Ethanol Dependence Abolishes Monoamine and GIRK (Kir3) Channel Inhibition of Orbitofrontal Cortex Excitability

Sudarat Nimitvilai¹, Marcelo F Lopez², Patrick J Mulholland^{1,2} and John J Woodward^{*,1,2}

¹Department of Neuroscience, Medical University of South Carolina, Charleston, SC, USA; ²Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, SC, USA

Alcohol abuse disorders are associated with dysfunction of frontal cortical areas including the orbitofrontal cortex (OFC). The OFC is extensively innervated by monoamines, and drugs that target monoamine receptors have been used to treat a number of neuropsychiatric diseases, including alcoholism. However, little is known regarding how monoamines affect OFC neuron excitability or whether this modulation is altered by chronic exposure to ethanol. In this study, we examined the effect of dopamine, norepinephrine, and serotonin on lateral OFC neuronal excitability in naive mice and in those exposed to chronic intermittent ethanol (CIE) treatment. All three monoamines decreased current-evoked spike firing of IOFC neurons and this action required G_{α} -coupled D2, α_2 -adrenergic, and 5HT_{1A} receptors, respectively. Inhibition of firing by dopamine or the D2 agonist quinpirole, but not norepinephrine or serotonin, was prevented by the GABA_A receptor antagonist picrotoxin. GABA-mediated tonic current was enhanced by dopamine or the D1 agonist SKF81297 but not quinpirole, whereas the amplitude of spontaneous IPSCs was increased by quinpirole but not dopamine. Spiking was also inhibited by the direct GIRK channel activator ML297, whereas blocking these channels with barium increased firing and eliminated the inhibitory actions of monoamines. In the presence of ML297 or the G-protein blocker GDP- β -S, DA induced a further decrease in spike firing, suggesting the involvement of a non-GIRK channel mechanism. In neurons from CIE-treated mice, spike frequency was nearly doubled and inhibition of firing by monoamines or ML297 was lost. These effects occurred in the absence of significant changes in expression of $G_{i/o}$ or GIRK channel proteins. Together, these findings show that monoamines are important modulators of IOFC excitability and suggest that disruption of this process could contribute to various deficits associated with alcohol dependence.

Neuropsychopharmacology (2017) 42, 1800–1812; doi:10.1038/npp.2017.22; published online 15 March 2017

INTRODUCTION

Monoamine neurotransmitters such as norepinephrine (NE), serotonin (5HT), and dopamine (DA) are important regulators of neuronal function and are critical for a wide variety of affective behaviors including reward, attention, emotion, memory, and learning. Alterations in the levels or function of monoamines and their receptors, either by disease or genetic differences, are thought to contribute to symptoms associated with many psychiatric diseases including addiction (Kirby *et al*, 2011; Narendran *et al*, 2014; Volkow *et al*, 2001). Indeed, drugs that target monoamine systems have long been used to treat affective and attention disorders (Rothman *et al*, 2008; Volkow *et al*, 2002).

The ability of these compounds to partially alleviate symptoms of psychiatric diseases is thought to largely reflect actions on neurons and circuits within the frontal cortex. Of these, the orbitofrontal cortex (OFC) is known to play an important role in integrating information from sensory

modalities in order to guide behavior based on optimal choices and outcomes. Thus, regions of the OFC mediate aspects of choice behavior (Roesch and Olson, 2007; Schoenbaum *et al*, 1998), 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). Dysfunction of the OFC is associated with numerous neuropsychiatric diseases including obsessive-compulsive disorder and symptoms of OFC deficiency are often observed in individuals suffering from alcohol and drug abuse disorders (Fortier *et al*, 2008; Verdejo-Garcia *et al*, 2006). In a previous report, we showed that alcohol-dependent mice have impaired performance on a reversal learning task (Badanich *et al*, 2011) that in both rodents (Brown *et al*, 2007) and primates (Jedema *et al*, 2011) requires the OFC. Withdrawal from repeated cycles of chronic intermittent ethanol exposure was also shown to enhance the intrinsic excitability of OFC neurons (Nimitvilai *et al*, 2016), suggesting that dysregulation of processes that modulate excitability may contribute to dysfunction associated with alcohol use disorders. Understanding the mechanisms that regulate OFC excitability is thus important for developing therapeutic interventions designed to restore normal control of neuronal activity.

*Correspondence: Dr JJ Woodward, Department of Neuroscience, Medical University of South Carolina, 67 President Street, IOP456N, Charleston, SC 29425, USA, Tel: 843 792 5225, Fax: 843 792 7353, E-mail: woodward@musc.edu

Received 10 August 2016; revised 18 January 2017; accepted 21 January 2017; accepted article preview online 31 January 2017

The OFC has been shown to be extensively innervated by monoamine neurotransmitters (Agster *et al.*, 2013; Homayoun and Moghaddam, 2008; Robbins and Arnsten, 2009) and dysregulation of monoamine function has been suggested to underlie deficits in certain OFC-dependent behaviors (Clarke *et al.*, 2005; Kheramin *et al.*, 2004; Winstanley *et al.*, 2006). However, virtually nothing is known regarding the functional effects of monoamines on the excitability of OFC neurons or whether this modulation is affected by prior exposure to ethanol. In this study, we used whole-cell patch-clamp electrophysiology to examine the effects of DA, NE, and 5HT on the intrinsic excitability of lateral OFC (lOFC) neurons and determined whether the actions of these modulators are altered in ethanol-dependent mice. The results demonstrate that monoamine neurotransmitters reduce excitability via activation of G protein-coupled inwardly rectifying potassium (GIRK; Kir3 family) channels and that this regulation is lost in mice made dependent on alcohol.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) (<https://www.jax.org/strain/00064>) at 9 weeks of age. They were group-housed (4/cage) and allowed to acclimatize to the colony room for at least 1 week in a temperature- and humidity-controlled AAALAC-approved facility. Animals were maintained on a 12 h light/dark cycle with lights off at 0900 h and had *ad libitum* access to food and water. 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.

Chronic Intermittent Ethanol Exposure

After acclimatization, mice were exposed to four cycles of chronic intermittent ethanol (CIE group) vapor or air (control) in inhalation chambers as previously described (Badanich *et al.*, 2011; Nimitvilai *et al.*, 2016). Briefly, each cycle consisted of daily exposure to ethanol vapor for 16 h followed by 8 h 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. The average BEC during the 4 weeks of CIE exposure was 196.76 ± 9.69 mg/dl. Mice were killed 3, 7, or 14 days following the final vapor exposure (ethanol or air) and used for electrophysiological and biochemical studies. Full details are provided in Supplementary Materials.

Brain Slice Electrophysiology

Whole-cell patch-clamp electrophysiology recordings were carried out in brain slices containing the lOFC as previously described (Badanich *et al.*, 2013). Briefly, recordings were localized to deep layers of the lOFC and current-clamp recordings used a potassium gluconate internal solution (in mM; 120 K-Gluconate, 10 KCl, 10 HEPES, 2 MgCl₂, 1 EGTA, 2 NaATP, and 0.3 NaGTP, adjusted to 294 mOsm, pH = 7.4).

In some experiments, GDP- β -S (500 μ M) was included in the potassium gluconate internal solution to examine a role for G-protein activation on AP spiking. For voltage-clamp experiments, a cesium chloride internal solution (in mM; 120 CsCl, 10 HEPES, 2 MgCl₂, 1 EGTA, 2 NaATP, 0.3 NaGTP, and 1 QX-314, adjusted to 294 mOsm, pH = 7.4) was used. To measure spontaneous inhibitory postsynaptic currents (sIPSCs), the glutamate NMDA antagonist DL-AP5 (100 μ M) and AMPA antagonist CNQX (10 μ M) were added to the aCSF perfusion solution to isolate GABA_A-mediated currents, and the neuron was voltage clamped at -70 mV. Spontaneous events were detected using a template matching algorithm and a threshold amplitude of 10 pA (AxographX software, Axograph, Sydney, Australia). 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 running on a Macintosh G4 computer (Apple, Cupertino, CA). Events were filtered at 4 kHz and digitized at a sampling rate of 10 kHz. Full details can be found in Supplementary Materials.

Drugs

Dopamine hydrochloride was purchased from Alfa Aesar (Ward Hill, MA). Serotonin hydrochloride, α -methyl-5HT, SKF81297, quinpirole hydrochloride, sulpiride, SCH23390, WAY100635, yohimbine, picrotoxin, naftopidil hydrochloride, nomifensine, DL-AP5, and CNQX were purchased from Tocris Biosciences (Minneapolis, MN). ML297 was purchased from Abcam (Cambridge, MA). GDP- β -S was purchased from Santa Cruz Biotechnology (Dallas, TX). Norepinephrine, strychnine, barium, and all reagents used to prepare aCSF, sucrose-containing and internal pipette solution were purchased from Sigma (St Louis, MO).

Western Blotting

OFC tissue punches were collected from control and CIE-treated mice at 3 days following the last exposure and western blotting was performed according to standard immunoblotting procedures described previously (McGuier *et al.*, 2015). Full details can be found in Supplementary Materials.

