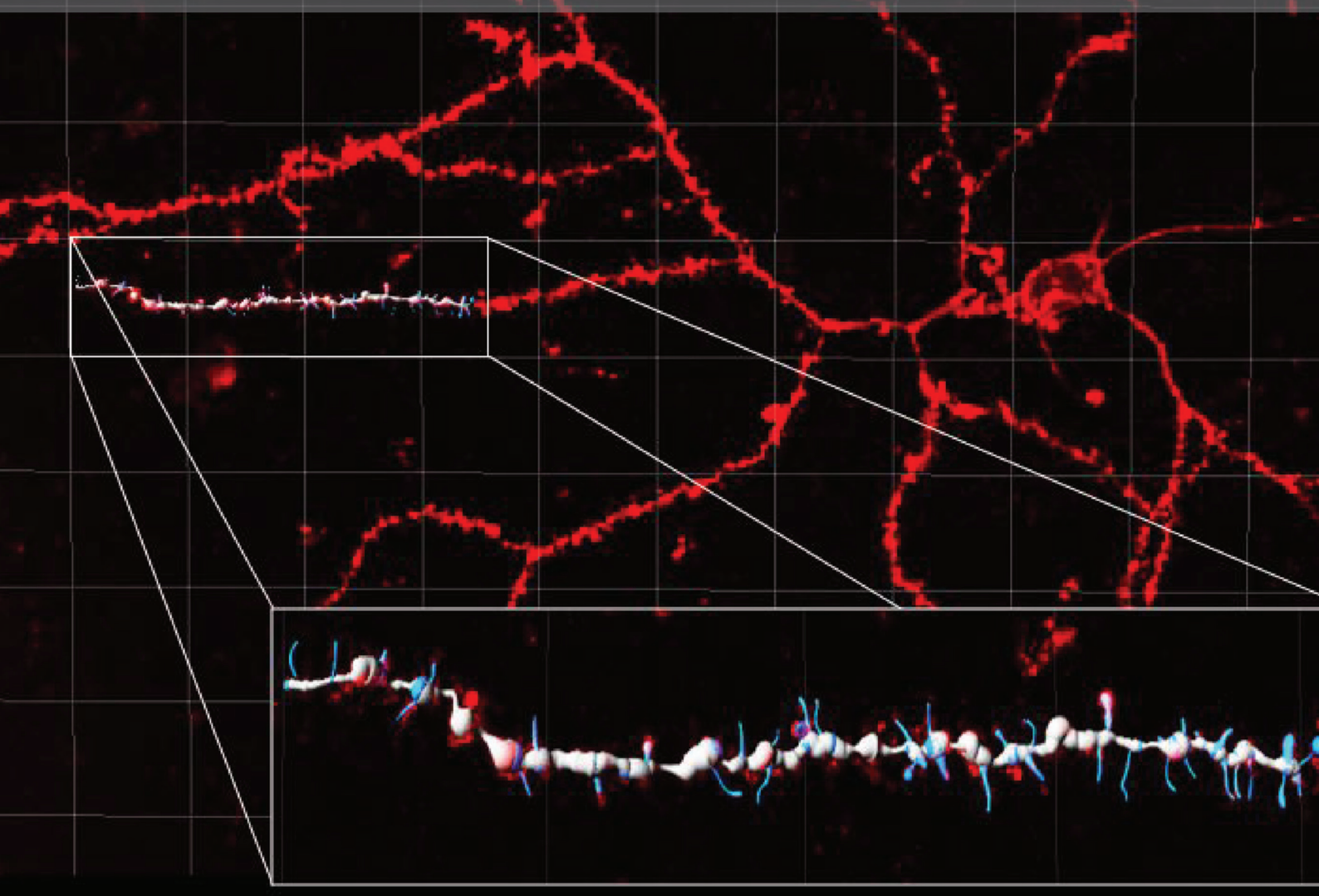
NEURONAL AND GLIAL STRUCTURAL PLASTICITY INDUCED BY DRUGS OF ABUSE

EDITED BY: M. Foster Olive and Justin Gass
PUBLISHED IN: Frontiers in Pharmacology



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ISSN 1664-8714
ISBN 978-2-88919-598-5
DOI 10.3389/978-2-88919-598-5

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NEURONAL AND GLIAL STRUCTURAL PLASTICITY INDUCED BY DRUGS OF ABUSE

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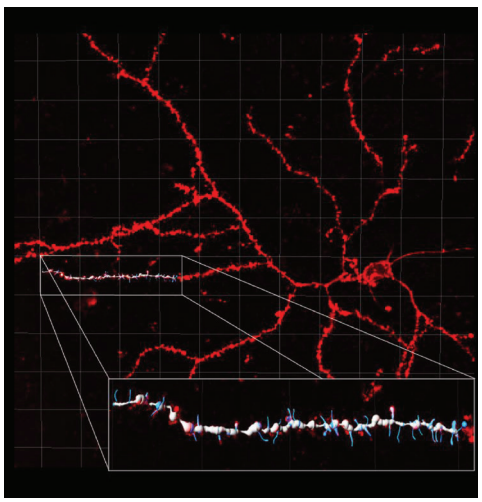

Cover photo: 3-D reconstruction of a DiI-labeled neuron in the neocortex. Boxed inset shows a selected dendritic segment for computer-assisted analysis of dendritic spine density and morphology.
Image by Amber LaCrosse, Sara B. Taylor, and M. Foster Olive, Department of Psychology, Arizona State University

Drugs of abuse induce a host of alterations in brain structure and function, ranging from changes in gene expression and epigenetic processes to aberrant synaptic plasticity to volumetric changes in discrete brain regions. These alterations can be drug class-specific, and are not confined to neurons, as drugs of abuse also induce molecular and cellular alterations in various glial cell types such as astrocytes and microglia. The phenomenon of drug-induced plasticity includes changes in dendritic branching and architecture, dendritic spine density and morphology, astrocyte-neuronal interactions, dysregulation of glutamatergic and GABAergic signaling, and alterations in myelination or microglial phenotype. This drug-induced "rewiring" of the brain at numerous levels can contribute to the development, maintenance, and persistence of the addicted state, as well as associated deficits in normal cognitive functioning. The aim of this Research Topic is to collect recent and important findings related to the structural alterations produced by drug of abuse in neurons, glial, and other cell types of the central nervous system.

Citation: Olive, M. F., Gass, J., eds. (2015). Neuronal and Glial Structural Plasticity Induced by Drugs of Abuse. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-598-5

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frontiers in Pharmacology

EDITORIAL
published: 15 May 2015
doi: 10.3389/fphar.2015.00088

Editorial: structural plasticity induced by drugs of abuse

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Keywords: addiction, plasticity, glutamate, neurotrophin, GABA, homer, oligodendrocyte, cytoskeleton

The roots of addiction are often attributed to the ability of repeated drug use to compromise proper functioning of the central nervous system. Drug-induced functional changes in the brain occur on many levels, including altered expression of specific genes via genomic and epigenetic mechanisms, induction of synaptic plasticity and other cellular adaptations, and volumetric changes in discrete brain regions (Luscher and Malenka, 2011). These alterations can be drug class-specific and occur in both neuronal and non-neuronal cell populations. Drug-induced plasticity, or "rewiring" of the brain contributes to the development, maintenance, and persistence of the addicted state (Kalivas and O'Brien, 2008). The goal of this Research Topic is to assimilate recent findings related to plasticity and structural alterations produced by drug of abuse in neurons, glia, and other cell types of the brain.

The mesolimbic dopamine system, originating in the ventral tegmental area (VTA) of the midbrain and projecting rostrally to the nucleus accumbens (NAc) and prefrontal cortex (PFC), mediates the acute reinforcing and incentive salience of abused drugs, as well as their aversive properties. As reviewed by Vashchinkina et al. (2014) mesolimbic dopamine neurons are highly regulated by intrinsic and extrinsic inhibitory GABAergic neurons. Vashchinkina et al. (2014) hypothesize that abused drugs induce structural and functional mesolimbic adaptations differentially via endogenous (e.g., THIP and neurosteroids) vs. exogenous (e.g., benzodiazepines and alcohol) modulators of $GABA_A$ receptors, as well as via synaptic vs. extrasynaptic $GABA_A$ receptors in the VTA. Collo et al. (2014) review evidence that drug-induced plasticity in mesolimbic dopaminergic neurons is mediated by common dopamine and brain-derived neurotrophic factor (BDNF) signaling pathways, specifically those recruiting MEK-ERK1/2, and PI3K-Akt-mTOR. Cadet and Bisagno (2014) review evidence for substantial plasticity in non-neuronal cell types in brain reward circuitry, including changes in astrocytic glutamate transporter expression, increases in pro-inflammatory cytokine production by microglia, and dysregulated oligodendrocytic myelin production. This latter topic is further elaborated on by Somkuwar et al. (2014) in their review of the proteoglycan neuron-glial antigen 2 (NG2), its expression by oligodendrocyte progenitor cells in the brain reward circuitry, and its interaction with stress-related neuromodulators. In addition, Bajo et al. (2015) provide evidence that interleukin-1β alters both basal and ethanol-facilitated GABAergic transmission in the central nucleus of the amygdala, part of the extended amygdala circuitry which is implicated in ethanol's central effects.

Drugs of abuse also alter the dynamics and microstructure of both dendrites and dendritic spines, which can be interpreted as cellular (mal)adaptations that reinforce the addiction cycle (Luscher and Malenka, 2011; Gipson et al., 2014). DePoy et al. (2014) show that repeated exposure of early adolescent mice to cocaine produces lasting reductions in orbitofrontal cortex dendritic arbor length and complexity and as well as impaired reversal learning. In addition, these investigators show that while mice carrying a heterozygous deletion of the actin cytoskeleton stabilizing protein p190RhoGAP

Edited and reviewed by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 March 2015*
Accepted: *10 April 2015*
Published: *15 May 2015*

Citation:
Olive MF and Gass JT (2015) Editorial: structural plasticity induced by drugs of abuse. Front. Pharmacol. 6:88. doi: 10.3389/fphar.2015.00088

exhibit enhanced vulnerability to cocaine-induced hyperlocomotion, orbitofrontal dendritic complexity in cocaine-naïve mice is intact. These findings suggest that orbitofrontal dendrite structure is impacted by repeated cocaine during adolescence, but pre-existing structural dendritic deficiencies do not account for increased behavioral sensitivity to this drug.

Glutamate is the predominant excitatory amino acid in the central nervous system and mediates both normal and maladaptive cellular plasticity. Several articles in this Research Topic provide novel findings on the role of glutamate in addictive processes. Weiland et al. (2015) demonstrate that antibiotics with the glutamate and GABA modulating properties (ceftriaxone and cefazolin, respectively) attenuate cue-primed reinstatement of alcohol-seeking. Griffin et al. (2015) demonstrate that chronic intermittent ethanol exposure results in increased extracellular levels of glutamate in the NAc in ethanol-dependent mice, but these effects are not a result of locally dysregulated sodium-dependent and independent glutamate transporter function, suggesting that extrinsic corticostriatal glutamatergic pathways may contribute to this hyperglutamatergic state. Finally, McGuier et al. (2015) show that deletion of the excitatory postsynaptic scaffolding protein Homer2 is associated with an increased density of long, thin dendritic spines in NAc core medium spiny neurons, yet unexpectedly these structural modifications are not modified by repeated alcohol exposure.

Together, this body of work indicates complex interactions between drugs of abuse, endogenous neuromodulators and their signaling targets, and the mechanisms underlying the functional and structural plasticity in the brain. Research into this complexity is only in its infancy, and needs to be pursued at multiple levels in order to better understand and treat addictive disorders.

Acknowledgments

The authors wish to acknowledge the funding support of NIH grants AA020537, AA013852, DA024355, DA025606, and DA037741.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 02 September 2014
doi: 10.3389/fphar.2014.00204

Glial-neuronal ensembles: partners in drug addiction-associated synaptic plasticity

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Keywords: synaptic plasticity, drug abuse, microglia, astroglia, oligodendroglia, inflammation

INTRODUCTION

Drug addiction is manifested by a compulsive drive to take licit or illicit substances despite repeated severe adverse consequences (Volkow et al., 2012). Addiction is also accompanied by a vicious cycle of binges, abstinence, and relapses. Almost all drugs of abuse trigger euphoric feelings consequent to a rapid increase of dopamine levels in the mesolimbic system. Even after long periods of abstinence, addicts remain vulnerable to drug craving and/or relapses that can be triggered by stimuli previously associated with drugs (Koob and Volkow, 2010). These features of addiction suggest that drugs might cause a form of persistent neuroplasticity that is acutely responsive to environmental stimuli, with consequent compulsive drug-seeking and taking behaviors.

Neural functions require the coordinated interactions of multiple neuronal cell types and a diverse population of glial cells. The three major glial cell types in the brain, astrocytes, oligodendrocytes, and microglia, communicate with each other and with neurons by using neurotransmitters, other small molecules, and gap junctions (Araque et al., 2014). Oligodendrocytes increase the speed of electrical transmission through nerve axons by forming the axonal myelin sheath and clustering ion channels at nodes of Ranvier (Nave, 2010). Microglia prune synapses in part by monitoring synaptic transmission (Schafer et al., 2013; Wake et al., 2013). Astrocytes can regulate synaptic transmission between neurons by modifying the concentration of extracellular potassium, controlling local blood flow, by releasing and/or taking up neurotransmitters or neuromodulators, by delivering nutrients to neurons, and by altering the geometry and volume of the brain extracellular space (Araque et al., 2014).

This brief summary of glial functions suggests that these cells might play important roles in the long-term manifestations of substance use disorders, both in terms of addiction to these agents and their long-term neuropsychiatric consequences. In what follows, we discuss some recent findings that support the thesis that glial cells are part and parcel of the plastic mechanisms that are induced by drugs of abuse.

BRAIN INFLAMMATION TRIGGERED BY DRUGS OF ABUSE

Gliosis and inflammatory responses are significant pathological features of substance use disorders (Cadet et al., 2014a). Inflammation is a natural response to damage and/or infection that are mediated by pro-inflammatory cytokines including interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα) (Glass et al., 2010). In the brain, microglial cells are the main orchestrators of these neuroinflammatory responses (Jeong et al., 2013). However, other cells including astrocytes, endothelial cells, perivascular and meningeal macrophages, and even neurons, can also produce pro-inflammatory mediators (Van Wagoner et al., 1999; Jeong et al., 2013). These factors appear to mediate some detrimental effects of inflammation on neurogenesis (Sierra et al., 2014).

