t(8) = 1.614$, $p=0.1453$). (C) 5HT caused a significant reduction in spike firing in air control mice (two-tailed paired t -test, $t(11) = 2.466$, $*p=0.0313$), but not those in the 3D-WD group (two-tailed paired t -test, $t(7) = 0.811$, $p=0.4441$). (D) The GIRK channel activator ML297 (10 μ M) significantly reduced firing in IOFC neurons from air-exposed mice (two-tailed paired t -test, $t(16) = 6.648$, $***p<0.0001$) but not those in the 3D-WD group (two-tailed paired t -test, $t(13) = 0.8796$, $p=0.395$).

Table 1

Electrophysiological Characteristics of IOFC neurons from female mice

Condition	RMP (mV)	Input resistance (M Ω)	AP threshold (mV)	AP height (mV)	AP width (ms)	AP rise (ms)	AHP (mV)
Naïve	-68.62 $\pm$ 0.71	61.60 $\pm$ 1.76	-40.49 $\pm$ 0.93	50.42 $\pm$ 1.06	1.18 $\pm$ 0.03	0.34 $\pm$ 0.06	14.29 $\pm$ 0.46
Air	-67.50 $\pm$ 1.89	57.70 $\pm$ 1.61	-41.73 $\pm$ 0.84	51.99 $\pm$ 1.24	1.22 $\pm$ 0.03	0.32 $\pm$ 0.01	14.35 $\pm$ 0.36
CIE	-66.95 $\pm$ 1.35	61.97 $\pm$ 2.26	-42.60 $\pm$ 0.77	53.03 $\pm$ 0.98	1.21 $\pm$ 0.03	0.30 $\pm$ 0.01	14.60 $\pm$ 0.29

Abbreviations: RMP, resting membrane potential; AP, action potential; AHP, after-hyperpolarization. Values are mean $\pm$ SEM. One-way ANOVA was used to compare differences in electrophysiological characteristics between naïve, air and CIE female mice. No significant differences in the RMP, input resistance, AP threshold, AP height, AP width or AP rise between the groups ($p > 0.05$).