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 repeated measures ANOVA, two-way ANOVA, and *t*-test) as indicated. Comparisons were considered significantly different when $p < 0.05$.

RESULTS

Monoamines Decrease the Excitability of Lateral OFC Neurons through G_{i/o} Linked Receptors

Dopamine. Recordings were carried out in large, regular spiking pyramidal neurons located in deep layers of the

Figure 1 Acute dopamine application decreases current-induced spiking of lateral OFC neurons via D2 receptors. Representative traces show decreased action potential (AP) spiking in the presence of 50 μ M DA as compared with control. (a) Effect of dopamine (10 and 50 μ M) on AP spiking (mean $\pm$ SEM) induced by a series of current injections (60–200 pA) in IOFC neurons. In comparison with control, DA produced a significant decrease in basal firing rates of IOFC neurons (two-way ANOVA: main effect of DA, $F_{(2, 390)} = 42.14$, $p = 0.0002$; *post hoc* comparison, control vs DA, $***p < 0.001$). (b) Low concentrations (1 and 100 nM) of DA did not alter the AP spiking of IOFC neurons (two-way ANOVA: main effect of DA, $F_{(2, 171)} = 0.036$, $p = 0.737$; *post hoc* comparison, control vs DA, $p > 0.99$). (c) In the presence of a monoamine transporter inhibitor nomifensine (10 μ M), DA at 1 nM (two-way ANOVA, $F_{(2, 216)} = 13.9$, $q = 5.27$, $***p < 0.0001$) produced a significant reduction in firing. (d) The D1 receptor blocker SCH23390 (10 μ M) had no effect on DA-induced inhibition; a significant reduction of spiking was observed when a combination of DA and SCH23390 was applied (two-way ANOVA, $F_{(2, 375)} = 16.25$, $***p < 0.0001$). SCH23390 alone did not alter the cell firing. (e) The D2 receptor blocker sulpiride (10 μ M) suppressed DA-induced inhibition of evoked spiking so that the number of spikes in response to a series of injected current were not significantly different from sulpiride baseline (two-way ANOVA, $F_{(2, 240)} = 11.58$, $q = 3.078$, $p > 0.05$). Sulpiride by itself produced a small but significant increase in firing rate (two-way ANOVA, $F_{(2, 240)} = 11.58$, $q = 6.89$, $p < 0.001$). (f) The D2 receptor agonist quinpirole produced a significant decrease in number of spikes in a concentration-dependent manner (two-way ANOVA: main effect of DA, $F_{(2, 210)} = 19.46$, $p < 0.001$; *post hoc* comparison, control vs quinpirole, $***p < 0.001$).

IOFC. Supplementary Table 1 shows values for resting membrane potential and input resistance in the absence and presence of monoamines. In current-clamp mode, injections of current evoked increasing numbers of action potentials (APs) in IOFC neurons (Figure 1a). Action potential firing was reduced by dopamine in a concentration-dependent manner (two-way ANOVA: main effect of dopamine, $F_{(2, 390)} = 42.14$, $p = 0.0002$) and this effect was reversed upon

washout. The *post hoc* comparisons reported significant differences in spiking in response to either 10 μ M ($q = 8.426$, $p < 0.0001$; $N = 9$) or 50 μ M dopamine ($q = 12.99$, $p < 0.0001$; $N = 8$). Concentrations of dopamine below 10 μ M had little effect on firing (Figure 1b; two-way ANOVA, $F_{(2, 171)} = 0.3058$, $p = 0.737$). To assess whether this reflects active uptake in the slice, recordings were carried out in the presence of the nonspecific monoamine transporter inhibitor

Figure 2 Acute application of norepinephrine or serotonin decreases current-induced spiking of lateral OFC neurons via α_2 -adrenergic or 5HT_{1A} receptors, respectively. Representative traces show decreased action potential (AP) spiking in the presence of 50 μ M NE or 5HT as compared with control. (a) Effect of NE (10 and 50 μ M) on AP spiking (mean $\pm$ SEM) induced by a series of current injections (60–200 pA) in IOFC neurons. NE significantly decreased current-evoked spiking in a concentration-dependent manner (two-way ANOVA: main effect of NE, $F_{(2, 255)} = 60.11$, $***p < 0.0001$; *post hoc* comparison, control vs NE, $***p < 0.0001$). (b) The α_2 -adrenergic receptor blocker yohimbine (10 μ M) by itself did not alter cell firing (two-way ANOVA, $F_{(2, 495)} = 2.401$, $q = 0.544$, $p = 0.0917$), but it suppressed NE-induced inhibition of spiking so that the level of reduction was not statistically different from those produced by yohimbine alone (two-way ANOVA, $F_{(2, 495)} = 2.401$, $q = 2.11$, $p = 0.0917$). (c) The α_1 -adrenergic receptor blocker naftopidil (10 μ M) did not alter spike firing (two-way ANOVA, $F_{(14, 140)} = 0.7912$, $p = 0.6766$) and did not suppress NE inhibition of spike firing; a significant reduction in AP spiking was found when a combination of NE and naftopidil was applied (two-way ANOVA, $F_{(14, 140)} = 0.7912$, $**p < 0.01$). (d) Effect of 5HT (10 and 50 μ M) on AP spiking (mean $\pm$ SEM) induced by a series of current injections (60–200 pA). 5HT produced a significant decrease in current-evoked spiking in a concentration-dependent manner (two-way ANOVA: main effect of 5HT, $F_{(2, 300)} = 55$, $***p < 0.0001$; *post hoc* comparison, control vs 5HT, $***p < 0.0001$). (e) The 5HT_{1A} receptor antagonist WAY100635 (100 nM) attenuated spike inhibition by 50 μ M 5HT so that the number of spikes mediated by WAY100635 plus 5HT did not differ from those mediated by WAY100635 alone (two-way ANOVA, $F_{(2, 345)} = 11.04$, $q = 2.231$, $p > 0.05$). The WAY100635 by itself slightly decreased AP firing of IOFC neurons (two-way ANOVA, $F_{(2, 345)} = 11.04$, $q = 4.298$, $**p < 0.01$). (f) In comparison with control, no changes in AP spiking induced by an agonist of 5HT₂ receptor α -methyl-5HT at 10 and 50 μ M was observed (two-way ANOVA, $F_{(2, 315)} = 2.52$, $p > 0.05$).

nomifensine (10 μ M). With nomifensine present (Figure 1c), firing was significantly decreased by dopamine concentrations as low as 1 nM (two-way ANOVA, $F_{(2, 216)} = 13.9$, $q = 2.55$, $p < 0.05$; $N = 10$) and 100 nM (two-way ANOVA, $F_{(2, 216)} = 13.9$, $q = 5.27$, $p < 0.0001$; $N = 7$), suggesting that monoamine transporters actively regulate extracellular levels of dopamine in the cortical slice. Nomifensine itself caused a small but significant decrease in AP spiking (two-way ANOVA, $F_{(1, 12)} = 15.2$, $p = 0.002$; $N = 13$; data not shown).

To determine whether dopamine inhibition of firing was receptor dependent, action potentials were recorded in the presence of D1 or D2 receptor antagonists. In the presence of the D1 receptor antagonist SCH23390, 50 μ M dopamine still produced a significant reduction in AP spiking of IOFC neurons (Figure 1d; two-way ANOVA, $F_{(2, 375)} = 16.25$, $q = 6.621$, $p < 0.0001$; $N = 8$). SCH23390 alone did not alter cell firing (two-way ANOVA, $F_{(2, 375)} = 16.25$, $q = 0.759$, $p > 0.05$). In contrast, dopamine (50 μ M) had no effect on

spiking in the presence of the D2 receptor antagonist sulpiride (Figure 1e; two-way ANOVA, $F_{(2, 240)} = 11.58$, $q = 3.078$, $p > 0.05$; $N = 6$). Alone, sulpiride produced a slight, but significant, increase in firing rate (two-way ANOVA,

$F_{(2, 240)} = 11.58$, $q = 6.89$, $p < 0.0001$), suggesting that these receptors may be tonically active in the slice. In concert with these findings, activation of D2 receptors directly with the selective agonist quinpirole also significantly reduced firing