Brain inflammation is also associated with an increased production of reactive oxygen species (ROS) and nitric oxide (NO), followed by the propagation of free radicals that damage cells (Cadet and Brannock, 1998; Krasnova and Cadet, 2009). Indeed, various psychostimulants, including amphetamine and methamphetamine, can produce ROS in dopaminergic nerve terminal regions (Krasnova and Cadet, 2009; Shiba et al., 2011). Acute or repeated cocaine administration also generates ROS in dopaminergic rat brain structures (Dietrich et al., 2005). MDMA also produces reactive nitrogen species in the rat that contribute to its neurotoxicity (O'Shea et al., 2014).

DRUGS ABUSE-INDUCED ALTERATIONS IN GLIAL CELLS

Astroglia

Astrocytes play diverse roles in the regulation of synaptic transmission. They clear synaptic transmitters from the cleft through the activity of transporters and can recycle glutamate through a glutamine intermediate to the synaptic terminal (Haydon et al., 2009). Astrocytes can also release glutamate (D'Ascenzo et al., 2007; Jourdain et al., 2007), the NMDA receptor co-agonist D-serine (Mothet et al., 2005), and ATP (Cotrina et al., 2000). Hydrolysis of ATP to adenosine is responsible for an adenosine 1 (A1) receptor-mediated presynaptic inhibition of excitatory synaptic transmission (Volterra and Meldolesi, 2005).

GFAP-positive glial cells in the mPFC contain cystine/glutamate antiporters (Pow, 2001) that maintain extracellular non-synaptic glutamate levels and provide functional support to neurons by

regulating extracellular potassium and the reuptake of glutamate at synapses (Wigley et al., 2007). Interestingly, non-synaptic glutamate derived from cystine/glutamate antiporters has been reported to modulate synaptic glutamate release and to regulate cocaine-induced drug seeking in rats (Moran et al., 2005). Moreover, down-regulation of the cystine/glutamate exchanger was reported to account for chronic cocaine-induced reduction in basal glutamate levels (Baker et al., 2003). Furthermore, the sodium-dependent glutamate uptake and the membrane level of the primary glial glutamate transporter (GLT1) were recently reported to be reduced in the nucleus accumbens upon withdrawal from self-administered cocaine (Scofield and Kalivas, 2014). It is important to note that Narita et al. (2006) have indicated that astrocyte-, but not microglia-, related soluble factors were able to amplify both methamphetamine- and morphine-dependent rewarding effects. Astrocytic control of glutamatergic signaling during abstinent periods may also critically impact reinstatement of drug-seeking behaviors (Turner et al., 2013). These conclusions stem from studies in which a glia-selective dominant-negative SNARE protein was expressed in mice and subsequently used to assess the contribution of glial transmission on cocaine-induced behaviors. The authors were able to demonstrate that glial transmission is necessary for reinstatement of drug-seeking behaviors triggered by cocaine or associated cues (Turner et al., 2013).

Although much remains to be done to clarify the role of astrocytes in drug-induced behaviors, some of their behavioral effects might be consequences to their production of trophic factors that can impact adult neurogenesis (Barkho et al., 2006). This idea is supported by the evidence that exposure to drugs of abuse can influence neurogenesis (Mandyam and Koob, 2012). It is important to also note that gliogenesis in the mPFC is altered after self-administration of various drugs of abuse (Mandyam and Koob, 2012). Taken together, the impact of drugs of abuse on both neurogenesis and gliogenesis might create an environment that is permissive to the generation and persistence of long-term memories associated with the addictive process.

It is also important to note that the glial-derived neurotrophic factor (*GDNF*) has also been studied in animal models of addiction (Yan et al., 2007, 2013; Lu et al., 2009). This factor provides trophic support to dopamine neurons and modulates midbrain microglial activation (Rocha et al., 2012). GDNF-dependent neuroadaptations in midbrain VTA neurons appear to play an important role in the development of incubation of cocaine craving (Lu et al., 2009). GDNF expression may be also associated with enduring vulnerability to reinstatement of METH-seeking behavior (Yan et al., 2007, 2013). More studies need to be conducted to elucidate how GDNF might influence dopaminergic functions in other brain regions after chronic exposure to psychostimulants and other drugs of abuse.

Oligodendroglia

Oligodendrocytes are cell types responsible for providing myelin for rapid propagation of action potentials (Nave, 2010). The brain contains an abundant class of progenitor cells that express the chondroitin sulfate proteoglycan, NG2, and the alpha receptor for platelet-derived growth factor (PDGFαR) (Nishiyama, 2007). These $NG2^{+}$glial cells are called oligodendrocyte precursor cells (OPCs) because they generate oligodendrocytes during early postnatal development (Nishiyama, 2007). OPCs remain abundant in the adult CNS and retain the ability to differentiate into oligodendrocytes (Kang et al., 2010). They can regenerate oligodendrocytes after their degeneration through chemical- or autoimmune-mediated demyelination (Franklin and Ffrench-Constant, 2008). Oligodendrocytes can also regulate axonal function via their influence on neuron-glial interactions (Fields, 2014)

At present, there is very little information available on the potential effects of drugs of abuse on oligodendrocytes. For example, Lin et al. (2013) used a 3.0-Tesla MR scanner to study the brains of 34 heavy smokers and compared them to those of 34 age- and sex-matched controls. They found that heavy smokers had lower fractional anisotropy in the left anterior corpus callosum, an area that corresponded to the genu and rostral body of the corpus callosumm. These smokers did not show any area of increased anisotropy. They reported further that these smokers showed decreased axial diffusivity and increased radial diffusivity, but no changes in mean diffusivity. The authors suggested that their observations might the results of axonal loss and disrupted myelin integrity (Fields, 2014). Importantly, regression analysis revealed that these changes were related to the duration of smoking, thus suggesting that long-term exposure to nicotine and/or other factors in smoke might damage or impair the functions of oligodendrocytes. These observations are consistent with previous observations in chronic cigarette smokers (Paul et al., 2008). It is also important to mention that nicotine can cause significant increases in myelin genes in the prefrontal cortex, caudate putamen, and the nucleus accumbens of rats exposed to the drug *in utero* (Cao et al., 2013). The impact of these changes on neuronal functions will need to be investigated further.

Animal studies have also revealed certain white matter abnormalities after extended cocaine use. For example, George et al. (2008) investigated memory functions in rats that had 6-h access preday to cocaine. These rats escalated their intake of cocaine and exhibited working memory deficits. In addition, there was a significant correlation between decreased NG2-positive cells and cognitive impairments in these rats. Other investigators have also reported that chronic cocaine can cause decreased level of white matter proteins in the mouse nucleus accumbens (Kovalevich et al., 2012). Opioids also appear to affect the functions of oligodendrocytes. Specifically, perinatal exposure to buprenorphine has been shown to influence brain myelination, in that some doses of the buprenorphine were associated with reduced nuber of myelinatied axons (Sanchez et al., 2008). Buprenorphine also caused an inverted U-type increases in myelin basic proteins (MBPs), with the highest doses causing normalization of the levels of MBPs (Eschenroeder et al., 2012). Low doses of bupenorphine also increeased morphological complexity and increased the percentage of pre-oligodendrocytes that reach maturity. These differentiating effects appear

to be mediated by stimulation of mu-opioid receptors (Eschenroeder et al., 2012). In contrast, higher does of the drug might exert their influence through the nociceptin/orphanin FQ (NOP) receptor (Eschenroeder et al., 2012). These observations suggest that further evaluation of oligodendryte functions in adults being treated with opioid agents are necessary.

These animal studies are consistent with the suggestion that myelin dysfunction might account for some of the deficits in white matter integrity described in studies of humans addicted to various substances (Cadet et al., 2014a). More studies are needed to elucidate if electrophysiological abnormalities observed in some models of addiction might be secondary to drug-induced myelin dysfunction and associated abnormalities in conduction of action potentials to synaptic areas.

Microglia

Microglial cells are the immune cells that reside in the brain parenchyma (Sierra et al., 2014). They are exceptional sensors of their microenvironment and respond by undergoing remarkable changes in morphology and gene expression (Aguzzi et al., 2013). During pathological insults, activated microglial cells thicken and retract their processes, extend filopodia, proliferate and migrate. They also release factors and compounds that can influence neuronal survival. These factors include proinflammatory cytokines, trophic factors, and ROS. They also phagocytose pathogens, degenerating cells, and debris (Schafer et al., 2013). Of relatedness to our present discussion, reactive microgliosis has been detected in several regions of the brains of methamphetamine addicts who had been abstinent for several years (Sekine et al., 2008). These results had suggested that methamphetamine exposure had engendered a process that had enduring effects on the proliferation of reactive microglial cells. These studies in humans found parallelism in preclinical studies documenting that methamphetamine induces microglial activation in the brain (Thomas et al., 2004; Raineri et al., 2012). Along with microglial activation, methamphetamine can increase striatal mRNA expression levels of IL-6 family pro-inflammatory cytokines, leukemia inhibitory factor, oncostatin m, and IL-6 (Robson et al., 2013). These observations are consistent with the idea that the drug might cause neuronal dysfunction via microglia-secreted pro-inflammatory and toxic factors.

In addition to their toxic effects, microglia can alter neuronal excitability by affecting both inhibitory and excitatory synaptic transmission (Sierra et al., 2014). Tremblay et al. (2010) showed that microglia normally contact spines, synaptic terminals, and synaptic clefts in the cortex (Tremblay et al., 2010). Microglia can also regulate basal glutamatergic and GABAergic synaptic transmission in the context of brain injury by a mechanism that involves the increased production of ATP that stimulates the release of brain-derived neurotrophic factor (BDNF) from microglial cells (Tsuda et al., 2003; Davalos et al., 2005). BDNF is a neurotrophin that regulates neuronal survival and differentiation. BDNF also modulates neuronal activity and synaptic plasticity (Santos et al., 2010). Because neurons and microglia express BDNF (Trang et al., 2011), this protein may influence a vast array of functions in the brain. Of specific relationship to our discussion, it has been shown that infusion of BDNF into subcortical structures such as the nucleus accumbens and ventral tegmental area enhances cocaine-induced behavioral sensitization and cocaine seeking (Lu et al., 2004; Graham et al., 2007). In contrast, BDNF infusion into the dorsomedial prefrontal cortex following cocaine self-administration attenuates relapse to cocaine seeking after abstinence; cue- and cocaine prime-induced reinstatement of cocaine-seeking were similarly affected (Whitfield et al., 2011). Some of the effects of cocaine on BDNF appear to be mediated via induced expression of microRNA 212 (Hollander et al., 2010), with the magnitude of BDNF expression being dependent on a homeostatic interaction of microRNA 212 and MeCP2 in the dorsal striatum (Im et al., 2010). Methamphetamine self-administration also causes increased BDNF expression at both mRNA and protein levels (Cadet et al., 2014b). However, since these studies did not clarify the principal sources of BDNF expression, it remains to be determined the extent to which microglial cells might be influencing these drug-induced changes in BDNF expression.

CONCLUSIONS

Addiction of licit and illicit substances can be viewed as maladaptive plastic responses to exposure to agents that impact the expression of various genes and proteins in the brain. Some of these proteins are known to be involved in developmental processes that are dormant during adulthood (Cadet, 2009; Cadet et al., 2014b). Drug-induced elevated expression of some of these proteins could have induced glial proliferation, neuronal dedifferentiation, as well as structural and dysfunctional interactions between glial and neuronal cells (Cadet, 2009). Because glial cells are such an integral part of global neuronal function, it will be very important to develop tool sets that can differentiate the short-term impact of drug-induced dysfunctions of glial cells that might negatively impact long-term brain functions. This is important in view of the fact that many neurodegenerative disorders including Parkinson's disease are thought to be secondary to glia-dependent neuroinflammatory responses (Rogers et al., 2007; Tansey and Goldberg, 2010). These statements implicate a need for novel approaches to the treatment of human addicts that emphasize the development of protective agents that could cause a return of their brains toward baseline homeostasis.

ACKNOWLEDGMENTS

The Intramural Research Program of the National Institute on Drug Abuse (NIDA), NIH, and DHHS supports this paper. Veronica Bisagno is supported by grants PIP11420100100072 and PICT 2012-0924, Argentina.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 July 2014; accepted: 15 August 2014; published online: 02 September 2014.
*Citation: Cadet JL and Bisagno V (2014) Glial-neuronal ensembles: partners in drug addiction-associated synaptic plasticity. Front. Pharmacol. **5**:204. doi: 10.3389/fphar.2014.00204*
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 October 2014
doi: 10.3389/fphar.2014.00228

Adolescent cocaine exposure simplifies orbitofrontal cortical dendritic arbors

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Cocaine and amphetamine remodel dendritic spines within discrete cortico-limbic brain structures including the orbitofrontal cortex (oPFC). Whether dendrite structure is similarly affected, and whether pre-existing cellular characteristics influence behavioral vulnerabilities to drugs of abuse, remain unclear. Animal models provide an ideal venue to address these issues because neurobehavioral phenotypes can be defined both before, and following, drug exposure. We exposed mice to cocaine from postnatal days 31–35, corresponding to early adolescence, using a dosing protocol that causes impairments in an instrumental reversal task in adulthood. We then imaged and reconstructed excitatory neurons in deep-layer oPFC. Prior cocaine exposure shortened and simplified arbors, particularly in the basal region. Next, we imaged and reconstructed orbital neurons in a developmental-genetic model of cocaine vulnerability—the *p190rhogap+/−* mouse. p190RhoGAP is an actin cytoskeleton regulatory protein that stabilizes dendrites and dendritic spines, and *p190rhogap+/−* mice develop rapid and robust locomotor activation in response to cocaine. Despite this, oPFC dendritic arbors were intact in drug-naïve *p190rhogap+/−* mice. Together, these findings provide evidence that adolescent cocaine exposure has long-term effects on dendrite structure in the oPFC, and they suggest that cocaine-induced modifications in dendrite structure may contribute to the behavioral effects of cocaine more so than pre-existing structural abnormalities in this cell population.