Figure 3 Effects of dopamine and D1/D2 agonists on synaptic and extrasynaptic GABA_A currents. (a) Picrotoxin (Ptx) attenuates the inhibitory effects of DA and quinpirole, but not NE and 5HT. Effect of Ptx and DA on AP spiking (mean $\pm$ SEM) induced by a series of current injections (60–200 pA) in IOFC neurons. In comparison with Ptx baseline, no difference in basal firing rates of IOFC neurons was observed when DA was added in the presence of Ptx (two-way ANOVA, $F_{(14, 180)} = 0.0624$, $p > 0.9999$). (b) Numbers of spikes at the highest current injection are compared as the percent change from Ptx or strychnine baseline in response to each drug treatment condition. In comparison with Ptx alone, no difference in number of spikes was observed when Ptx was added together with DA (two-tail paired *t*-test, $t(6) = 2.396$, $p = 0.0536$) or quinpirole (two-tail paired *t*-test, $t(5) = 1.077$, $p = 0.3306$). However, NE (two-tail paired *t*-test, $t(5) = 4.113$, $*p = 0.0092$) or 5HT (two-tail paired *t*-test, $t(5) = 4.175$, $*p = 0.0087$) significantly decreased the cell firing as compared with Ptx baseline. With strychnine, DA produced a significant decrease in AP spiking as compared with strychnine alone (two-tail paired *t*-test, $t(5) = 3.614$, $*p = 0.0153$). (c) Representative traces of sIPSCs in the absence (baseline) and presence of DA, SKF81297 or quinpirole are shown. Graphs display individuals and mean ($\pm$ SEM) changes in sIPSC amplitude (d) and sIPSC frequency (e). In comparison with control, application of dopamine (50 μ M) did not alter sIPSC amplitude (c, two-tailed *t*-test, $t(10) = 0.6305$, $p = 0.5425$) or frequency (d, two-tail paired *t*-test, $t(10) = 0.5298$, $p = 0.6078$). Similarly, no change in sIPSC amplitude (c, two-tailed *t*-test, $t(7) = 0.1762$, $p = 0.8651$) or frequency (d, two-tail paired *t*-test, $t(7) = 0.4763$, $p = 0.6484$) was observed when SKF81297 was applied. Quinpirole (5 μ M) significantly enhances sIPSC amplitude (c, two-tailed *t*-test, $t(8) = 3.61$, $***p = 0.0069$), but had no effect on sIPSC frequency (d, two-tailed *t*-test, $t(8) = 0.735$, $p = 0.4834$). (f) Tonic currents before and during dopamine (50 μ M), SKF81297 (10 μ M), or quinpirole (5 μ M) application were measured. Dopamine induced a significant increase in the holding current of IOFC neurons (two-tailed *t*-test, $t(7) = 3.932$, $***p = 0.0057$) and this effect was blocked by picrotoxin (100 μ M; two-tailed *t*-test, $t(4) = 0.054$, $p = 0.9595$) or SCH23390 (two-tailed *t*-test, $t(6) = 1.457$, $p = 0.1953$), but not sulpiride (two-tailed *t*-test, $t(15) = 2.252$, $*p = 0.0397$). Similarly, SKF81297 significantly enhanced tonic current (two-tailed *t*-test, $t(7) = 3.945$, $***p = 0.0056$) and this effect was suppressed by picrotoxin (two-tailed *t*-test, $t(5) = 0.2097$, $p = 0.8422$). No change in tonic current was observed when quinpirole was applied (two-tailed *t*-test, $t(8) = 1.252$, $p = 0.2458$).

(Figure 1f; two-way ANOVA: main effect of quinpirole, $F_{(2, 210)} = 19.46$, $p < 0.0001$). The *post hoc* comparisons reported significant differences in spiking in response to 5 μM quinpirole ($q = 6.09$, $p < 0.0001$; $N = 8$).

Norepinephrine. Similar to dopamine, acute application of either 10 or 50 μM NE produced a robust inhibition of AP spiking in IOFC neurons (two-way ANOVA, main effect of NE, $F_{(2, 255)} = 60.11$, $p < 0.0001$) and this effect reversed after washout. The *post hoc* comparisons showed significant decreases in firing in response to either 10 μM ($q = 9.089$, $p < 0.0001$; $N = 7$) or 50 μM NE (Figure 2a; $q = 15.25$, $p < 0.0001$; $N = 6$). Application of the α -2 adrenergic receptor antagonist yohimbine (10 μM) alone did not alter the excitability of IOFC neurons (two-way ANOVA, $F_{(2, 495)} = 2.401$, $q = 0.544$, $p = 0.0917$) but it did suppress NE inhibition of spiking (Figure 2b; two-way ANOVA, $F_{(2, 495)} = 2.401$, $q = 2.11$, $p = 0.0917$; $N = 12$). In contrast, the reduction in firing by NE was not reduced by the α 1 adrenergic receptor antagonist naftopidil (10 μM) (Figure 2c; two-way ANOVA, $F_{(2, 104)} = 6.223$, $q = 3.306$, $p < 0.01$; $N = 6$) and by itself, naftopidil had no effect on spiking (two-way ANOVA, $F_{(2, 104)} = 6.223$, $q = 0.3779$, $p = 0.6766$).

Serotonin. As summarized in Figure 2d and similar to that observed for DA and NE, acute application of serotonin reversibly decreased AP spiking of IOFC neurons in a concentration-dependent manner (two-way ANOVA, main effect of 5HT, $F_{(2, 300)} = 55$, $p = 0.0314$; $N = 8$). The *post hoc* comparisons showed significant decreases in spiking in response to either 10 μM ($q = 6.831$, $p < 0.0001$) or 50 μM ($q = 14.64$, $p < 0.0001$) serotonin. This effect was blocked by the 5HT_{1A} antagonist WAY100635 (100 nM; two-way ANOVA, $F_{(2, 345)} = 11.04$, $q = 2.231$, $p > 0.05$, Figure 2e) that itself produced a small decrease in firing rate (two-way ANOVA, $F_{(2, 345)} = 11.04$, $q = 4.298$, $p < 0.01$). As there is also evidence for 5HT₂ receptors in the OFC, we tested whether selectively activating these receptors would influence IOFC neuronal excitability. There was no significant change in AP firing of IOFC neurons in the presence of 10 μM ($N = 9$) or 50 μM ($N = 6$) of the 5HT₂ agonist α -methyl-5HT (Figure 2f; two-way ANOVA, $F_{(2, 315)} = 2.52$, $p > 0.05$).

Involvement of GABA_A Receptors in Monoamine Inhibition of IOFC Neuronal Excitability

To explore potential mechanisms underlying monoamine inhibition of IOFC firing, we investigated whether GABA_A receptors were involved as previous studies report that monoamines can potentiate GABA_A-induced inhibition of neurons in the PFC (Zhou and Hablitz, 1999) and other brain areas (Hoerbelt et al, 2015; Janssen et al, 2009; Rainnie, 1999). Recordings were performed in the presence of a GABA_A receptor blocker picrotoxin (100 μM) in the aCSF, and the effect of each monoamine was reexamined. With picrotoxin present, 50 μM dopamine no longer inhibited current-evoked spiking of IOFC neurons (Figure 3a; two-way ANOVA, $F_{(1, 180)} = 0.0624$, $p = 0.99$; $N = 7$). Similarly, the inhibitory effect of the D2 receptor agonist quinpirole (5 μM) was also blocked by picrotoxin (Supplementary Figure S1a; two-way ANOVA, $F_{(1, 100)} = 0.011$, $p = 0.9153$; $N = 6$). In

contrast, the inhibition of AP spiking by 50 μM norepinephrine (Supplementary Figure S1b; two-way ANOVA, $F_{(1, 150)} = 101.4$, $p < 0.0001$; $N = 6$) or serotonin (Supplementary Figure S1c; two-way ANOVA, $F_{(1, 300)} = 31.4$, $p < 0.0001$; $N = 10$) was not affected by picrotoxin. In addition to GABA_A receptors, picrotoxin also inhibits glycine-activated chloride channels (Wang et al, 2006) and we previously reported a glycine-mediated inhibition of neuronal firing in the IOFC (Badanich et al, 2013). To investigate whether glycine receptors have a role in dopamine-mediated inhibition of spike firing, recordings were carried out in the presence of strychnine. There was no change in dopamine inhibition of IOFC firing by strychnine (1 μM) (Supplementary Figure S1d, two-way ANOVA, $F_{(1, 150)} = 56.83$, $p < 0.0001$; $N = 6$). Summarized in Figure 3b are the number of spikes at the highest current injection step in the presence of picrotoxin or strychnine and each monoamine (50 μM) and data are normalized to the picrotoxin or strychnine baseline.

To further probe how GABA_A receptors are involved in the dopamine inhibition of IOFC neuronal firing, we monitored sIPSCs and tonic current before and during exposure to dopamine, the D1 agonist SKF81297, or the D2 agonist quinpirole. In these experiments, a cesium chloride internal solution was used and voltage-clamp recordings were carried out in the presence of glutamate receptor antagonists (CNQX and AP5) to isolate GABA_A-mediated events. Interestingly, application of dopamine (50 μM) had no significant effect on sIPSC amplitude (Figure 3c and d; two-tail paired *t*-test, $t(10) = 0.6305$, $p = 0.5425$) or frequency (Figure 3c and e; two-tail paired *t*-test, $t(10) = 0.5298$, $p = 0.6078$) when compared with data from control recordings. Similarly, the D1 agonist SKF81297 (10 μM) did not alter sIPSC amplitude (two-tail paired *t*-test, $t(7) = 0.1762$, $p = 0.8651$) or frequency (two-tail paired *t*-test, $t(7) = 0.4763$, $p = 0.6484$) when compared with the control baseline. In contrast, the D2 agonist quinpirole (5 μM) significantly increased the amplitude of sIPSCs (Figure 3c and d; two-tail paired *t*-test, $t(8) = 3.61$, $p = 0.0069$), whereas it had no effect on frequency (Figure 3c and e; two-tail paired *t*-test, $t(8) = 0.735$, $p = 0.4834$).

To assess whether these dopamine receptor agonists also show differential effects on tonic currents mediated by extrasynaptic GABA_A receptors, we measured the holding current before and during local application of DA, SKF81297, or quinpirole. DA (50 μM) induced a significant increase in the holding current of IOFC neurons (Figure 3f; two-tailed *t*-test, $t(7) = 3.932$, $p = 0.0057$) and this effect was blocked by picrotoxin (100 μM ; two-tailed *t*-test, $t(4) = 0.054$, $p = 0.9595$). Similarly, the D1 agonist SKF81297 (10 μM) significantly increased tonic current (two-tailed *t*-test, $t(7) = 3.945$, $p = 0.0056$) and this increase was also suppressed by picrotoxin (two-tailed *t*-test, $t(5) = 0.2097$, $p = 0.8422$). However, there was no change in tonic current during application of quinpirole (5 μM) to the patched neuron (two-tailed *t*-test, $t(8) = 1.252$, $p = 0.2458$). Blocking D1 receptors with SCH23390 attenuated DA enhancement of tonic current (two-tailed *t*-test, $t(6) = 1.457$, $p = 0.1953$), whereas blocking D2 receptors with sulpiride had no effect on DA-induced tonic currents (two-tailed *t*-test, $t(15) = 2.252$, $p = 0.0397$).

Figure 4 Inhibition of GIRK channels attenuates the inhibitory effects of monoamines. (a) Number (mean $\pm$ SEM) of spikes from IOFC neurons plotted against a series of current injections. The GIRK channel inhibitor barium (100 μ M) caused a slight but significant increase in spiking when compared with control (two-way ANOVA, $F_{(1, 560)} = 27.2$, $***p < 0.0001$). (b) Numbers of spikes at the highest current injection are compared as percent change from barium baseline in response to each drug treatment condition. In comparison with barium alone, no difference in number of spikes was observed when barium was added together with DA (two-tail paired t -test, $t(4) = 0.85$, $p = 0.4438$), NE ($n = 6$, two-tail paired t -test, $t(5) = 1.52$, $p = 0.1889$), or 5HT (two-tail paired t -test, $t(5) = 0.86$, $p = 0.4292$). (c–e) Number (mean $\pm$ SEM) of spikes from IOFC neurons plotted against a series of current injections. A GIRK channel activator ML297 (10 μ M) significantly decreased AP spiking in comparison with control baseline (two-way ANOVA, $F_{(1, 192)} = 24.15$, $p < 0.0001$). In comparison with ML297 baseline, DA (c, two-way ANOVA, $F_{(1, 144)} = 16.59$, $**p < 0.001$) but not NE (d) or 5HT (e) produced a significant decrease in AP spiking. (f) Numbers of spikes at the highest current injection are compared as a percent change from ML297 baseline in response to each drug treatment condition. With ML297, DA further decreased spike firing and this decrease was significantly different from ML297 baseline (two-tail paired t -test, $t(9) = 3.281$, $**p = 0.0095$). No difference in AP spiking was observed when NE (two-tail paired t -test, $t(6) = 0.2247$, $p = 0.8297$) or 5HT (two-tail paired t -test, $t(10) = 1.881$, $p = 0.0894$) was added in the presence of ML297.

Role of GIRK Channels in the Inhibitory Effects of Monoamines

Many $G_{i\alpha}$ -coupled receptors are linked to stimulation of GIRK (Kir3 family) channels that are major regulators of neuronal excitability (Dascal, 1997; Hille, 1992). As D2, 5HT_{1A}, and $\alpha 2$ -adrenergic receptors are all $G_{i\alpha}$ coupled, we examined whether modulating GIRK channel activity would interfere with the inhibitory effects of monoamines. As shown in Figure 4a, a low concentration of barium (100 μ M) chosen to primarily inhibit GIRK channels (McDaid et al, 2008; Hurst et al, 1995; van Welie et al, 2005) produced a small, but significant, increase in AP spiking of IOFC

neurons (two-way ANOVA, $F_{(1, 560)} = 27.2$, $p < 0.0001$; $N = 30$). Data in Figure 4b show the number of spikes at the highest current injection step (220 pA) in the presence of barium and each monoamine (50 μ M) and are normalized to the barium baseline. No significant difference in spike number from barium control recordings was observed for dopamine (two-tail paired t -test, $t(4) = 0.85$, $p = 0.4438$), serotonin (two-tail paired t -test, $t(5) = 0.86$, $p = 0.4292$), or norepinephrine (two-tail paired t -test, $t(5) = 1.52$, $p = 0.1889$). We then tested whether ML297, a direct activator of GIRK channels, would decrease cell firing and whether it occluded the inhibitory effects of each monoamine. As shown in Figure 4c–e, ML297 (10 μ M, applied in

Figure 5 OFC neurons are hyperexcitable after withdrawal from CIE exposure and resistant to the inhibitory effects of the GIRK activator ML297 and monoamines. (a) Number (mean $\pm$ SEM) of spikes from IOFC neurons from air- and CIE-treated mice plotted against a series of current injections (60–200 pA). In comparison with air control, basal firing rates of IOFC neurons were significantly enhanced in 3-day withdrawal (3D-WD) and 7D-WD CIE groups (two-way ANOVA: main effect of CIE, $F_{(4, 364)} = 31.77$, $***p < 0.0001$; *post hoc* comparison, air vs 3D-WD CIE $***p < 0.0001$, air vs 7D-WD CIE $***p < 0.0001$). No difference in AP spiking between 14D-WD CIE and air control groups was observed ($p > 0.05$). (b) CIE exposure produced a significant decrease in ML297-induced inhibition in AP spiking at the 3-day withdrawal so that the mean number of spikes in the presence of ML297 did not differ from control baseline (two-tail paired *t*-test, $t(10) = 0.1316$, $p = 0.8979$). Significant inhibition by ML297 was observed in air-exposed mice (two-tail paired *t*-test, $t(16) = 7.086$, $***p < 0.0001$), 7D-WD (two-tail paired *t*-test, $t(17) = 3.226$, $***p = 0.005$) and 14D-WD (two-tail paired *t*-test, $t(7) = 4.459$, $**p = 0.0029$) CIE groups. (c) Representative western blots and optical density (mean $\pm$ SEM) of G_{i/o} protein and GIRK subunits in IOFC neurons from air and 3D-WD groups. No difference in expression of G_{i/o} (two-tailed *t*-test, $t(9) = 0.3727$, $p = 0.718$), GIRK1 (two-tailed *t*-test, $t(10) = 0.9863$, $p = 0.3473$), or GIRK2 (two-tailed *t*-test, $t(18) = 0.9218$, $p = 0.3688$) protein between air- and 3D-WD CIE-exposed groups were observed. (d) DA caused a significant reduction in number of spikes in air control (two-tail paired *t*-test, $t(4) = 6.236$, $**p = 0.0034$), but not in 3D-WD (two-tail paired *t*-test, $t(5) = 0.2606$, $p = 0.8048$) and 7D-WD (two-tail paired *t*-test, $t(5) = 1.266$, $p = 0.2613$) CIE groups. (e) NE significantly reduced evoked spiking in air control (two-tail paired *t*-test, $t(6) = 5.296$, $**p = 0.0018$), but not in 3D-WD (two-tail paired *t*-test, $t(4) = 0.8409$, $p = 0.4477$) and 7D-WD (two-tail paired *t*-test, $t(3) = 0.5315$, $p = 0.6319$) CIE groups. (f) A significant decrease in spiking by 5HT was observed in air control (two-tail paired *t*-test, $t(5) = 2.626$, $*p = 0.0467$), but not in 3D-WD (two-tail paired *t*-test, $t(4) = 1.713$, $p = 0.1619$) and 7D-WD (two-tail paired *t*-test, $t(4) = 1.422$, $p = 0.228$) CIE groups. (b, d–f) Numbers of spikes at the highest current injection are compared as percent change from control baseline (without drug) in response to each treatment condition.