Keywords: psychostimulant, orbital, sholl, adolescence, addiction

INTRODUCTION

Cocaine addiction is characterized by maladaptive decision making, a loss of control over drug consumption, and habit-like drug seeking despite adverse consequences. These cognitive changes likely reflect the effects of repeated drug exposure on prefrontal cortical neurobiology that then further promote drug use (Jentsch and Taylor, 1999; Everitt and Robbins, 2005; Torregrossa et al., 2011; Lucantonio et al., 2012). Additionally, *pre-existing* neurobehavioral characteristics in drug-naïve individuals may contribute to drug vulnerabilities (Ersche et al., 2012). Rodents provide an ideal model system to isolate vulnerability factors in drug-naïve organisms and also characterize the *consequences* of cocaine exposure because like humans, rodents will readily self-administer cocaine and engage in complex decision making, as well as relapse-like behavior. Also as in humans, individual differences in behavioral response strategies can serve as phenotypic predictors of addiction-like behaviors such as drug seeking following periods of abstinence (Deroche-Gamonet et al., 2004). Finally, even experimenter-administered, rather than self-administered, cocaine can induce behavioral phenotypes in rodents that are relevant to addiction etiology in humans, e.g., increased propensity to engage in reward-seeking habits (Schoenbaum and Setlow, 2005; Gourley et al., 2013c; Hinton et al., 2014).

We recently sought to identify the long-term consequences of cocaine exposure during adolescence, when drug use is often initiated in humans, and when the prefrontal cortex is still developing (Casey et al., 2000; Giedd, 2004; Paus et al., 2008). We found that >7 weeks following subchronic exposure during adolescence, cocaine-exposed mice preferentially engaged habit-like response strategies at the expense of flexible action-outcome-based strategies to acquire food reinforcers (Hinton et al., 2014). Inactivation of the orbitofrontal cortex (oPFC) blocks goal-directed action selection in the same task (Gourley et al., 2013a), and adolescent cocaine exposure eliminates dendritic spines in this region (Gourley et al., 2012). Whether adolescent cocaine exposure regulates oPFC dendrite arbor structure is, to our knowledge, unresolved.

Such gross remodeling could contribute to persistent maladaptive decision making following adolescent cocaine exposure and potentially to increased risk of dependence (O'Brien and Anthony, 2005) or decreased likelihood of seeking treatment (Kessler et al., 2001) in individuals who initiate cocaine use in adolescence.

We first quantified the effects of adolescent cocaine exposure on behavioral flexibility in an oPFC-dependent instrumental reversal task and on dendrite arbor structure in adult excitatory deep-layer oPFC neurons. Next, we aimed to evaluate whether *pre-existing* morphological abnormalities in the same neuron population were associated with behavioral vulnerabilities. The model we selected for this experiment was the *p190rhogap*+/− mouse. These mice are deficient in p190RhoGAP, a principal Src substrate in the brain that regulates cell structure through interactions with the RhoA GTPase (Brouns et al., 2000, 2001). Drug-naïve *p190rhogap*+/− mice appear at baseline to be behaviorally unremarkable (Gourley et al., 2012, 2013b). Nonetheless, they are highly sensitive to cocaine such that a single injection elicits a sensitization-like response (Gourley et al., 2012), making them an ideal candidate model by which to isolate pre-existing structural factors associated with subsequent cocaine vulnerability.

Our findings suggest that adolescent cocaine exposure simplifies excitatory oPFC dendritic arbors. By contrast, dendrite arbors appear grossly normal in *p190rhogap*+/− mice and thus, do not obviously account for behavioral vulnerabilities to cocaine in these animals.

MATERIALS AND METHODS

SUBJECTS

All mice were bred on a C57BL/6 background, and those used in anatomical studies expressed *thy1*-derived yellow fluorescent protein (YFP; Feng et al., 2000) to enable dendrite imaging. YFP-expressing p190RhoGAP-deficient mice (*p190rhogap*+/−) or *p190rhogap*+/+ littermates were also used. *p190rhogap*+/− mice have a ∼32–40% reduction in p190RhoGAP protein expression (Brouns et al., 2000). Mice were maintained on a 12-h light cycle (0700 on), and provided food and water *ad libitum* unless otherwise noted. Procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Emory and Yale University Institutional Animal Care and Use Committees, as appropriate.

ADOLESCENT COCAINE EXPOSURE

Cocaine or saline (Sigma) was administered for five consecutive days starting at postnatal day (P) 31 (10 mg/kg, *i.p.*, 1 ml/100 g). Then, mice were left undisturbed until P56, at which point they were euthanized for anatomical studies or tested in an instrumental reversal learning task.

INSTRUMENTAL REVERSAL LEARNING

Mice with a history of adolescent cocaine exposure were food-restricted as adults to ∼93% of their original body weight and trained to nose poke for food reinforcement (20 mg grain-based pellets; Bioserv) using illuminated Med-Associates conditioning chambers. Training was initiated with a continuous reinforcement schedule; 30 pellets were available for responding on each of two distinct nose poke recesses located on opposite sides of a single wall within the chambers, resulting in 60 pellets/session. Sessions ended when all 60 pellets were delivered or at 135 min. Responding on a center aperture was not reinforced. Following 7 days of training, mice were required to "reverse" their responding to this center aperture to continue to obtain reinforcement. Responding on the previously active apertures was no longer reinforced. These "reversal" sessions were 25 min in duration and used a variable ratio two schedule of reinforcement (Gourley et al., 2010). Responses on the active and inactive nose poke apertures were quantified, as were head entries into the magazine where pellets were delivered. One cocaine-exposed mouse consistently generated values two standard deviations above the group mean and was excluded.

LOCOMOTOR MONITORING

In 8-week-old mice, we used a within-subjects design to compare the locomotor response to cocaine between *p190rhogap*+/− and *p190rhogap*+/+ littermates. Mice were administered cocaine (10 mg/kg, *i.p.*, 1 ml/100 g) for five sequential days, and then left undisturbed for 7–10 days at which point a "challenge" injection was administered (10 mg/kg, *i.p.*, 1 ml/100 g).

Locomotor activity was monitored using customized Med-Associates chambers equipped with 16 photobeams. Mice were first habituated to the chambers for 1 h following a saline injection, then cocaine was administered. Total photobeam breaks following the cocaine injection were normalized to those generated in the 30 min following saline injection. During the challenge session, all mice were habituated to the locomotor monitoring chambers for 1 h without injection, then saline was administered and mice were monitored for 30 min, and finally, cocaine was administered, and mice were monitored for an additional 30 min. Cocaine-elicited photobeam breaks were normalized to those following saline in order to control for conditioned locomotor activation in response to injection. This experiment served to provide evidence that *p190rhogap*+/− mice are hyper-sensitive to cocaine, as we have previously reported (Gourley et al., 2012), but importantly, mice used for anatomical studies were cocaine-naïve because our goal was to evaluate pre-existing factors that might be associated with cocaine vulnerability.

DENDRITIC ARBOR RECONSTRUCTION AND MEASUREMENT

Mice were euthanized, and fresh brains were submerged in 4% paraformaldehyde for 48 h, then transferred to 30% w/v sucrose, followed by sectioning into 50 μm-thick coronal sections on a microtome held at −15°C. These relatively thin sections allow us to image whole deep-layer neurons without background fluorescence that would otherwise obstruct reconstruction. Little is known regarding the typical morphology of these neurons. This may be because traditional Golgi impregnation can spare deep-layer oPFC (Kolb et al., 2008), and while it is conceivable that we under-count dendrites that may have been truncated along the rostro-caudal plane, neurons with clear dendritic arbor truncations were excluded from the analyses.

Neurons were imaged on a spinning disk confocal (VisiTech International, Sunderland, UK) on a Leica microscope. Z-stacks

were collected with a 20× 1.4 NA objective using a 1-μm step size, sampling above and below the neuron. After imaging, we confirmed at 10× that the image was collected from the oPFC. Most images were collected from the lateral oPFC, however, the ventral subregion was also sampled. Neurons contained at least two basal dendritic arbors and a distinct intact apical dendrite, all with at least second-order branching.

Neurons were reconstructed in three dimensions by a single experimenter blind to group using Neurolucida (MBF Biosciences). Total dendritic material was measured for apical and basal arbors. To assess dendrite complexity, a 3-D version of a Sholl analysis (Wellman et al., 2007) was performed by measuring the number of dendritic intersections within 10-μm concentric spheres radiating from the soma. Four to 11 neurons/mouse from the *p190rhogap*+/− population and four to eight neurons/mouse from the cocaine-exposed population were imaged, reconstructed, and analyzed. Group sizes were six to seven mice in the cocaine-exposed population and four to six mice in the *p190rhogap*+/− population.

STATISTICAL ANALYSES

For instrumental conditioning studies, responding on the active and inactive apertures and magazine head entry rates were compared by ANOVA with repeated measures and group as the independent variable. For locomotor assessments, cocaine-elicited photobeam breaks (calculated as potentiation from baseline) were compared by ANOVA with repeated measures and group as the independent variable. Locomotor counts on the challenge day were compared between groups by unpaired *t*-test.

For anatomical studies, each mouse contributed a single value—the mean of its multiple neurons—to the analyses. Dendrite lengths were compared between groups by unpaired *t*-test. Sholl intersections were compared by ANOVA with repeated measures. In the case of interactions, *post hoc* comparisons were generated using Tukey's *t*-tests; the results of *post hoc* comparisons are indicated graphically. $p < 0.05$ was considered significant. In one instance, a Kolmogorov–Smirnov comparison was also applied to total dendrite length; in this case, each neuron was considered an independent sample.

RESULTS

Here we aimed to quantify the effects of adolescent cocaine exposure on oPFC dendrite morphology. We first, however, confirmed that adolescent cocaine had long-term behavioral consequences. Mice were exposed to subchronic cocaine during early adolescence, from P31 to P35 (Spear, 2000), then left undisturbed until adulthood. Cocaine exposure is thought to confer a bias toward inflexible, maladaptive decision-making strategies, so we tested mice in an instrumental "reversal learning" task that is sensitive to chronic cocaine exposure or lesions of the oPFC in adult mice (Krueger et al., 2009; Gourley et al., 2010).

Mice with a history of subchronic saline or cocaine exposure were able to acquire a nose poke response for food reinforcement as adults (interaction $F < 1$; **Figure 1A**). Qualitatively, cocaine appeared to modestly decrease overall responding, but this effect did not reach significance [main effect $F_{(1,21)} = 3.9$, $p = 0.06$], and response rates were equivalent at the end of training. When the response requirement was "reversed" such that mice were required to respond on an aperture at a separate location in the conditioning chamber, mice with a history of cocaine exposure generated fewer responses [main effect of cocaine $F_{(1,20)} = 4.1$, $p < 0.05$; **Figure 1B**]. We identified no effects of cocaine on responding on the previously reinforced aperture or magazine head entry rate (both $F < 1$; **Figure 1C**). This pattern recapitulates the effects of prolonged cocaine exposure in adult mice (Krueger et al., 2009), as well as lesions of the lateral oPFC (Gourley et al., 2010).

We next analyzed the effects of adolescent cocaine exposure on dendrite structure in the adult lateral oPFC. Representative deep-layer neurons from adult mice are shown (**Figure 2A**). Note

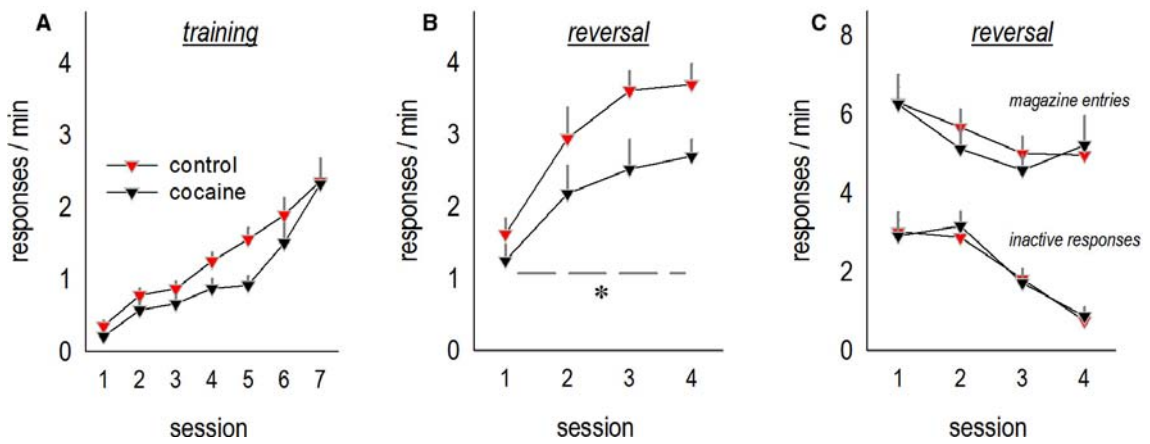

FIGURE 1 | Adolescent cocaine exposure impairs instrumental reversal learning in adulthood. (A) Mice were exposed to cocaine or saline from P31 to P35, then left undisturbed until adulthood, at which point they were trained to nose poke for food reinforcers. **(B)** When the location of the reinforced aperture within the chamber was then "reversed," cocaine-exposed mice generated fewer responses on the now-active aperture. **(C)** By contrast, cocaine exposure did not impact response inhibition on the previously reinforced apertures or head entries into the food-associated magazine. Means + SEMs, *$p < 0.05$, main effect of cocaine.