the superfusate for 10 min) significantly decreased firing as compared with the control drug-free baseline (two-way repeated measures ANOVA, $F_{(1, 43)} = 64.78$, $p < 0.0001$; $N = 44$). Adding DA in the presence of ML297 (Figure 4c) further decreased spiking, whereas neither NE (Figure 4d) nor 5HT (Figure 4e) produced any additional decrease in

firing as compared with the ML297 baseline. Summarized in Figure 4f are the number of spikes at the highest current injection step in the presence of ML297 and each monoamine (data from Figure 4c–e) and are normalized to the ML297 baseline. With ML297, spiking produced by NE (two-tail paired *t*-test, $t(7) = 0.4472$, $p = 0.6682$) or 5HT (two-tail

paired *t*-test, $t(10) = 1.813$, $p = 0.0998$) was not different from spiking produced by ML297 alone. In contrast, DA further decreased AP spiking and this decrease was significantly different from ML297 baseline (two-tail paired *t*-test, $t(8) = 4.6$, $p = 0.0018$). We then tested whether blocking G-protein activation prevents monoamine-induced inhibition of spiking. In the presence of GDP- β -S (500 μ M, applied intracellularly through patch pipette), 5HT (50 μ M) did not significantly inhibit cell firing (Supplementary Figure S2b and d; two-way repeated measures ANOVA, $F_{(9, 54)} = 1.689$, $p = 0.1144$; $N = 7$). In contrast, DA (50 μ M) reduced current-evoked spiking in the presence of GDP- β -S (Supplementary Figure S2c and d; two-way repeated measures ANOVA, $F_{(9, 54)} = 4.946$, $p < 0.0001$; $N = 9$), although the magnitude of inhibition was less than that without GDP- β -S in the pipette (see Figure 1a for comparison). GDP- β -S by itself had no effect on AP spiking over a 20 min time course (Supplementary Figure S2a, two-way repeated measures ANOVA, $F_{(18, 90)} = 0.8849$, $p = 0.5975$; $N = 6$). Together with results from the barium and ML297 recordings, these findings are consistent with the idea that $G_{i/o}$ -coupled monoamine receptors reduce excitability of IOFC neurons via activation of GIRK channels and suggest the involvement of an additional non-GIRK mechanism in the inhibitory actions of DA.

CIE Exposure Attenuates ML297 and Monoamine Inhibition of Firing

We previously demonstrated that withdrawal from chronic intermittent ethanol treatment increases the excitability of IOFC neurons for up to 10 days after the last ethanol exposure (Nimitvilai *et al*, 2016). In the present study, we replicated this finding, added data from a 14-day withdrawal group, and examined whether CIE exposure alters monoamine inhibition of current-evoked spiking. As reported previously (Nimitvilai *et al*, 2016), CIE-exposed mice showed enhanced AP spiking as compared with air controls (Figure 5a; two-way ANOVA: main effect of CIE, $F_{(4, 364)} = 31.77$, $p < 0.0001$). The *post hoc* comparisons revealed significant differences in spiking for 3-day ($q = 8.338$, $p < 0.0001$; $N = 17$) and 7-day ($q = 7.189$, $p < 0.0001$; $N = 15$) withdrawal (WD) groups. At 14 days after withdrawal, spiking from CIE-treated mice was not different from air controls ($q = 0.771$, $p > 0.05$; $N = 9$).

We then tested whether CIE exposure altered the inhibition of firing by direct activation of GIRK channels. Shown in Figure 5b are the number of spikes at the highest current injection step in the presence of ML297 that are normalized to control baseline (without drug). The GIRK activator ML297 (10 μ M) significantly decreased spiking in neurons from air-exposed mice (two-tail paired *t*-test, $t(16) = 7.086$, $p < 0.0001$), but had no effect on firing in IOFC neurons from the 3D-WD CIE group (two-tail paired *t*-test, $t(10) = 0.1316$, $p = 0.8979$). In the 7D-WD group, inhibition of spiking by ML297 was significantly different from the control ($n = 18$, two-tail paired *t*-test, $t(17) = 3.226$, $p = 0.005$), but the level of inhibition was still less than that seen in neurons from air-exposed animals. By 14 days of withdrawal, ML297 inhibition of spiking was similar to that of air controls (two-tail paired *t*-test, $t(7) = 4.459$, $p = 0.0029$). Multiple GIRK channel subtypes have been identified, and in

the brain, GIRK1, GIRK2, and GIRK3 subunits are moderately or highly expressed whereas that of GIRK4 is low or undetectable (Chan *et al*, 1997; Karschin *et al*, 1996; Kobayashi *et al*, 1995). The expressions of G_i and G_o proteins that link GPCRs to GIRKs are also highly expressed in brain including the frontal cortex (Cruz *et al*, 2008; Goodfellow *et al*, 2009, 2014; Saenz del Burgo *et al*, 2008). To determine whether CIE exposure alters the expression of these proteins, western blotting was performed using antibodies against $G_{i/o}$ protein subunits and GIRK channels including GIRK1 that is the target of ML297 (Days *et al*, 2010). At the 3-day WD time where ML297 had no effect on firing, air- and CIE-treated mice expressed similar amounts of $G_{i/o}$ proteins (two-tailed *t*-test, $t(9) = 0.3727$, $p = 0.718$), GIRK1 (two-tailed *t*-test, $t(10) = 0.9863$, $p = 0.3473$), and GIRK2 (two-tailed *t*-test, $t(18) = 0.9218$, $p = 0.3688$) channel subunits (Figure 5c).

Although CIE exposure blunted the inhibitory effect of the direct channel activator ML297 on OFC neuron firing, no changes in expression of $G_{i/o}$ or GIRK channel subunits were observed, suggesting that receptor-mediated effects on excitability might be unaffected. Thus, in an additional set of air- and CIE-treated mice, we tested the effect of DA, NE and 5HT on current-evoked spiking of IOFC neurons. As previously observed (Figures 1 and 2), AP spiking was significantly reduced by DA (Figure 5d; two-tail paired *t*-test, $t(4) = 6.236$, $p = 0.0034$), NE (Figure 5e; two-tail paired *t*-test, $t(6) = 5.296$, $p = 0.0018$), or 5HT (Figure 5f; two-tail paired *t*-test, $t(5) = 2.626$, $p = 0.0467$) in neurons from air control groups. However, DA, NE, and 5HT inhibition of evoked firing was completely abolished in neurons from CIE-treated mice (Figures 5d–f, respectively). Unlike that found for ML297, the loss of monoamine inhibition of excitability persisted for up to 7 days following withdrawal from CIE exposure.

DISCUSSION

We reported previously that ethanol, applied acutely, reduces the intrinsic excitability of IOFC neurons (Badanich *et al*, 2013), and that firing is enhanced in alcohol-dependent mice (Nimitvilai *et al*, 2016). The results of the present study demonstrate that the enhanced excitability of IOFC neurons in CIE-treated mice is accompanied by a loss of monoamine inhibition of firing normally observed in control animals. Specifically, DA, NE, and 5HT each decreased current-evoked firing of IOFC neurons from control animals and results from pharmacological studies suggest that this action was mediated primarily via $G_{i\alpha}$ -coupled D2, α_2 , and 5HT_{1A} receptors, respectively. These receptors all stimulate inhibitory GIRK channels consistent with the findings showing that the GIRK channel blocker barium occluded monoamine-induced inhibition of firing and that ML297, a direct GIRK channel activator, itself reduced firing. Overall, these results suggest that monoamines likely play a key role in modulating the intrinsic excitability of IOFC neurons and that loss of this action following chronic ethanol exposure may contribute to the impairment of OFC-dependent behaviors observed in alcohol-dependent individuals.

In this study, IOFC neurons from CIE mice were resistant to monoamine or ML297-induced inhibition of firing and this occurred in the absence of obvious changes in GIRK1,

GIRK2, or $G_{i/o}$ subunit expression. Although we cannot rule out that there may have been local or cell-type-specific changes in these proteins that may have been missed in the tissue punch samples, these findings suggest that CIE may induce changes in monoamine receptors or in processes that couple these receptors to their GIRK and G-protein counterparts. Although possible, it seems unlikely that CIE could cause identical changes in the expression of specific G_i -linked monoamine receptors among three different neurotransmitter families, suggesting that downstream events common to each of these receptors may be a more likely site of action. GIRK channels (Huang *et al*, 1998; Xiao *et al*, 2003) and some members of the transient receptor potential family (Huang *et al*, 1998; Xiao *et al*, 2003) require the membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP2) for activation or regulation. Acute ethanol modulation of these channels has been shown to be sensitive to manipulations in PIP2 (Huang *et al*, 1998; Xiao *et al*, 2003) and following CIE, PIP2 activation of phospholipase C in NG108 neuroglia cells to ethanol is blunted (Katsura *et al*, 1994; Pandey, 1996). Similar findings have been reported in ethanol-dependent mice (Katsura *et al*, 1994; Pandey, 1996). Chronic ethanol exposure and withdrawal has also been shown to upregulate the expression of calmodulin (Katsura *et al*, 1994; Pandey, 1996), phospholipase C, and selective protein kinase C isozymes (Katsura *et al*, 1994; Pandey, 1996) in several brain regions and cultured neurons. Upregulation of these signaling molecules is linked to destabilization of GIRK-PIP2 interactions and PIP2 depletion on channel activity (Katsura *et al*, 1994; Pandey, 1996). Although not measured in this study, depletion or uncoupling of PIP2 from its downstream targets could account for the global loss of GIRK channel function in CIE-treated animals even though overall channel expression was not changed. Interestingly, the loss of monoamine inhibition in CIE-treated mice persisted for at least 1 week, whereas the ability of ML297 to reduce firing recovered by the 7-day WD period. This differential recovery of action suggests that the loss of monoamine inhibition of excitability following CIE exposure may involve multiple sites in the receptor/G-protein/GIRK channel cascade that links monoamines to control of firing. In addition, we reported previously that acute ethanol reduces firing of IOFC neurons via activation of ionotropic glycine receptors (Badanich *et al*, 2013) and that following CIE exposure, this effect is lost along with a blunting of inhibition by the glycine transport inhibitor sarcosine, reduced expression of the GlyT1 transporter, and a functional downregulation of small conductance (SK) potassium channels (Nimitvilai *et al*, 2016). When combined with the findings of the current study, these results demonstrate that CIE exposure and withdrawal induces time-dependent changes in multiple mediators of IOFC excitability that renders these neurons hyperexcitable. Restoring control over firing of OFC neurons in ethanol-dependent subjects thus likely requires the targeting of multiple neurotransmitter systems and effectors.

Whereas the effects of NE and 5HT on IOFC firing appeared to result from activation of GIRK channels, the actions of DA were more complex. Both DA and a specific D2 receptor agonist quinpirole decreased spike firing in IOFC neurons, and these effects were blocked by picrotoxin, suggesting the involvement of GABA_A receptors. Many

studies have reported on DA ability to modulate synaptic GABA-mediated inhibition in the PFC, with some reporting a D2-mediated decrease in GABA release (Retaux *et al*, 1991) or evoked GABAergic synaptic response (Penit-Soria *et al*, 1989) and others showing increases in extracellular GABA (Grobin and Deutch, 1998) or GABAergic sIPSCs (Penit-Soria *et al*, 1987; Zhou and Hablitz, 1999) following exposure to a D2 agonist. In the adult but not juvenile rat PFC, activation of D2 receptors enhances the firing of fast-spiking interneurons that inhibit pyramidal neuron excitability (Gorelova *et al*, 2002; Williams and Goldman-Rakic, 1995) and D1-mediated activation of GABAergic interneurons results in attenuation of spontaneous PFC neuronal firing (Gorelova *et al*, 2002; Williams and Goldman-Rakic, 1995). DA also has effects on extrasynaptic GABA_A receptors that generate a tonic inhibitory current in various brain regions. For example, in nucleus accumbens medium spiny neurons (MSNs), activation of D1 receptors increases tonic current, whereas acute or prolonged activation of D2 receptors has no effect or decreases tonic current, respectively (Maguire *et al*, 2014). In the dorsal striatum, the effect of DA on tonic current is age dependent, mediated mainly by the $\alpha 5$ GABA subunit in D2-MSNs in immature mice and by the δ -subunit in D1-MSNs in mature animals (Ade *et al*, 2008; Janssen *et al*, 2009; Santhakumar *et al*, 2010). In the IOFC of adult rats, D1 receptor activation increases the amplitude of NMDA EPSCs and D2 receptor agonists decrease NMDA EPSCs (Thompson *et al*, 2016). In contrast, Wallace *et al* (2014) reported that DA had no effect on mono- or polysynaptic responses of electrically evoked field responses recorded from slices containing caudal areas of the OFC. In this study, we found that DA or the D1 agonist SKF81297 had no effect on sIPSCs but increased a tonic current that was suppressed by picrotoxin. In contrast, the D2 agonist quinpirole increased sIPSC amplitude but had no effect on GABA-mediated tonic inhibition. Although both D1- and D2-mediated events would be expected to reduce excitability, blocking D1 receptors did not prevent DA inhibition of current-evoked spiking, whereas picrotoxin prevented inhibition of firing by either DA or the D2 agonist quinpirole. D2 activation of fast-spiking interneurons in the IOFC, similar to that found in adult rat mPFC (Gorelova *et al*, 2002; Williams and Goldman-Rakic, 1995), could explain these results, although the effects of DA on spiking were also blocked by barium suggesting a direct postsynaptic GIRK-mediated mechanism. In addition, unlike NE or 5HT, DA induced an additional inhibition of firing in the presence of the direct GIRK channel activator ML297 or the G-protein blocker GDP- β -S, again suggesting multiple mechanisms for DA modulation of OFC firing. How these actions influence monoamine regulation of OFC activity *in vivo* is not yet clear, although results from studies using indirect monoamine agonists suggest that both increases and decreases can occur. For example, OFC spiking measured *in vivo* was mainly enhanced following experimenter administered amphetamine (Homayoun and Moghaddam, 2008), whereas bidirectional changes in OFC spiking were observed in rats that received cocaine noncontingently or via self-administration or during presentation of cocaine-predictive cues (Guillen *et al*, 2010). These results suggest that both local and circuit-based mechanisms ultimately determine the

net result of monoamine receptor activation on OFC neuron firing.

We note that in the present study, micromolar concentrations of DA, NE, and 5HT were required to alter firing of IOFC neurons, similar to findings reported for 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) and for the OFC (Wallace et al, 2014). However, extracellular concentrations of monoamines in the PFC are estimated to be in the nanomolar range (Mallo et al, 2008; Moghaddam et al, 1990), calling into question the physiological relevance of the high concentrations used in brain slice studies. This apparent discrepancy may reflect active uptake of monoamines, and/or the slow penetration of these substances into the relatively thick (~300 μ m) slices used in these experiments. Although both NE transporters and serotonin transporters appear to be highly expressed in the PFC (Morón et al, 2002), expression of the DA transporters is minimal (Freed et al, 1995; Sesack et al, 1998). Nevertheless, DA in the PFC can be broken down by enzymatic means (catechol-O-methyltransferase) and DA is actively taken up by the other monoamine transporters, especially that for NE (Di Chiara et al, 1992; Morón et al, 2002; Mundorf et al, 2001; Pani et al, 1990; Yamamoto and Novotney, 1998). In the present study, dopamine applied in the presence of a concentration of the nonspecific monoamine transporter inhibitor predicted to block all monoamine transporters (Tuomisto, 1977) significantly decreased IOFC neuronal firing at concentrations as low as 1–100 nM. These findings suggest that active transport regulates extracellular levels of monoamines even in acute slices of the OFC. As there is evidence for an increase in monoamine uptake rate following CIE (Di Chiara et al, 1992; Yamamoto and Novotney, 1998) and inhibition of monoamine transporter attenuates excessive drinking and impulsivity in high-alcohol-preferring animals (Di Chiara et al, 1992; Yamamoto and Novotney, 1998), it will be important to determine whether CIE also affects monoamine transporter activity in the OFC.

Imbalance or dysfunction of monoamine neurotransmission has been linked to functional abnormalities in the OFC and deficits in behavioral flexibility that require an intact OFC (Pardey et al, 2013; Wischhof et al, 2011; Zeeb et al, 2010). For example, low levels of serotonin within the OFC impaired performance on a visual discrimination reversal task in marmosets and rodents (Clarke et al, 2004; Masaki et al, 2006), and caused disruption on a detour reaching task in monkeys (Walker et al, 2006). In addition, animals receiving systemic administration of haloperidol, a potent DA receptor blocker with higher affinity for D2-like receptors, or the D2/D3 receptor antagonist raclopride, require more trials and produce more errors in order to reach the performance criterion in the reversal (Ridley et al, 1981; Lee et al, 2007). Reductions in D2 receptors have also been associated in alcoholism and other substance abuse disorders (Noble, 2000). We reported previously that ethanol-dependent mice exhibit deficits in reversal learning (Badanich et al, 2011) and drink more alcohol compared with control animals (den Hartog et al, 2016). The present study showed that CIE induced a loss of D2, 5HT_{1A}, and α 2 receptor modulation of intrinsic excitability, suggesting that a loss of monoamine receptor activity within the OFC would

be expected to adversely impact the organization of neural networks and cognitive functions mediated by each monoamine, and thus may play a role in behavioral inflexibility and cognitive deficits associated with chronic alcohol exposure.

In conclusion, the results of the present study suggest that monoamines may play a key role in regulating the intrinsic excitability of IOFC neurons, through modulation of GABA-mediated events and activation of G_{iα}-coupled GIRK channels (summarized in a model; Supplementary Figure S3). Maintaining optimal levels of monoamine signaling is likely critical for support of cognitive functions that require an intact OFC and loss of these regulatory processes may contribute to the pathology of various neuropsychiatric disorders including schizophrenia, obsessive-compulsive disorder, as well as alcohol and substance abuse disorders. The profound loss of DA-, NE-, and 5HT-mediated control of IOFC excitability following CIE exposure also suggests that treatments focused on specific receptor subtypes may not be effective in restoring the balance of monoamine signaling in alcohol-dependent individuals especially during the early stages of abstinence.

FUNDING AND DISCLOSURE

This work was supported by P50AA010761 and R37AA009986 to JJW; UO1 AA020929 to MFL; and U01AA020930 to PJM. The authors declare no conflict of interest.