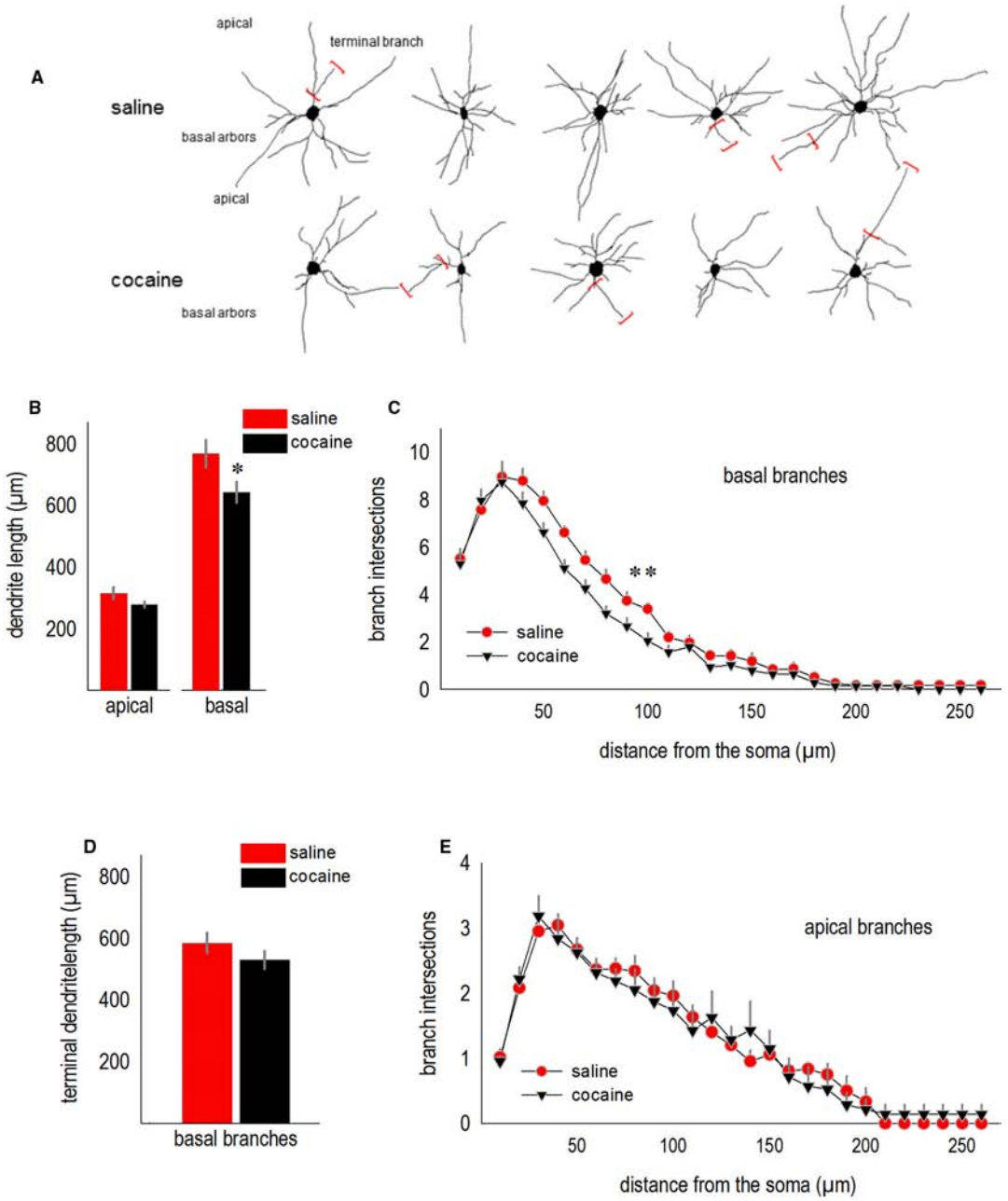

FIGURE 2 | Cocaine exposure during adolescence simplifies oPFC dendrite arbors in adulthood. (A) Representative deep-layer oPFC pyramidal neurons are shown. Terminal branches are indicated by red brackets. **(B)** Total dendrite length in the apical tree was not significantly affected, but basal lengths were reduced. **(C)** Simultaneously, dendritic arbors on the basal tree were simplified, as indicated by fewer Sholl intersections 40–100 µm from the soma. **(D)** The total length of basal terminal branches did not significantly differ between groups, consistent with arbor simplification in relatively close proximity to the soma in **(C)**. **(E)** Sholl intersections for apical arbors did not differ. Means + SEMs. $*p < 0.05$ vs. saline, $**p < 0.05$ for 40–100 µm from the soma.

the relatively stellate shape of oPFC pyramidal neurons compared to the more classically pyramidal shape of neurons in other subregions of the prefrontal cortex (further discussed in Kolb et al., 2008; see also Liston et al., 2006; Bortolato et al., 2011).

Total basal arbor length was reduced in cocaine-exposed mice ($t11 = 2.3$, $p < 0.05$; **Figure 2B**). Basal arbors were also less complex, as indicated by fewer Sholl intersections 40–100 µm from the soma [interaction $F_{(25,275)} = 1.9$, $p < 0.01$; **Figure 2C**]. As another metric of dendrite length, we measured terminal branches, the segments following the last bifurcations of each dendrite. In this case, terminal branch lengths did not differ ($t11 = -1.3$, $p > 0.2$; **Figure 2D**), consistent with evidence from

the Sholl analysis that a history of adolescent cocaine exposure simplifies dendrite arbors in close proximity to the soma (again, **Figure 2C**).

Despite differences in basal arbor length and complexity, apical arbors were not significantly affected (for length, $t11 = 1.9$, $p = 0.09$; for Sholl intersections, Fs < 1; **Figures 2B,E**).

We next evaluated dendrite complexity in a developmental-genetic model of cocaine vulnerability—*p190rhogap+/−* mutant mice. We selected these mice because p190RhoGAP is a cytoskeleton regulatory protein implicated in postnatal dendrite stability in the brain (Sfakianos et al., 2007), and we have previously reported that *p190rhogap+/−* mice display augmented sensitivity to cocaine (Gourley et al., 2012). Specifically, mice develop a sensitization-like response even after exposure to a single relatively low dose, and locomotor activity remains exaggerated over the course of several daily cocaine administrations, an effect that we recapitulate here by administering 10 mg/kg cocaine to mice daily for 5 days [main effect of genotype $F_{(1,20)} = 7.4$, $p = 0.01$; **Figure 3A**]. Mice were then left undisturbed for 1 week, after which they were habituated to the locomotor monitoring chambers for 1 h, then injected with saline and monitored for 30 min, then finally, injected with cocaine. Wild type mice generated 1.4 times as many photobeam breaks following low-dose cocaine "challenge" relative to saline. By contrast, *p190rhogap+/−* littermates broke >4-fold more photobeams following cocaine exposure ($t19 = -2.1$, $p = 0.05$; **Figure 3A**).

Adult drug-naive *p190rhogap+/−* mice were crossed with mice expressing YFP, generating YFP-expressing wild type and *p190rhogap+/−* offspring, and allowing us to potentially identify structural predictors of cocaine vulnerable *prior to drug exposure*. When oPFC dendrites from drug-naïve *p190rhogap+/−* mice were imaged and reconstructed, however, we identified no differences in total dendrite length (apical, $t8 = 1.2$, $p = 0.3$; basal, $t8 = 0.8$, $p = 0.5$; **Figure 3B**). By contrast, basal dendrite lengths differed in *cocaine-exposed* mice above, so as an additional, potentially more nuanced measure, we compared dendrite lengths using a Kolmogorov–Smirnov analysis in which the basal dendrite length from each neuron was considered an independent sample. Even here, we again did not identify differences between wild type and *p190rhogap+/−* mice ($D = 0.2$, $p = 0.3$; **Figure 3C**). Consistent with this outcome, basal arbor complexities did not differ, as determined by Sholl intersections (interaction $F < 1$;

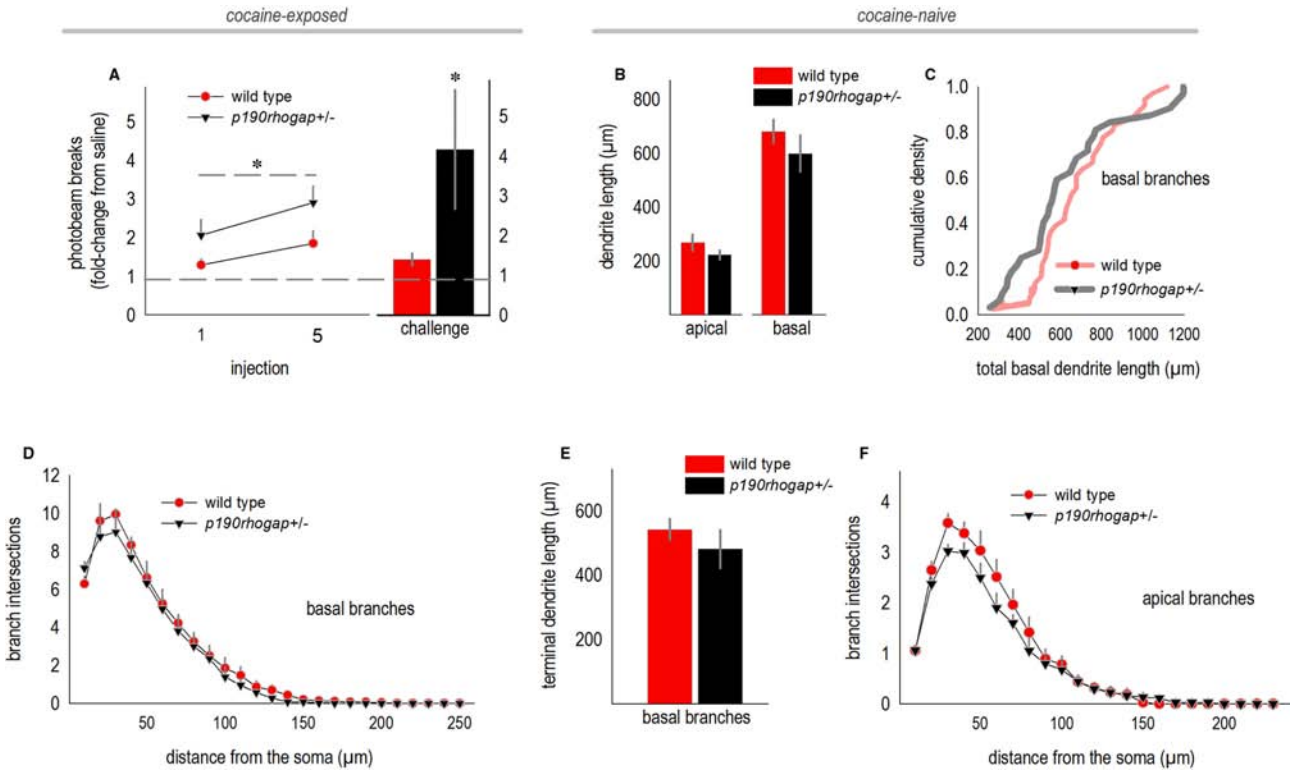

FIGURE 3 | oPFC dendrites in drug-naïve *p190rhogap+/−* mice are intact. **(A)** *p190rhogap+/−* mice are hyper-sensitive to cocaine, as indicated by increased locomotor activity following cocaine injection, relative to saline injection (dashed line at 1), and relative to littermate wild type mice. The bar graph shows that following a washout period, cocaine injection elicited greater photobeam breaks in *p190rhogap+/−* mice than in wild type littermates. **(B)** We next assessed the morphology of deep-layer oPFC neurons in drug-naïve mice. Length of the apical and basal dendrites did not differ between drug-naïve groups, despite behavioral vulnerabilities to cocaine. **(C)** Basal dendrites are represented another way—here, total dendrite length for each neuron is represented in a cumulative density function. Again, we identified no significant differences between groups. **(D)** Sholl analyses of basal arbors also revealed no differences in complexity. **(E)** Additionally, the length of the terminal arbors did not differ. **(F)** Sholl analyses indicated that the complexity of apical arbors also did not differ. Means + SEMs. *$p \leq 0.05$ vs. wild type.

Figure 3D), and the length of the terminal branches did not differ ($t8 = -1$, $p = 0.3$; **Figure 3E**).

When we quantified Sholl intersections for the apical arbor, neither interactions nor main effects were detected [interaction $F = 1$; main effect $F_{(1,22)} = 1.4$, $p = 0.3$; **Figure 3F**]. Qualitatively, wild type mice appeared to have more complex arbors, but this impression was driven by a single mouse.

DISCUSSION

The ability of neurons to integrate into networks and regulate behavior is determined in part by the size, shape, and complexity of dendrites. Dendrites can be remarkably plastic—for example, oPFC dendritic arbors remodel following stressor exposure (Liston et al., 2006; Dias-Ferreira et al., 2009) and environmental enrichment (Comeau et al., 2010). Some such modifications may play a role in mood disorders and other psychopathologies involving cortico-striatal circuits (e.g., cocaine addiction), but the characterization of structural modifications that—like drug craving in addiction—persist beyond the period of active drug exposure remains incomplete. We used transgenic mice expressing *thy1*-derived YFP to isolate and reconstruct dendritic arbors of excitatory deep-layer oPFC neurons. We report that arbors remodeled in response to subchronic cocaine exposure in adolescence and were simplified in adulthood. By contrast, dendritic arbors in drug-naïve *p190rhogap+/−* mutant mice—a model of cocaine vulnerability (Gourley et al., 2012)—were intact, suggesting that the *response* to cocaine, rather than pre-existing structural deficiencies *per se*, is associated with behavioral sensitivity to further drug exposure in these mice.

PREFRONTAL CORTICAL DENDRITES REORGANIZE IN RESPONSE TO COCAINE

The effects of amphetamine-like psychostimulants such as cocaine on neural structure have been intensively studied since the seminal reports of Robinson and Kolb (1997, 1999) describing drug-induced dendrite and dendritic spine elaboration in the nucleus accumbens and medial prefrontal cortex. Within the prefrontal cortex, the vast majority of subsequent research has remained focused on medial wall structures, largely sparing the oPFC; this is despite overwhelming evidence implicating oPFC function in addiction etiology (e.g., see Lucantonio et al., 2012). Currently available data indicate that amphetamine and cocaine *reduce* dendritic spine density in the oPFC (Kolb et al., 2004; Crombag et al., 2005; Muhammad and Kolb, 2011a,b; Gourley et al., 2012; but see Ferrario et al., 2005), but effects on dendrite structure remain unclear. We report novel evidence that cocaine exposure simplifies oPFC dendrite arbors, particularly in the basal region. Notably, chronic ethanol exposure does not remodel excitatory oPFC neurons (Holmes et al., 2012; DePoy et al., 2013), thus the present effects may be selective to stimulants, or potentially cocaine specifically.

In the parietal cortex, amphetamine exposure blocks the dendrite-elaborating effects of environmental enrichment, consistent with our current findings, although amphetamine alone has no consequences (Kolb et al., 2003). Nonetheless, we found evidence of long-term dendrite *simplification* following cocaine. How might we reconcile this apparent contradiction? One difference, in addition to the anatomical, is that cocaine was administered here during the equivalent of adolescence, a period of vulnerability to the development of dependence in humans (Anthony and Petronis, 1995; O'Brien and Anthony, 2005). Recent studies using small-animal magnetic resonance imaging complement ours, revealing that adolescent (though not adult) cocaine exposure results in cortical thinning (Wheeler et al., 2013). Interestingly, overall oPFC volume is *increased* following adolescent cocaine exposure; this increase could conceivably reflect glial responses to cocaine (Bowers and Kalivas, 2003; Haydon et al., 2009), though further investigations are necessary.

Here, oPFC neurons in mice with a history of adolescent cocaine exposure were simplified, particularly in the basal region. This is notable given that adolescent psychostimulant exposure in non-human primates *also* simplifies basal arbors in deep-layer prefrontal cortex (Selemon et al., 2007). A strong trend for a reduction in dendritic spine density was also reported by Selemon et al. (2007); similarly, oPFC dendritic spines are eliminated following adolescent cocaine exposure in the mouse (Gourley et al., 2012). The amygdala projects to deep-layer prefrontal cortex in both rodents and primates, with neurons terminating on dendritic spine heads of both apical and basal branches (Gabbott et al., 2006; Ghashghaei et al., 2007). Our findings thus indicate that the structural effects of adolescent psychostimulant exposure in critical cortico-amygdala circuits implicated in addiction (see Torregrossa et al., 2011) translate across rodent–primate species.

In tandem with arbor simplification, cocaine exposure impaired performance in an oPFC-dependent instrumental reversal task. In this task, mice are trained to nose poke for food reinforcers in a chamber with multiple response operandi. Once mice have acquired the reinforced response, the location of the reinforced aperture is "reversed," in this case, from the lateral walls of the testing chamber to a center aperture, and mice must redirect responding to this previously non-reinforced aperture. Lesions of the lateral, but not medial, oPFC delay response acquisition, as does chronic cocaine exposure in adult mice (Krueger et al., 2009; Gourley et al., 2010). Conversely, instrumental reversal learning in drug-naïve mice is associated with subsequent cocaine self-administration patterns, with poor reversal performance predictive of higher rates of cocaine-reinforced responding (Cervantes et al., 2013). We report that even *subchronic* cocaine exposure in adolescent mice impaired response acquisition several *weeks* following drug exposure. Similarly, in a water maze reversal, early-adolescent cocaine exposure impairs response acquisition up to 10 days following exposure (Santucci et al., 2004). Together with multiple reports that cocaine exposure also occludes reversal learning based on stimulus–outcome associative contingencies (see Lucantonio et al., 2012), these findings highlight the long-term negative impact of cocaine on oPFC function, resulting in inflexible habit-like response strategies.

The oPFC also appears to regulate behavioral sensitivity and resilience to contextual stimuli associated with cocaine. For example, prolonged oPFC inactivation enhances context-induced reinstatement of cocaine seeking in rats, sparing drug-seeking behaviors induced by other conditioned stimuli (Fuchs et al., 2004; Lasseter et al., 2009). Thus, the *healthy* oPFC may gate the influence of contextual cues associated with drugs of abuse; repeated

cocaine exposure could degrade this function through repeated stimulation of the dopamine D1 receptor, for example (Lasseter et al., 2014), simplification of neural structure (**Figure 1**), and/or imbalance between D1 and D2, given that D2 is highly expressed on basal arbors that were eliminated here (Brock et al., 1992).

oPFC DENDRITE MORPHOLOGY IN DRUG-NAÏVE COCAINE-VULNERABLE MICE IS INTACT

Structural remodeling in the central nervous system is orchestrated by Rho family GTPases including RhoA (Rho), Rac1, and Cdc42, which coordinate the actin cytoskeletal rearrangements required for dendrite elaboration or simplification. Rho activation decreases branch extensions in multiple neural systems (e.g., Li et al., 2000; Wong et al., 2000), and interference with Rho activity promotes arbor growth (e.g., Sin et al., 2002; Couch et al., 2010) or activity-dependent remodeling of dendritic spines (Murakoshi et al., 2011). In the *adolescent* hippocampus, Rho activation causes dendritic arbor retraction, reducing overall length and complexity (Sfakianos et al., 2007).

Rho is inhibited endogenously by p190RhoGAP, which is activated by integrin receptor binding to extracellular matrix proteins (Arthur et al., 2000; Hernandez et al., 2004; Moresco et al., 2005; Bradley et al., 2006). *p190rhogap-/-* mice are not viable, and while *p190rhogap+/−* mice appear superficially normal, they exhibit significant vulnerabilities to genetic and chemical perturbations. For example, simultaneous heterozygosity for mutations in both p190RhoGAP and the cytoskeletal regulatory protein Arg kinase results in increased Rho activity and hippocampal dendritic arbor destabilization, accompanied by novel object recognition deficits (Sfakianos et al., 2007). Further, mice deficient in p190RhoGAP or the upstream effectors β1-integrin or Arg kinase are hypervulnerable to cocaine, generating a sensitization-like response following a single injection (Gourley et al., 2009, 2012; Warren et al., 2012).

The *p190rhogap+/−* mouse provides an opportunity to characterize neural morphology in an organism that is behaviorally vulnerable to cocaine *prior to cocaine exposure*. Throughout, however, we identified no differences in the size or complexity of excitatory deep-layer oPFC neurons between mutants and *p190rhogap+/+* littermates. These findings suggest that although cocaine exposure remodels the same neuron population, *pre-existing* deficiencies in dendrite arbors do not obviously account for drug vulnerability. Previously, we characterized dendritic spine density on excitatory oPFC neurons in naïve *p190rhogap+/−* mice and *p190rhogap+/−* mice exposed to a subthreshold dose of the stress hormone corticosterone (Gourley et al., 2013b). Corticosterone reduced oPFC spine density in *p190rhogap+/−* mice, and these structural deficiencies emerged in concert with anhedonic-like behavior. Thus, p190RhoGAP may regulate the structural *response* of oPFC neurons to varied pathological insults. In line with this perspective, ethanol activates p190RhoGAP and thereby decreases actin stress fiber density in neonate astrocytes exposed to ethanol (Selva and Egea, 2011).

While we did not identify structural *predictors* of cocaine vulnerability in the oPFC, it is important to note that our findings do not preclude the possibility that pre-existing morphological or physiological characteristics in other models contribute to drug vulnerabilities. Additionally, pre-existing characteristics of cell populations in other brain regions—e.g., in the striatum, amygdala, or other regions of the frontal cortex (Ersche et al., 2012; Winhusen et al., 2013)—may significantly impact drug vulnerability even prior to active drug exposure.

SUMMARY

The present results contribute to the general perspective that psychostimulant-induced neural remodeling has meaningful behavioral implications. These include potentially adaptive consequences. For example, cocaine-induced dendritic spine proliferation in the nucleus accumbens has been associated with behavioral resilience (e.g., Smith et al., 2014), and blockade of certain cocaine-induced dendritic spine modifications in the oPFC and nucleus accumbens can *increase—rather than occlude*—sensitivity to subsequent cocaine exposure (Toda et al., 2006; Pulipparacharuvil et al., 2008; Gourley et al., 2012). Meanwhile, the *correction* of long-term or metaplastic modifications following prolonged cocaine exposure may have behavioral benefits (Shen et al., 2009; Giza et al., 2013). Animal models provide an ideal venue to disentangle these issues, and to determine neurobiological vulnerability and resiliency factors using both prospective and retrospective approaches.

ACKNOWLEDGMENTS

This work was supported by the Emory University Research Council, DA 034808, DA 036737, GM 100411, and CA 133346. The Yerkes National Primate Research Center is supported by the Office of Research Infrastructure Programs/OD P51OD11132.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2014; accepted: 23 September 2014; published online: 27 October 2014.

Citation: DePoy LM, Perszyk RE, Zimmermann KS, Koleske AJ and Gourley SL (2014) Adolescent cocaine exposure simplifies orbitofrontal cortical dendritic arbors. Front. Pharmacol. 5:228. doi: 10.3389/fphar.2014.00228

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

MINI REVIEW ARTICLE
published: 25 November 2014
doi: 10.3389/fphar.2014.00259

Structural plasticity in mesencephalic dopaminergic neurons produced by drugs of abuse: critical role of BDNF and dopamine

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Mesencephalic dopaminergic neurons were suggested to be a critical physiopathology substrate for addiction disorders. Among neuroadaptive processes to addictive drugs, structural plasticity has attracted attention. While structural plasticity occurs at both pre- and post-synaptic levels in the mesolimbic dopaminergic system, the present review focuses only on dopaminergic neurons. Exposures to addictive drugs determine two opposite structural responses, hypothrophic plasticity produced by opioids and cannabinoids (in particular during the early withdrawal phase) and hypertrophic plasticity, mostly driven by psychostimulants and nicotine. *In vitro* and *in vivo* studies identified BDNF and extracellular dopamine as two critical factors in determining structural plasticity, the two molecules sharing similar intracellular pathways involved in cell soma and dendrite growth, the MEK-ERK1/2 and the PI3K-Akt-mTOR, via preferential activation of TrkB and dopamine D3 receptors, respectively. At present information regarding specific structural changes associated to the various stages of the addiction cycle is incomplete. Encouraging neuroimaging data in humans indirectly support the preclinical evidence of hypotrophic and hypertrophic effects, suggesting a possible differential engagement of dopamine neurons in parallel and partially converging circuits controlling motivation, stress, and emotions.

Keywords: morphology, cocaine, D3 receptor, dendrites, nicotine, ERK, mTOR

INTRODUCTION

Structural plasticity in neurons can be defined as a series of measurable changes in the morphologically defined components of the neuron, i.e., numbers, size, and composition of soma, dendrites, axons, and synapses, occurring over time and in response to changes in the cell environment. Structural plasticity can also be seen as one aspect of neuroadaptation, a general process present in neurons of specific neural circuits when responding to repeated physiologic stimuli, pathologic agents, or effective doses of pharmacologic substance, including addictive drugs (Ikemoto and Bonci, 2014). These stimuli act by engaging molecular mechanisms that are critical for cell growth and survival, their impact on the cell morphology being defined by the stimulus intensity and by genetic and epigenetic predisposing factors that constitute the neuroadaptive potential of the cells.

Since 1990s neuroadaptation and plasticity have been recognized to be relevant in addiction disorders characterized by chronic misuse of neuroactive substances (Koob, 1992; Nestler, 1992; Di Chiara, 1995; Everitt et al., 2001). In mammals, pharmacologic agents characterized by their addictive properties, for example psychostimulants (e.g., cocaine, amphetamines), opioids (e.g., heroin and morphine), nicotine, cannabinoids, and alcohol, were found to engage dopaminergic neurons of the mesocorticolimbic contingent located in the ventral tegmental area (VTA) (Koob, 1992; Di Chiara, 1995; Koob and Le Moal, 2005; Chen et al., 2010). These neurons produce dopamine (DA) as principal neurotransmitter, project to cortical and limbic brain structures and are involved in regulation of motivation, reward, motor response selection, mood, and arousal. While a large body of experimental findings supports the role for dopaminergic neurotransmission in mediating the addictive properties of these drugs (Koob, 1992; Di Chiara, 1995; Koob and Le Moal, 2005; Kalivas and O'Brien, 2008; Chen et al., 2010), less research was dedicated to the structural changes occurring during exposure to addictive drugs or following their withdrawal. The initial interest on structural plasticity was focused on glutamatergic and GABAergic neurons of nucleus accumbens and prefrontal cortex, i.e., on neurons located in terminal fields of the mesencephalic dopaminergic system (Robinson and Kolb, 1997, 2004; Russo et al., 2010) rather than on their presynaptic side. In dopaminergic neurons structural plasticity was indirectly inferred on the basis of changes in the expression of "marker" proteins though to be involved in structural changes, such as axonal neurofilaments (Nestler, 1992), a phenomenon only later confirmed using morphological techniques (Sklair-Tavron et al., 1996). In fact, by definition, structural plasticity requires morphologic evidence. Dopaminergic neurons are generally identified by immunocytochemistry or immunofluorescence with selective antibodies that recognize tyrosine hydroxylase (TH) or dopamine transporter (DAT; Köhler and Goldstein, 1984). When applied to the *post-mortem* study in mammalian brains, TH immunocytochemistry allows reliable estimate of soma size and neuron counts in substantia nigra (SN, also identified as A9) and VTA (also identified as A10). Conversely, a proper analysis of the dendrite length and branching is not possible, due to the

complex overlapping of the dendritic arborizations of adjacent dopaminergic neurons. Visualization of dendrites and dendritic spines of a single neuron requires different approaches, such as the classical Golgi-Cox staining (Juraska et al., 1977) associated with immunohistochemistry (Spiga et al., 2011) intracellular injection with Lucifer Yellow via micropipettes (Sklair-Tavron et al., 1996) or diolistic gene gun delivery of fluorescent dyes (Shen et al., 2008). Dopaminergic neurons can be studied *in vitro* using primary cell cultures from the ventral mesencephalon of rodent embryos or newborns (Shimoda et al., 1992; Collo et al., 2008). The *in vitro* approach allows the simultaneous evaluation of soma size, dendritic arborization, dendritic spines, neurochemistry, and intracellular molecular signaling due to their sparse distribution in the culture dish and their standardized control conditions (Collo et al., 2008, 2012).

In this article we summarize the evidence of structural plasticity occurring in mesencephalic dopaminergic neurons following exposure to addictive drugs, focusing on soma, and dendritic arborization rather than synapses and addressing the key molecular intracellular signaling involved.

STRUCTURAL PLASTICITY IN DOPAMINERGIC NEURONS AS CELLULAR NEUROADAPTATION: OPPOSITE EFFECTS OF OPIOIDS AND PSYCHOSTIMULANTS

Structural plasticity includes hyperplastic and hypoplastic phenomena, i.e., the increase or decrease of number and size of morphologically defined components of the neuron. In the brain reward circuit, drugs of addiction produce both hyperplastic and hypoplastic phenomena, the former generally associated to psychostimulants, the latter to the use of opioids (for review see Russo et al., 2009, 2010). Since chronic exposure to both opioids and psychostimulants produces behavioral sensitization, compulsive drug taking, and relapse after extinction, at the time of their initial discovery these changes appeared somewhat contradictory, casting some doubts about the relevance of structural plasticity in addictive behavior. Recent findings regarding the role of withdrawal state (Spiga et al., 2010; Mazei-Robison et al., 2011), region-specific changes of synaptic spines (Russo et al., 2010) and differential regulation of endogenous neurotrophins, in particular brain derived neurotropic factors (BDNF) (Russo et al., 2009; Koo et al., 2012), have been advocated as key factors in disentangling this paradox; some possible explanations will be reviewed later in this article.

Chronic exposures to opioids reduce soma size and dendrites of the dopaminergic neurons located in the VTA of adult rodents without reducing the number of neurons (Sklair-Tavron et al., 1996; Spiga et al., 2003; Russo et al., 2007). Opioid-induced hypotrophic effects on soma were observed following either passive dosing or self-administration of heroin or morphine and persist for several weeks during withdrawal. Functionally, chronic exposure to opioids is known to increase VTA neural firing while reduction is observed during withdrawal (Diana et al., 1995; Koo et al., 2012).