REFERENCES

- Ade KK, Janssen MJ, Ortinski PI, Vicini S (2008). Differential tonic GABA conductances in striatal medium spiny neurons. *J Neurosci* 28: 1185–1197.
- Agster KL, Mejias-Aponte CA, Clark BD, Waterhouse BD (2013). Evidence for a regional specificity in the density and distribution of noradrenergic varicosities in rat cortex. *J Comp Neurol* 521: 2195–2207.
- Badanich K, Mulholland P, Beckley J, Trantham-Davidson H, Woodward J (2013). Ethanol reduces neuronal excitability of lateral orbitofrontal cortex neurons via a glycine receptor dependent mechanism. *Neuropsychopharmacology* 38: 1176–1188.
- Badanich KA, Becker HC, Woodward JJ (2011). Effects of chronic intermittent ethanol exposure on orbitofrontal and medial prefrontal cortex-dependent behaviors in mice. *Behav Neurosci* 125: 879–891.
- Bissonette GB, Martins GJ, Franz TM, Harper ES, Schoenbaum G, Powell EM (2008). Double dissociation of the effects of medial and orbital prefrontal cortical lesions on attentional and affective shifts in mice. *J Neurosci* 28: 11124–11130.
- Brown KL, Calizo LH, Goodlett CR, Stanton ME (2007). Neonatal alcohol exposure impairs acquisition of eyeblink conditioned responses during discrimination learning and reversal in weanling rats. *Dev Psychobiol* 49: 243–257.
- Chan KW, Sui JL, Vivaudou M, Logothetis DE (1997). Specific regions of heteromeric subunits involved in enhancement of G protein-gated K⁺ channel activity. *J Biol Chem* 272: 6548–6555.
- Clarke HF, Dalley JW, Crofts HS, Robbins TW, Roberts AC (2004). Cognitive inflexibility after prefrontal serotonin depletion. *Science* 304: 878–880.
- Clarke HF, Walker SC, Crofts HS, Dalley JW, Robbins TW, Roberts AC (2005). Prefrontal serotonin depletion affects reversal learning but not attentional set shifting. *J Neurosci* 25: 532–538.

- Cruz HG, Berton F, Sollini M, Blanchet C, Pravetoni M, Wickman K et al (2008). Absence and rescue of morphine withdrawal in GIRK/Kir3 knock-out mice. *J Neurosci* **28**: 4069–4077.
- Dascal N (1997). Signalling via the G protein-activated K⁺ channels. *Cell Signal* **9**: 551–573.
- Days E, Kaufmann K, Romaine I, Niswender C, Lewis M, Utley T et al (2010). Discovery and characterization of a selective activator of the G-protein activated inward-rectifying potassium (GIRK) channel. *Probe Reports from the NIH Molecular Libraries Program*, Bethesda, MD, USA.
- den Hartog C, Zamudio-Bulcock P, Nimitvilai S, Gilstrap M, Eaton B, Fedarovich H et al (2016). Inactivation of the lateral orbitofrontal cortex increases drinking in ethanol-dependent but not non-dependent mice. *Neuropsychopharmacology* **107**: 451–459.
- Di Chiara G, Tanda GL, Frau R, Carboni E (1992). Heterologous monoamine reuptake: lack of transmitter specificity of neuron-specific carriers. *Neurochem Int* **20**: 231S–235S.
- Di Pietro NC, Seamans JK (2011). Dopamine and serotonin interactively modulate prefrontal cortex neurons in vitro. *Biol Psychiatry* **69**: 1204–1211.
- Fortier CB, Steffen EM, Lafleche G, Venne JR, Disterhoft JF, McGlinchey RE (2008). Delay discrimination and reversal eyeblink classical conditioning in abstinent chronic alcoholics. *Neuropsychology* **22**: 196–208.
- Freed C, Revay R, Vaughan RA, Kriek E, Grant S, Uhl GR et al (1995). Dopamine transporter immunoreactivity in rat brain. *J Comp Neurol* **359**: 340–349.
- Gonzalez-Islas C, Hablitz JJ (2001). Dopamine inhibition of evoked IPSCs in rat prefrontal cortex. *J Neurophysiol* **86**: 2911–2918.
- Goodfellow NM, Benekareddy M, Vaidya VA, Lambe EK (2009). Layer II/III of the prefrontal cortex: Inhibition by the serotonin 5-HT_{1A} receptor in development and stress. *J Neurosci* **29**: 10094–10103.
- Goodfellow NM, Sargin D, Ansorge MS, Gingrich JA, Lambe EK (2014). Mice with compromised 5-HTT function lack phosphotyrosine-mediated inhibitory control over prefrontal 5-HT responses. *J Neurosci* **34**: 6107–6111.
- Gorelova N, Seamans JK, Yang CR (2002). Mechanisms of dopamine activation of fast-spiking interneurons that exert inhibition in rat prefrontal cortex. *J Neurophysiol* **88**: 3150–3166.
- Grobin AC, Deutch AY (1998). Dopaminergic regulation of extracellular gamma-aminobutyric acid levels in the prefrontal cortex of the rat. *J Pharmacol Exp Ther* **285**: 350–357.
- Guillen K, Kravitz AV, Moorman DE, Peoples LL (2010). Orbitofrontal and insular cortex: neural responses to cocaine-associated cues and cocaine self-administration. *Synapse* **64**: 1–13.
- Hille B (1992). G protein-coupled mechanisms and nervous signaling. *Neuron* **9**: 187–195.
- Hoerbel P, Lindsley TA, Fleck MW (2015). Dopamine directly modulates GABA_A receptors. *J Neurosci* **35**: 3525–3536.
- Homayoun H, Moghaddam B (2008). Orbitofrontal cortex neurons as a common target for classic and glutamatergic antipsychotic drugs. *Proc Natl Acad Sci USA* **105**: 18041–18046.
- Huang CL, Feng S, Hilgemann DW (1998). Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gbetagamma. *Nature* **391**: 803–806.
- Hurst RS, Latorre R, Toro L, Stefani E (1995). External barium block of Shaker potassium channels: evidence for two binding sites. *J Gen Physiol* **106**: 1069–1087.
- Janssen MJ, Ade KK, Fu Z, Vicini S (2009). Dopamine modulation of GABA tonic conductance in striatal output neurons. *J Neurosci* **29**: 5116–5126.
- Jedema HP, Carter MD, Dugan BP, Gurnsey K, Olsen AS, Bradberry CW (2011). The acute impact of ethanol on cognitive performance in rhesus macaques. *Cereb Cortex* **21**: 1783–1791.
- Karschin C, Dissmann E, Stuhmer W, Karschin A (1996). IRK(1–3) and GIRK(1–4) inwardly rectifying K⁺ channel mRNAs are differentially expressed in the adult rat brain. *J Neurosci* **16**: 3559–3570.
- Katsura M, Ohkuma S, Chen DZ, Kuriyama K (1994). Ethanol-induced alteration in activities of cerebral phosphatidylinositol 4,5-bisphosphate-specific and cytosolic phospholipase C in the brain: analysis using NG 108-15 cells and brains from ethanol-inhaled mice. *Neurochem Int* **24**: 541–547.
- Kheramin S, Body S, Ho MY, Velazquez-Martinez DN, Bradshaw CM, Szabadi E et al (2004). Effects of orbital prefrontal cortex dopamine depletion on inter-temporal choice: a quantitative analysis. *Psychopharmacology (Berl)* **175**: 206–214.
- Kirby LG, Zeeb FD, Winstanley CA (2011). Contributions of serotonin in addiction vulnerability. *Neuropharmacology* **61**: 421–432.
- Kobayashi T, Ikeda K, Ichikawa T, Abe S, Togashi S, Kumanishi T (1995). Molecular cloning of a mouse G-protein-activated K⁺ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, 2 and 3) mRNAs in mouse brain. *Biochem Biophys Res Commun* **208**: 1166–1173.
- Lee B, Groman S, London ED, Jentsch JD (2007). Dopamine D₂/D₃ receptors play a specific role in the reversal of a learned visual discrimination in monkeys. *Neuropsychopharmacology* **32**: 2125–2134.
- Maguire EP, Macpherson T, Swinny JD, Dixon CI, Herd MB, Belelli D et al (2014). Tonic inhibition of accumbal spiny neurons by extrasynaptic alpha4betadelta GABA_A receptors modulates the actions of psychostimulants. *J Neurosci* **34**: 823–838.
- Mallo T, Koiv K, Koppel I, Raudkivi K, Uustare A, Rinken A et al (2008). Regulation of extracellular serotonin levels and brain-derived neurotrophic factor in rats with high and low exploratory activity. *Brain Res* **1194**: 110–117.
- Masaki D, Yokohama C, Kinoshita S, Tsuchida H, Nakatomi Y, Yoshimoto K et al (2006). Relationship between limbic and cortical 5-HT neurotransmission and acquisition and reversal learning in a go/no-go task in rats. *Psychopharmacology (Berl)* **189**: 249–258.
- McAlonan K, Brown VJ (2003). Orbital prefrontal cortex mediates reversal learning and not attentional set shifting in the rat. *Behav Brain Res* **146**: 97–103.
- McDaid J, McElvain MA, Brodie MS (2008). Ethanol effects on dopaminergic ventral tegmental area neurons during block of Ih: involvement of barium-sensitive potassium currents. *J Neurophysiol* **100**: 1202–1210.
- McGuier N, Padula A, Lopez M, Woodward J, Mulholland P (2015). Withdrawal from chronic intermittent alcohol exposure increases dendritic spine density in the lateral orbitofrontal cortex in mice. *Alcohol* **49**: 21–27.
- Moghaddam B, Roth RH, Bunney BS (1990). Characterization of dopamine release in the rat medial prefrontal cortex as assessed by in vivo microdialysis: comparison to the striatum. *Neuroscience* **36**: 669–676.
- Morón JA, Brockington A, Wise RA, Rocha BA, Hope BT (2002). Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. *J Neurosci* **22**: 389–395.
- Mundorf ML, Joseph JD, Austin CM, Caron MG, Wightman RM (2001). Catecholamine release and uptake in the mouse prefrontal cortex. *J Neurochem* **79**: 130–142.
- Narendran R, Mason NS, Paris J, Himes ML, Douaihy AB, Frankle WG (2014). Decreased prefrontal cortical dopamine transmission in alcoholism. *Am J Psychiatry* **171**: 881–888.
- Nimitvilai S, Arora DS, Brodie MS (2012). Reversal of dopamine inhibition of dopaminergic neurons of the ventral tegmental area is mediated by protein kinase C. *Neuropsychopharmacology* **37**: 543–556.
- Nimitvilai S, Lopez M, Mulholland P, Woodward J (2016). Chronic intermittent ethanol exposure enhances the excitability and synaptic plasticity of lateral orbitofrontal cortex neurons and induces a tolerance to the acute inhibitory actions of ethanol. *Neuropsychopharmacology* **41**: 1112–1127.