Reduced soma size and neural firing were also observed during withdrawal from cannabinoids in rodents (Diana et al., 1998; Spiga et al., 2010). These effects are partially determined by endogenous opioids since acute morphine attenuates the behavioral cannabinoid withdrawal syndrome in mice (Lichtman et al., 2001). Interestingly, chronic exposure to cannabinoids *per se* does not produce change of soma sizes of VTA dopaminergic neurons. Lack of change in the soma size was also recently showed in rats trained to chronically self-administer cocaine, nicotine, and alcohol when sacrificed in presence of drugs (Mazei-Robison et al., 2011). These data do not rule out the possibility of changes during withdrawal or crash after drug taking "binges," both conditions associated to a functional hypodopaminergic state (Weiss et al., 1992; Melis et al., 2005; Zhang et al., 2012); so far structural effects were not studied.

Cocaine and amphetamine exposures *in vivo* increase dendrite arborization and spines in VTA (Mueller et al., 2006; Sarti et al., 2007). *In vitro* studies on primary cultures of mesencephalic neurons from mouse embryos corroborate this evidence. Dose-dependent increases of soma size, maximal dendrite length, and number of primary dendrites were observed (Collo et al., 2008, 2012). Interestingly, also nicotine was shown to increase structural plasticity of dopaminergic neurons *in vitro*, effect blocked by mecamylamine and dihydro-β-erythroidine but not methyllycaconitine, suggesting the involvement of α4β2 nicotinic receptor (nAChR; (Collo et al., 2013). These nicotinic hetero-receptors expressed in dopaminergic neurons control DA release and are critical for the reinforcing effects of nicotine *in vivo* (Picciotto et al., 1998). Consistently, dopaminergic neurons from the mesencephalon of α4 nAChR-subunit knock out (KO) mice did not show nicotine-induced plasticity (Collo et al., 2013).

Prenatal exposure to either cocaine or nicotine during the last gestational phase (E17-21) was associated with significant increase of soma size of dopaminergic neurons in newborns and young mice (Collo et al., 2012, 2013). Prenatal exposures to cocaine and amphetamines produce long-term changes in the behavior and neurochemistry of the mesencephalic dopaminergic system of offspring assessed as adults (Crozatier et al., 2003; Lloyd et al., 2013), suggesting a possible association between dopaminergic structural plasticity and liability to develop addiction.

CRITICAL ROLE OF THE BDNF-TrkB SIGNALING IN DETERMINING STRUCTURAL PLASTICITY OF DOPAMINERGIC NEURONS EXPOSED TO ADDICTIVE DRUGS

Neurotrophic factors that bind to the tropomyosin-related kinase B (TrkB) receptor were shown to be of importance in the development of the central nervous system (CNS) and in shaping neuronal morphology of dopamine neurons and other brain circuits (for a review see Ohira and Hayashi, 2009). In particular, BDNF-TrkB signaling has been extensively studied as critical mediator of the structural changes produced by addictive drugs (Russo et al., 2009; Koo et al., 2012). Mesencephalic dopaminergic neurons significantly express BDNF since prenatal time (Baquet et al., 2005). Still present in adult life, BDNF expression can be transiently increased by psychostimulants in VTA dopaminergic neurons (Graham et al., 2007). These increases consolidate and persist over time during abstinence (Pu et al., 2006) and during extinction of drug self-administration and in craving incubation paradigms (Grimm et al., 2003). Infusion of BDNF in VTA induces long-lasting potentiation of cocaine seeking

during abstinence (Lu et al., 2004), while BDNF immunoneutralization attenuates the cocaine addictive behavioral effects (Graham et al., 2007). To our knowledge, direct evidence of structural changes in dopaminergic neurons during withdrawal, abstinence and incubation with psychostimulants is lacking in literature. However, in consideration of the well-known BDNF neurotrophic properties on dendrites and soma size, it is possible to speculate that some structural plasticity could occur. Interestingly, GDNF, another neurotrophic factor, increases in VTA during cocaine withdrawal and mediates incubation of cocaine craving (Lu et al., 2009), further supporting possible structural effects.

Almost opposite effects were observed with opioids: morphine reduces BDNF expression in VTA neurons; low BDNF levels were associated with reduced soma size, and local infusion with BDNF normalizes soma size (Sklair-Tavron et al., 1996; Russo et al., 2009). Recent studies using conditional KO mice and optogenetic technology showed that morphine-induced low levels of BDNF in the VTA are associated to hypersensitization of VTA dopaminergic neurons to morphine, whose administration increases firing and DA release, producing conditioned place preference (Koo et al., 2012). Conversely, acute withdrawal and abstinence are associated with increased BDNF expression and TrkB-mediated plasticity changes that are essentials for negative reinforcing effects of morphine withdrawal (Vargas-Perez et al., 2014). Interestingly, the opioid effects on DA release are indirect, mediated by GABAergic inhibitory neurons under glutamatergic control (Bonci and Williams, 1997; Vargas-Perez et al., 2009; Jalabert et al., 2011), suggesting a role also for these neurotransmitters.

The main intracellular pathways activated by BDNF-TrkB signaling are the MEK-ERK, the PI3K-Akt-mTORC1, the PLCy-DAG-PKC/Ca^{2+}, and NFkB pathways, all involved in cell survival and growth (Kumar et al., 2005; Russo et al., 2009). These pathways are not only activated by BDNF but also by G-protein coupled receptors (e.g., Girault et al., 2007). Recent evidence indicates that cocaine and nicotine activate both MEK-ERK and Akt-mTORC1 pathways in primary cultures of dopaminergic neurons (Collo et al., 2012, 2013). Phosphorylation in these two pathways was found critical for structural plasticity since pretreatments with selective inhibitors for ERK, PI3K, and mTORC1 block the increase of soma size and dendritic arborization produced by psychostimolants and nicotine (Collo et al., 2013). Conversely, morphine exposure was associated with reduction in Akt levels and phosphorylation, attenuating mTOR-dependent phosphorylation (Russo et al., 2007; Mazei-Robison et al., 2011). The central role of the PI3K-Akt-mTOR pathway in determining soma size of mesencephalic dopaminergic neurons is exemplified by the phosphatase and tensin homolog (PTEN) KO mice. PTEN is a negative regulator of PI3K whose null mutation leads to a constitutive preferential state of activation of Akt-mTORC1 pathway; the result is a massive increase in soma size of dopaminergic neurons already visible in newborns, that persists in adult mice (Diaz-Ruiz et al., 2009). Other mechanisms affecting dendrite and soma size include the modulation of Ca^{2+} levels and the cAMP production, the latter not operated by BDNF. A large body of evidence indicates that Ca^{2+}-dependent AMPA and NMDA glutamate receptors regulate dendrite growth in pyramidal neurons and interneurons (Hamad et al., 2011). In dopaminergic neurons NMDA-dependent axonal growth was described as related to CaMKII phosphorylation (Schmitz et al., 2009), while preliminary *in vitro* data indicate a critical role for AMPA receptors. In GABAergic neurons located in the VTA, chronic activation of the cAMP-PKA-CREB was associated with reduced firing and soma size in dopaminergic neurons during morphine withdrawal (Bonci and Williams, 1997; Koo et al., 2012), suggesting an indirect involvement in structural plasticity.

Interestingly, structural changes of soma size and dendritic arborization of dopaminergic neurons are not specific of addictive drugs. In a recent article a reduction of soma size in the VTA was observed in male rats after single and repeated mating episodes (Pitchers et al., 2014). Naloxone treatment reversed soma size reduction and attenuated the longer-term expression of experience-induced facilitation of sexual behavior without affecting its rewarding properties. In another study, an increase of the number, size, and dendritic spines of mesencephalic dopaminergic neurons was associated to exercise and intense motor behavior in rats exposed to moderate dose of dopaminergic neurotoxins (Real et al., 2013), supporting a role for neurotrophic BDNF-TrkB signaling in behaviorally induced structural plasticity.

DOPAMINE AS NEUROTROPHIC FACTOR: ROLE OF D3 RECEPTOR SIGNALING IN STRUCTURAL PLASTICITY OF DOPAMINERGIC NEURONS

In addition to its role as a neurotransmitter, DA can act as neurotrophic factor. When released in the extracellular space, DA binds to postsynaptic receptors, producing structural plasticity: for example DA increases TrkB phosphorylation via D1 receptor (Iwakura et al., 2008). DA also binds to presynaptic D3, $D2_S$, and D5 receptors located on dopaminergic neurons (Zhang and Sulzer, 2012). Functional studies in mutant mice indicate that D2 and D3 receptors are complementary in regulating phasic and tonic dopamine release from dopaminergic nerve terminals in caudate and nucleus accumbens (Le Foll et al., 2005b; Maina and Mathews, 2010). The intracellular pathways activated by the presynaptic DA receptors and related to structural plasticity are only partially understood, being the majority of studies performed in non-dopaminergic cells. Converging findings indicated a primary role for D3 receptors in dopaminergic structural plasticity via phosphorylation of MEK-ERK1/2 and PI3K-Akt-mTORC1 pathways (Cussac et al., 1999; Beom et al., 2004; Collo et al., 2012, 2013). Conversely, D2s receptors inhibit MEK-ERK1/2 pathway and are negatively coupled with adenylate-cyclase (Van-Ham et al., 2007). PLCy activation and β-arrestin non-canonical pathways were described for the D2L splice variant present on postsynaptic neurons (Del'guidice et al., 2011). Finally, less data are available on D5 receptor, whose role has been related to functional plasticity (Schilström et al., 2006; Argilli et al., 2008).

Direct evidence linking D3 receptors and structural plasticity was recently obtained in primary cultures of dopaminergic neurons from mouse embryos. Repeated exposure with low doses

of D3-preferential agonists, such as quinpirole or 7OH-DPAT, increased soma size and the number and length of primary dendrites (Collo et al., 2008). These effects were also produced by drugs of addiction such as cocaine, amphetamine, nicotine, and ketamine, all known to increase extracellular levels of DA in the VTA. Pretreatments with DA D3 selective antagonist SB277011-A and the non-selective D2/D3 antagonist sulpiride resulted in a blockade of dendrite outgrowth and soma size (Collo et al., 2008, 2012). No structural plasticity was observed when treatments with psychostimulants or nicotine were performed in cell cultures from the mesencephalon of D3 KO mice (Collo et al., 2008, 2012, 2013). When nicotine was repeatedly administered to pregnant D3 KO mice during the last gestational phase, no effect was observed on the soma size of VTA neurons of newborns. Recent evidence suggests that D3 receptors work in concert with BDNF-TrkB signaling. *In vivo* experiment showed that D3 receptor expression depends on the levels of BDNF (Guillin et al., 2003) and that cocaine exposure increases the synthesis of both BDNF and D3 receptors (Le Foll et al., 2005a), while morphine increases the expression of D3 receptors only (Spangler et al., 2003), marking a difference between the two addictive drugs (**Figure 1**).

HUMAN EVIDENCE OF ADDICTIVE DRUG INDUCED STRUCTURAL PLASTICITY

No direct human evidence of structural plasticity induced by addictive drugs in dopaminergic neurons is currently available. *Post-mortem* studies in cocaine users revealed a 16% reduction of melanized dopaminergic neurons with no reported change of soma size (Little et al., 2009), while a reduction of TH levels in dopaminergic terminals of the striatum was found in heroin addicts (Kish et al., 2001).

In vivo neuroimaging studies, which lack cellular resolution, showed reduced 6-FDOPA uptake in the dopaminergic terminals of the striatum of cocaine addicts during 10–30 days of abstinence (Wu et al., 1997). Interestingly, another marker of dopaminergic terminals in striatum, i.e., DAT levels, was found reduced in methamphetamine addicts (Chang et al., 2007) and in tobacco and marijuana smokers (Leroy et al., 2012). Extracellular DA release estimated using the 11C-raclopride displacement techniques indicated a lower DA tone in ventral striatum of cocaine (Martinez et al., 2009) and marijuana users (Volkow et al., 2014), the latter correlated with enhanced stress reactivity and irritability, confirming a hypodopaminergic state. Structural MRI showed a volumetric increase in the left nucleus accumbens in marijuana users (Gilman et al., 2014), enlarged striatum in methamphetamine users (Chang et al., 2007) and reduction in nucleus accumbens, anterior cingulated and orbitofrontal cortex in children exposed *in utero* to opioids (Walhovd et al., 2007), all findings in line with preclinical observations.

CONCLUSION AND FUTURE RESEARCH

Addictive drugs induce structural plasticity in dopaminergic neurons. While a complete picture of structural changes associated to the different stages of the addiction cycle and its translational value in human is still lacking, differences among the main addictive drugs in producing either hypotrophic or hypertrophic response stand out, driven by the respective down or up regulations of BDNF and extracellular dopamine levels. These effects can be more

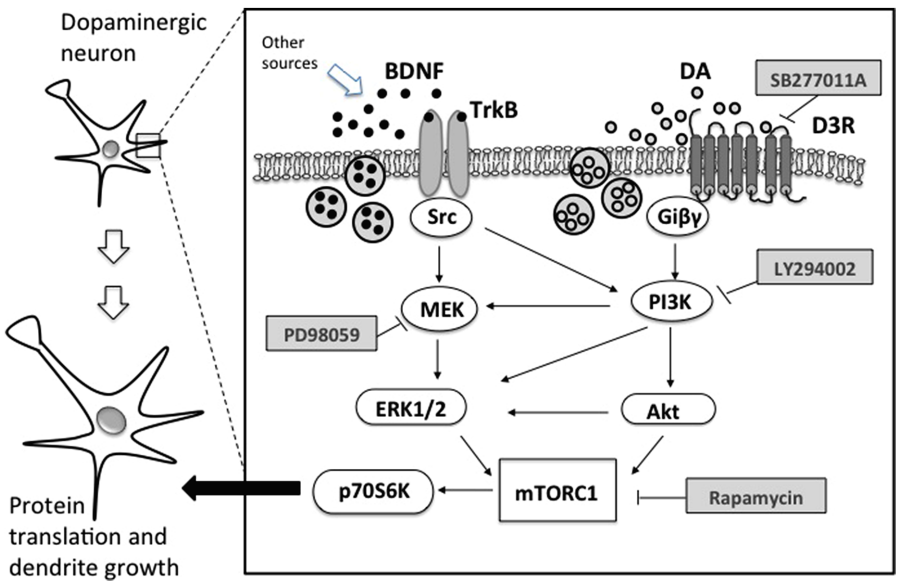

FIGURE 1 | Schematic representation of relevant intracellular pathways of BDNF and dopamine dependent structural plasticity in dopaminergic neurons. TrkB, tropomyosin-related kinase B; Src, proto-oncogene tyrosine protein kinase; MEK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; D3R, dopamine D3 receptor; Giβγ, G protein; PI3K, phosphatidylinositol 3-kinase; Akt, serine threonine kinase or protein kinase B; mTORC1, mammalian target of rapamycin complex 1; p70S6K, p70 ribosomal S6 protein kinase; PD98059, MEK inhibitor; LY294002, PI3K inhibitor; rapamycin, mTORC1 inhibitor; SB277011A, selective D3R inhibitor.

conspicuous during neural development, as shown in offspring following *in utero* exposure or *in vitro* using embryo-derived cell cultures. Overall, structural changes appear to be related to some differences in targeting of reward and stress circuits that work in parallel to control motivation (Koob, 2013; Ikemoto and Bonci, 2014). These long term structural changes can be seen as substrates of "memory" traces (Nestler, 2013) that would eventually constitute a liability for drug taking relapse.

ACKNOWLEDGMENTS

This manuscript was supported by the grant from ex 60%, University of Brescia to Ginetta Collo. The authors thank Emilio M. Pich for helpful discussion regarding the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 October 2014; paper pending published: 29 October 2014; accepted: 06 November 2014; published online: 25 November 2014.

*Citation: Collo G, Cavalleri L and Spano P (2014) Structural plasticity in mesencephalic dopaminergic neurons produced by drugs of abuse: critical role of BDNF and dopamine. Front. Pharmacol. **5**:259. doi: 10.3389/fphar.2014.00259*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

ORIGINAL RESEARCH ARTICLE
published: 23 February 2015
doi: 10.3389/fphar.2015.00028

Homer2 deletion alters dendritic spine morphology but not alcohol-associated adaptations in GluN2B-containing N-methyl-D-aspartate receptors in the nucleus accumbens

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Repeated exposure to ethanol followed by withdrawal leads to alterations in glutamatergic signaling and impaired synaptic plasticity in the nucleus accumbens (NAc) in both clinical and preclinical models of ethanol exposure. Homer2 is a member of a family of postsynaptic density (PSD) scaffolding proteins that functions in part to cluster N-methyl-D-aspartate (NMDA) signaling complexes in the PSD, and has been shown to be critically important for plasticity in multiple models of drug and alcohol abuse. Here we used *Homer2* knockout (KO) mice and a chronic intermittent intraperitoneal (IP) ethanol injection model to investigate a potential role for the protein in ethanol-induced adaptations in dendritic spine morphology and PSD protein expression. While deletion of *Homer2* was associated with increased density of long spines on medium spiny neurons of the NAc core of saline treated mice, ethanol exposure had no effect on dendritic spine morphology in either wild-type (WT) or Homer2 KO mice. Western blot analysis of tissue samples from the NAc enriched for PSD proteins revealed a main effect of ethanol treatment on the expression of GluN2B, but there was no effect of genotype or treatment on the expression other glutamate receptor subunits or PSD95. These data indicate that the global deletion of *Homer2* leads to aberrant regulation of dendritic spine morphology in the NAc core that is associated with an increased density of long, thin spines. Unexpectedly, intermittent IP ethanol did not affect spine morphology in either WT or KO mice. Together these data implicate Homer2 in the formation of long, thin spines and further supports its role in neuronal structure.

Keywords: dendritic spines, nucleus accumbens, structural plasticity, cellular morphology, Homer2, postsynaptic density

INTRODUCTION

Chronic intermittent ethanol exposure and withdrawal induce plasticity in glutamatergic synapses of the nucleus accumbens (NAc). These neuroadaptations include altered synaptic expression of N-methyl-D-aspartate (NMDA)-type glutamate receptor subunits (Gremel and Cunningham, 2009; Obara et al., 2009; Mulholland et al., 2011), increased extracellular glutamate levels in ethanol-seeking behaviors (Gass et al., 2014; Griffin et al., 2014), and morphological adaptations in the size and density of dendritic spines in the NAc (Zhou et al., 2007; Spiga et al., 2014). Furthermore, these glutamate-based neuroadaptations are thought to contribute to the severity of withdrawal symptoms, consumption of large amounts of ethanol (EtOH), and relapse-seeking behaviors (Kalivas, 2009).

Dendritic spine morphology and NMDA receptor expression are intimately associated with the form and function of the postsynaptic density (PSD), an area of dense protein scaffolding in the postsynaptic membrane (Sheng and Sala, 2001; Kristiansen et al., 2006; Cui et al., 2007; Ultanir et al., 2007). The Homer family of proteins (Homer1a/b/c, 2a/b, and Homer3a/b) plays an integral role in the structure of the PSD by tethering metabotropic glutamate (mGlu) receptors, IP_3 receptors, and ionotropic glutamate receptors to scaffolding proteins through an Enabled/VASP homology 1 (EVH1) domain (Xiao et al., 1998; Shiraishi-Yamaguchi and Furuichi, 2007). Homer-linked structures are thought to act as a signaling complex by linking various proteins in close proximity, ultimately facilitating signal transduction. For example, mGlu receptors, IP_3 receptors, and Shank are all critically involved in Ca^{2+} signaling at the PSD, and are bound in proximity to each other by Homer and other scaffolding proteins. Subsequently, activity-dependent reversible clustering of these Homer complexes could lead to structural and functional remodeling of the synapse (Shiraishi-Yamaguchi and Furuichi, 2007; Shiraishi-Yamaguchi et al., 2009). Evidence also suggests that Homer2 regulates expression of the GluN2A subunit of the NMDA receptor in the accumbens (Szumlinski et al., 2005). Because Homer2a/b is highly expressed throughout the CNS and is predominant in the PFC, NAc, and striatum (Shiraishi-Yamaguchi and Furuichi, 2007), this member of the Homer family is of particular

interest when studying neuronal plasticity at the synaptic level.

In models of cocaine addiction, Homer2 has been shown to regulate behavioral and biochemical sensitivity to the drug (Szumlinski et al., 2004). A plethora of recent study also implicate Homer2 in the maladaptive behavioral plasticity associated with ethanol reward and addiction (Szumlinski et al., 2005; Obara et al., 2009; Goulding et al., 2011; Cozzoli et al., 2012). Homer2a/b and mGlu1/5 expression are increased in the NAc after chronic intermittent intraperitoneal (IP) injections of ethanol (Szumlinski et al., 2005; Goulding et al., 2011). These studies suggest that ethanol exposure can produce relatively long-lasting adaptations of Homer2 expression and glutamatergic signaling in the NAc. In the present study, we investigated how the deletion of *Homer2* impacted dendritic spine morphology and protein expression in PSD-enriched tissue from the NAc using a chronic intermittent IP ethanol exposure model. These studies revealed that this chronic intermittent ethanol exposure paradigm resulted in increased GluN2B expression in both wild-type (WT) and knockout (KO) mice, and that *Homer2* deletion is associated with an increase in the density of long, thin spines. Taken together, these observations provide evidence that Homer2 plays a role in the regulation of dendritic spine morphology, and further suggests that homeostatic regulation of GluN2B in response to ethanol exposure is robust enough to overcome the absence of Homer2.

MATERIALS AND METHODS

CHRONIC INTERMITTENT IP ETHANOL EXPOSURE

Adult (8–10 weeks of age at the start of experimentation) male WT mice and mice with null mutations of *Homer2* (backcrossed with C57BL/6J mice for >6 generations) were generated and maintained by heterozygous mating as described previously (Szumlinski et al., 2005). Genotype for each mouse was determined in duplicate, and only mice with confirmed genotype were included in the study. Mice were group housed (3–4/cage) under a reverse 12 h light/dark cycle (lights on at 0200). Rodent chow (Harlan Teklad, Madison, WI, USA) and water were available *ad libitum*. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited facility with automated temperature, humidity, and light cycle control. Following a protocol previously reported to induce locomotor sensitization and conditioned place preference (Nocjar et al., 1999; Szumlinski et al., 2005), mice received intraperitoneal (IP) injections of vehicle (0.9% sterile saline) or 3 g/kg ethanol (in 0.9% sterile saline; 0.2 ml/g) every other day for a total of eight injections. All work was approved by the Institutional Animal Care and Use Committee and conducted according to the requirements of the NIH Guide for the Care and Use of Laboratory Animals (2011).

BLOOD ETHANOL CONCENTRATION

To determine genotypic differences in ethanol metabolism and any development of metabolic tolerance, blood samples were taken from the infraorbital sinus from WT and *Homer2* KO mice 1 h after the first and last ethanol injection. Blood ethanol concentrations (BECs; mg%) were analyzed using a modified version of a previously described colorimetric alcohol oxidase assay (Prencipe et al., 1987).

ETHANOL-INDUCED SEDATION

To assess the effect of deletion of Homer2 on the sedative and motor-impairing effects of ethanol, we utilized an ethanol-sedation test using previously described methods (Szumlinski et al., 2005). Knock out and WT mice received intraperitoneal (IP) injections of 5 g/kg ethanol. Once immobile, the mice were laid on their backs in their home cages and the time to regain their righting reflex as defined by the time taken to place all four paws on the cage floor was measured.

SUBCELLULAR FRACTIONATION AND WESTERN BLOTTING

Tissue punches were taken from the NAc core of WT and *Homer2* KO mice 1 h after the final ethanol or saline injection. Triton X-100 insoluble fractions that are enriched in PSD proteins were prepared as previously described (Mulholland et al., 2011). In brief, a Dounce homogenate was prepared and centrifuged at 12,000 × *g* for 20 min to obtain a membrane fraction. The pellet was resuspended in buffer containing 0.5% Triton X-100 and rotated at 4°C for 15 min. This fraction was then centrifuged at 12,000 × *g* for 20 min to yield soluble and insoluble fractions. The insoluble fraction was then sonicated in 2% LDS and stored at −80°C until analysis.

For western blot procedures samples were diluted with NuPAGE 4X LDS sample loading buffer (Invitrogen Corporation, Carlsbad, CA, USA; pH 8.5) containing 50 mM dithiothreitol, and samples were denatured for 10 min at 70°C. Five micrograms of each sample were separated using the Bis-Tris (375 mM resolving buffer and 125 mM stacking buffer, pH 6.4; 7.5% acrylamide) discontinuous buffer system with MOPS electrophoresis buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7). Protein was then transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After transfer, blots were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and then blocked with PBST containing 5% non-fat dried milk (NFDM) for 1 h at room temperature with agitation. The membranes were then incubated overnight at 4°C with primary antibodies diluted in PBST containing 0.5% NFDM and washed in PBST prior to 1 h incubation at room temperature with horseradish peroxidase conjugated secondary antibodies diluted 1:2000 in PBST. Membranes received a final wash in PBST and the antigen-antibody complex was detected by enhanced chemiluminescence using a ChemiDoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The bands were quantified by mean optical density using computer-assisted densitometry with ImageJ v1.41 (National Institutes of Health, USA). Because the use of loading controls [e.g., actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] for normalization in western blot experiments are subject to quantitation errors (Dittmer and Dittmer, 2006; Aldridge et al., 2008), normalization to a total protein stain (i.e., amido black) was used in these studies. Before each study, a series of western blots were performed using different titrations of sample and antibody to

establish the linear range for each target. GluN1 antibody was purchased from BD Pharmingen (1:4000; Catalog # 556308; San Jose, CA, USA). GluN2A and GluA1 antibodies were purchased from EMD Millipore (1:2000; Catalog # 07-732; Billerica, MA, USA). GluN2B and PSD95 antibodies were purchased from the UC Davis/NIH NeuroMab Facility (1:2000; Catalog # 75-097; Davis, CA, USA).

DENDRITIC SPINE LABELING, MORPHOLOGICAL CLASSIFICATION, AND ANALYSIS

Neuronal labeling and morphological classification of dendritic spines of medium spiny neurons (MSNs) in the NAc core were carried out using previously reported methods (Jung et al., 2013; McGuier et al., 2014). One hour after the final ethanol injection, mice were anesthetized with urethane (1.5 g/kg, IP) and perfused with 0.1 M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were blocked and post-fixed for 30 min. Next, 150 μm thick coronal slices were prepared using a vibratome. DiI coated tungsten particles (1.3 μm diameter) were delivered to the slices using a modified Helios Gene Gun (Bio-Rad; Hercules, CA, USA) fitted with a polycarbonate filter (3.0 μm pore size; BD Biosciences; San Jose, CA, USA). Slices were left overnight at 4°C in PB to allow the DiI to completely diffuse through labeled neurons and sections were post fixed in 4% PFA for 1 h at room temperature. After mounting with Prolong Gold Antifade mounting media (Life Technologies; Carlsbad, CA, USA), slices were imaged (1–4 dendritic sections/mouse; voxel size: 47 nm × 47 nm × 100 nm) using a Zeiss LSM 510 confocal microscope fitted with a 63× oil immersion objective (Plan-Apochromat, Zeiss, NA = 1.4, working distance = 190 μm). Images of sections of dendrites starting >75 μm away from the soma of MSNs in the NAc core were acquired and then deconvolved using AutoQuant (Media Cybernetics; Rockville, MD, USA). Imaris XT (Bitplane; Zurich, Switzerland) was used to generate a filament of the dendritic shaft and spines. Dendritic spines were identified using Imaris software and then classified into four categories (stubby, long, filopodia, and mushroom) based on the spine length and the width of the spine head and neck, where L is spine length, D_H is spine head diameter, and D_N is spine neck diameter. Long spines were identified as having a $L \geq 0.75$ μm and <3 μm, mushroom spines had a $L < 3.5$ μm, $D_H > 0.35$ μm and a $D_H > D_N$, stubby spines had a $L < 0.75$ μm, and filopodia were identified as having a $L \geq 3$ μm. Data on dendritic spine parameters were averaged for each dendritic section and were collated from the Imaris output via custom scripts written in Python. Dendritic spine data were then averaged for each mouse, and the data were analyzed using two-way ANOVAs and unpaired, two-tailed t-tests. All data are reported as mean ± SEM and statistical significance was established with $p < 0.05$.