- Noble EP (2000). The DRD2 gene in psychiatric and neurological disorders and its phenotypes. *Pharmacogenomics* **1**: 309–333.
- Pandey SC (1996). Acute and chronic ethanol consumption effects on the immunolabeling of Gq/11 alpha subunit protein and phospholipase C isozymes in the rat brain. *J Neurochem* **67**: 2355–2361.
- Pani L, Carboni S, Kusmin A, Gessa G, Rossetti Z (1990). Nimodipine inhibits cocaine-induced dopamine release and motor stimulation. *Eur J Pharmacol* **176**: 245–246.
- Pardey MC, Kumar NN, Goodchild AK, Cornish JL (2013). Catecholamine receptors differentially mediate impulsive choice in the medial prefrontal and orbitofrontal cortex. *J Psychopharmacol* **27**: 203–212.
- Penit-Soria J, Audinat E, Crepel F (1987). Excitation of rat prefrontal cortical neurons by dopamine: an *in vitro* electrophysiological study. *Brain Res* **425**: 263–274.
- Penit-Soria J, Retaux S, Maurin Y (1989). [Effects of the stimulation of D1 and D2 dopaminergic receptors on the electrically induced release of gamma-(3H)-aminobutyric acid in the prefrontal cortex of the rat]. *C R Acad Sci III* **309**: 441–446.
- Rainnie DG (1999). Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *J Neurophysiol* **82**: 69–85.
- Retaux S, Besson MJ, Penit-Soria J (1991). Opposing effects of dopamine D2 receptor stimulation on the spontaneous and the electrically evoked release of [3H]GABA on rat prefrontal cortex slices. *Neuroscience* **42**: 61–71.
- Ridley RM, Haystead TA, Baker HF (1981). An analysis of visual object reversal learning in the marmoset after amphetamine and haloperidol. *Pharmacol Biochem Behav* **14**: 345–351.
- Robbins TW, Arnsten AF (2009). The neuropsychopharmacology of fronto-executive function: monoaminergic modulation. *Annu Rev Neurosci* **32**: 267–287.
- Roesch MR, Olson CR (2007). Neuronal activity related to anticipated reward in frontal cortex: does it represent value or reflect motivation? *Ann N Y Acad Sci* **1121**: 431–446.
- Rothman RB, Blough BE, Baumann MH (2008). Dual dopamine/serotonin releasers: potential treatment agents for stimulant addiction. *Exp Clin Psychopharmacol* **16**: 458–474.
- Saenz del Burgo L, Cortes R, Mengod G, Zarate J, Echevarria E, Salles J (2008). Distribution and neurochemical characterization of neurons expressing GIRK channels in the rat brain. *J Comp Neurol* **510**: 581–606.
- Santhakumar V, Jones RT, Mody I (2010). Developmental regulation and neuroprotective effects of striatal tonic GABAA currents. *Neuroscience* **167**: 644–655.
- Schoenbaum G, Chiba AA, Gallagher M (1998). Orbitofrontal cortex and basolateral amygdala encode expected outcomes during learning. *Nat Neurosci* **1**: 155–159.
- Sesack SR, Hawrylak VA, Guido MA, Levey AI (1998). Cellular and subcellular localization of the dopamine transporter in rat cortex. *Adv Pharmacol* **42**: 171–174.
- Thompson JL, Yang J, Lau B, Liu S, Baimel C, Kerr LE et al (2016). Age-dependent D1-D2 receptor coactivation in the lateral orbitofrontal cortex potentiates NMDA receptors and facilitates cognitive flexibility. *Cereb Cortex* **26**: 4524–4539.
- Trantham-Davidson H, Burnett EJ, Gass JT, Lopez MF, Mulholland PJ, Centanni SW et al (2014). Chronic alcohol disrupts dopamine receptor activity and the cognitive function of the medial prefrontal cortex. *J Neurosci* **34**: 3706–3718.
- Tremblay L, Schultz W (1999). Relative reward preference in primate orbitofrontal cortex. *Nature* **398**: 704–708.
- Tuomisto J (1977). Nomifensine and its derivatives as possible tools for studying amine uptake. *Eur J Pharmacol* **42**: 101–106.
- van Welie I, Wadman WJ, van Hoof JA (2005). Low affinity block of native and cloned hyperpolarization-activated Ih channels by Ba²⁺ ions. *Eur J Pharmacol* **507**: 15–20.
- Verdejo-Garcia A, Bechara A, Recknor EC, Perez-Garcia M (2006). Executive dysfunction in substance dependent individuals during drug use and abstinence: an examination of the behavioral, cognitive and emotional correlates of addiction. *J Int Neuropsychol Soc* **12**: 405–415.
- Volkow ND, Chang L, Wang GJ, Fowler JS, Ding YS, Sedler M et al (2001). Low level of brain dopamine D2 receptors in methamphetamine abusers: association with metabolism in the orbitofrontal cortex. *Am J Psychiatry* **158**: 2015–2021.
- Volkow ND, Fowler JS, Wang GJ, Ding YS, Gatley SJ (2002). Role of dopamine in the therapeutic and reinforcing effects of methylphenidate in humans: results from imaging studies. *Eur Neuropsychopharmacol* **12**: 557–566.
- Walker SC, Mikheenko YP, Argyle LD, Robbins TW, Roberts AC (2006). Selective prefrontal serotonin depletion impairs acquisition of a detour-reaching task. *Eur J Neurosci* **23**: 3119–3123.
- Wallace J, Jackson RK, Shotton TL, Munjal I, McQuade R, Gartside SE (2014). Characterization of electrically evoked field potentials in the medial prefrontal cortex and orbitofrontal cortex of the rat: modulation by monoamines. *Eur Neuropsychopharmacol* **24**: 321–332.
- Wang DS, Mangin JM, Moonen G, Rigo JM, Legendre P (2006). Mechanisms for picrotoxin block of alpha2 homomeric glycine receptors. *J Biol Chem* **281**: 3841–3855.
- Williams GV, Goldman-Rakic PS (1995). Modulation of memory fields by dopamine D1 receptors in prefrontal cortex. *Nature* **376**: 572–575.
- Winstanley CA, Theobald DE, Dalley JW, Cardinal RN, Robbins TW (2006). Double dissociation between serotonergic and dopaminergic modulation of medial prefrontal and orbitofrontal cortex during a test of impulsive choice. *Cereb Cortex* **16**: 106–114.
- Wischhof L, Hollenstein KJ, Koch M (2011). Impulsive behaviour in rats induced by intracortical DOI infusions is antagonized by co-administration of an mGlu2/3 receptor agonist. *Behav Pharmacol* **22**: 805–813.
- Xiao J, Zhen XG, Yang J (2003). Localization of PIP2 activation gate in inward rectifier K⁺ channels. *Nat Neurosci* **6**: 811–818.
- Yamamoto BK, Novotney S (1998). Regulation of extracellular dopamine by the norepinephrine transporter. *J Neurochem* **71**: 274–280.
- Zeeb FD, Floresco SB, Winstanley CA (2010). Contributions of the orbitofrontal cortex to impulsive choice: interactions with basal levels of impulsivity, dopamine signalling, and reward-related cues. *Psychopharmacology (Berl)* **211**: 87–98.
- Zhou FM, Hablitz JJ (1999). Dopamine modulation of membrane and synaptic properties of interneurons in rat cerebral cortex. *J Neurophysiol* **81**: 967–976.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)