RESULTS

Homer2 KO AND REPEATED ETHANOL EXPOSURE

It has been reported that *Homer2* KO mice exhibit enhanced aversion to ethanol, fail to sensitize to repeated injections of ethanol, and display increased latency to regain a righting reflex (Szumlinski et al., 2005). In our initial set of studies, we sought to replicate these behavioral and metabolic experiments as a means of confirming the behavioral phenotype of *Homer2* deletion in our cohort of mice. After the first and last of eight IP injections of ethanol, the blood ethanol concentration (BEC) was determined in WT and KO mice. As shown in **Figure 1A**, there was no interaction of genotype and time in BECs of the mice after the eighth injection compared to the first injection [repeated-measures two-way ANOVA $F(1,9) = 4.8$; $p = 0.0552$; $n = 4$–7/group]. However, there was a main effect of time on BEC [$F(1,9) = 19.70$; $p = 0.0016$]. Also shown in **Figure 1B**, body weights did not differ by genotype or treatment when measured after the first and last injection. Lastly, in a latency to right task, WT and *Homer2* KO mice were administered a sedative dose of ethanol (5 g/kg IP) and the time until the mice regained its righting reflex was determined. In contrast to previously published work (Szumlinski et al., 2005), deletion of *Homer2* did not influence latency to right (**Figure 1C**; $n = 7$–8/genotype). Therefore, *Homer2* KO mice did not exhibit changes in ethanol metabolism or body weight after repeated ethanol exposure or latency to right after a challenge injection of acute ethanol.

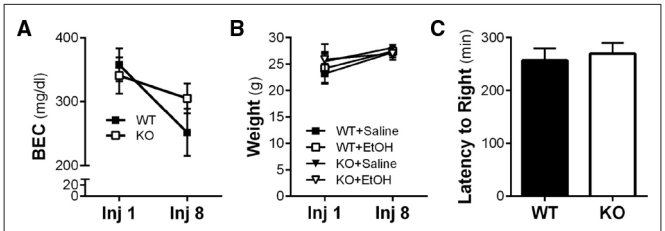

FIGURE 1 | Ethanol-induced behavior and metabolic adaptations are not altered by *Homer2* KO. (A) Neither WT nor KO mice developed metabolic tolerance to ethanol after the final IP injection and there was no effect of genotype on ethanol metabolism. **(B)** Wild-type and KO mouse weights were not different from each other and did not change during the course of the chronic intermittent ethanol IP injection procedure. **(C)** There was no effect of genotype on loss of righting reflex in response to a single 5 g/kg IP injection of ethanol.

REPEATED ETHANOL EXPOSURE, *Homer2*, AND PSD PROTEINS

Homer2 plays an integral role in the scaffolding network of the PSD, and repeated bouts of ethanol exposure and withdrawal are known to induce adaptations in the expression of glutamate receptors at synapses in the NAc (Zhou et al., 2007; Spiga et al., 2014; Uys et al., in press). Thus, we hypothesized that the absence of the Homer2 protein would prevent these changes in ethanol-induced neuroplasticity. To test this hypothesis, WT and KO mice were subjected to the chronic intermittent IP ethanol injection procedure, and a PSD-enriched faction was prepared 1 h after the last injection. As shown in **Figure 2**, western blot analysis revealed that there was no significant treatment effect or interaction of genotype on the expression levels of PSD95, GluN1, GluN2A, or GluA1, but there was a significant effect of ethanol on the expression of GluN2B in both WT and KO mice [main effect of treatment $F(1,27) = 8.315$, $p = 0.0076$]. These data indicate that while chronic intermittent IP injections of ethanol induces biochemical adaptations in GluN2B expression, this increase does not depend on Homer2 expression.

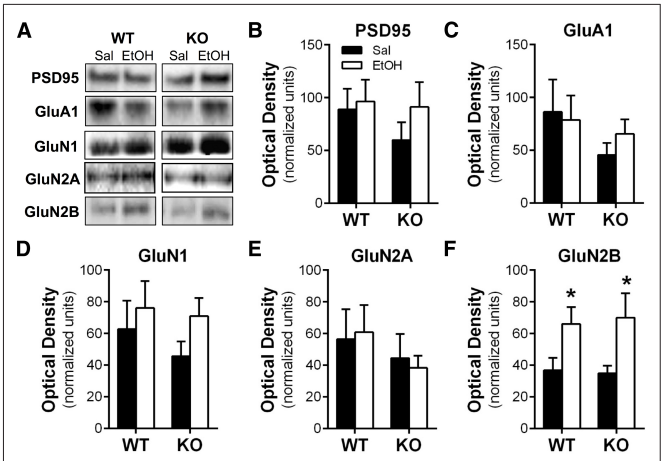

FIGURE 2 | Repeated chronic intermittent injections of ethanol induced NMDA receptor plasticity independent of genotype. (A) Representative images of western blots of the PSD-enriched tissue samples. There was no effect of ethanol treatment or genotype on the expression of **(B)** PSD-95, **(C)** GluA1, **(D)** GluN1, or **(F)** GluN2A. **(E)** Repeated intermittent injections of ethanol resulted in increased expression of GluN2B in both WT and *Homer2* KO mice (*$p < 0.05$).

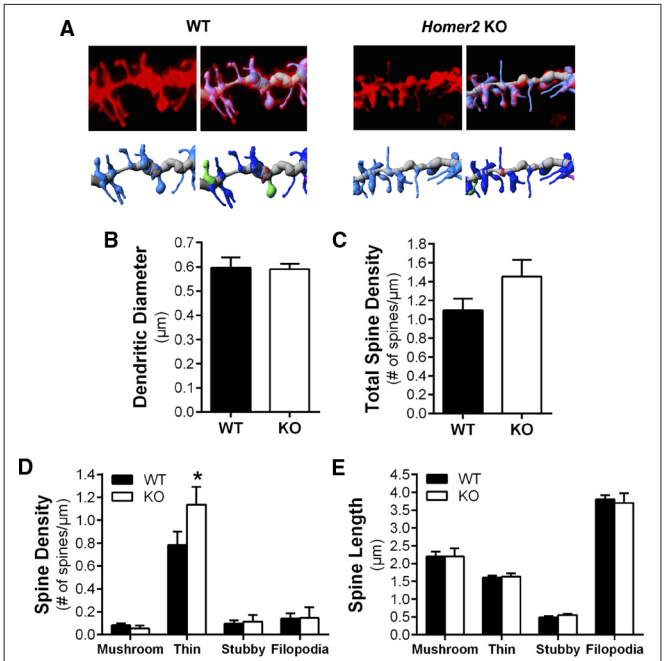

FIGURE 3 | Deletion of Homer2 increased long spine density compared to WT mice. (A) Representative images of diolistically labeled dendritic segments from WT saline and Homer2 KO saline treated mice. The dendritic shaft is shown in gray and spines are shown in light blue. Classified spines are b color (dark blue = long, thin; pink = filopodia; red = stubby; green = mushroom). **(B)** Dendritic diameter and **(C)** total spine density were not altered by genotype. **(D)** Analysis of spine subclasses revealed that long thin spine density is increased in KO mice compared WT (*$p < 0.05$). **(E)** There were no genotypic differences in spine length.

Homer2 AND DENDRITIC SPINES

Repeated cycles of ethanol exposure and withdrawal are known to induce adaptations in the morphology of dendritic spines in MSNs of the NAc core and shell (Zhou et al., 2007; Spiga et al., 2014; Uys et al., in press). Given that Homer2 is a key component of the PSD scaffold, we next investigated whether the genetic deletion of this protein altered structural plasticity by examining spine morphology in the MSNs of the NAc core. Analyses comparing saline-injected WT and KO mice revealed no differences in total spine density [two-tailed *t*-test, $t(15) = 1.682$, $p = 0.1133$, n = 6–11 mice/genotype, 11–23 dendritic segments/genotype; **Figures 3A,C**], or the diameter of the dendritic shaft [two-tailed *t*-test, $t(15) = 0.1011, 0.9208$; **Figure 3B**]. Dendritic spines can be classified into subclasses (stubby, mushroom, long, or filopodia) based on the morphological characteristics of the spine. Length and diameter of the spine neck and terminal point, and the shape of the spine have been associated with differential expression of glutamate receptors and Ca^{2+}- and cAMP-regulated signaling proteins (Zhou et al., 2007; Obara et al., 2009; Goulding et al., 2011; Cozzoli et al., 2012; Lum et al., 2014). Comparison of these spine parameters showed no difference in spine length [two-way ANOVA, $F(3,52) = 0.1606$, $p = 0.9224$, n = 6–11 mice/genotype, 11–23 dendritic segments/genotype; **Figure 3E**], while the density of long, thin spines was significantly increased in the KO mice compared to WT [two-way ANOVA, $F(3,60) = 2.551$, $p = 0.0640$; *post hoc* $p < 0.05$; n = 6–11/genotype, 11–23 dendritic segments/genotype; **Figure 3D**].

Wild-type and *Homer2* KO mice also received chronic intermittent IP ethanol injections to determine if Homer2 regulates ethanol-induced adaptations in dendritic spine morphology. In contrast to our hypothesis, chronic intermittent IP injections of ethanol had no effect on dendritic spine morphology or density, and there was no interaction between genotype and ethanol treatment on total spine density [two-way ANOVA, $F(1,27) = 0.6362$, $p = 0.4320$, n = 6–11 mice/group, 11–23 dendritic segments/group] or spine density by class [two-way ANOVAs, thin: $F(1,27) = 0.3708$, $p = 0.5477$; mushroom: $F(1,27) = 0.008345$, $p = 0.9279$; stubby: $F(1,27) = 0.04343$, $p = 0.8365$; filopodia: $F(1,27) = 0.4314$, $p = 0.5169$; n = 6–11 mice/group, 11–23 dendritic segments/group; **Figures 4A–F**]. In the case of long spine density, deletion of Homer2 lead to increased density regardless of treatment [two-way ANOVA, main effect of genotype $F(1,27) = 7.221$, $p = 0.0122$; n = 6–12 mice/treatment; **Figure 4C**]. Together these data indicate that Homer2 deletion increases density of long, thin spines.

DISCUSSION

This study investigated the involvement of the PSD scaffolding protein Homer2 in ethanol-induced biochemical and morphological adaptations of the NAc using a chronic intermittent IP ethanol injection model. These studies showed no effect of intermittent IP ethanol exposure or genotype on ethanol metabolism, weight, or latency to right when comparing WT and *Homer2* KO mice. The expression of GluN2B was elevated in the PSD from both WT and KO mice after repeated ethanol exposure, however there was no influence of genotype on protein expression. Finally, while *Homer2* KO mice exhibited an increase in the density of long, thin spines in the NAc core compared to WT mice, chronic intermittent IP injections of

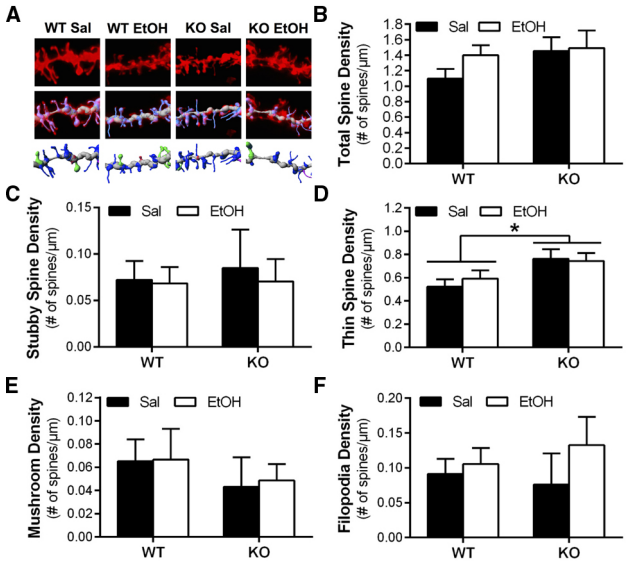

FIGURE 4 | Deletion of Homer2 is associated with increased long spine density compared to WT mice that is unaffected by repeated ethanol exposure. (A) Representative images of WT and Homer2 KO saline (sal) and ethanol (EtOH) treated mice showing a diolistically labeled dendrite segment (top), a labeled dendrite with a computer-generated filament (middle), and the filament with classified spines (blue = long thin, pink = filopodia, red = stubby, green = mushroom; bottom). **(B)** Total spine density was unaffected by genotype or treatment. **(C)** Class analyses of spine density showed no effect of treatment or genotype on stubby spine density. **(D)** There was a main effect of genotype on thin spine density ($^*p = 0.0122$), but no effect of treatment. Similar analyses showed no effect of treatment or genotype on the density of **(E)** mushroom spines or **(F)** filopodia. Representative images for WT Sal and KO Sal are the same as those used in **Figure 3**.

ethanol did not induce morphological adaptations in this brain region.

It has been suggested that the Homer family of proteins are potential key regulators of dendritic spine morphology (Sala et al., 2002, 2005). In cultured hippocampal neurons, Sala et al. (2002) showed that dendritic spines on immature neurons over-expressing Shank were shorter and displayed thicker heads that morphologically resembled more mature type spines. Furthermore this study showed that a Homer1b-Shank interaction was necessary for this phenomenon. Additional studies in HeLa cells have shown that Cupidin's (a Homer2a isoform) interaction with the small GTPase Cdc42 within a scaffolding network is required to prevent Cdc42-induced filopodia-like protrusion formation (Shiraishi-Yamaguchi et al., 2009). This group also showed that without Cupidin localized to the synapse in cultured hippocampal neurons, there was a marked decrease in miniature excitatory postsynaptic currents. Altogether these studies suggest that, at least in culture, Homer is necessary for spine maturation and synaptic function. In the present study, we observed that Homer2 deletion leads to an increase in the density of long spine on MSNs of the NAc core under basal conditions. This class of spine is characterized by its long, thin appearance, no significant head and neck separation, and a lack of a substantial PSD network (Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970; Uys et al., in press). Interestingly, long spines are associated with enhanced plasticity, and are believed to be precursors to the more stable and mature mushroom type "memory" spines (Holtmaat et al., 2006; Bourne and Harris, 2007; Arnsten et al., 2012), and recently it was reported that chronic ethanol exposure altered spine density in the NAc (Zhou et al., 2007; Spiga et al., 2014; Uys et al., in press). While we observed that Homer2 deletion did not alter the expression of PSD95 in PSD-enriched samples from the NAc, it is possible that the absence of Homer2 destabilizes PSD formation and promotes the formation of immature long spines. Indeed previous reports have shown a necessity of a Homer/c interaction to induce spine head enlargement via recruitment of IP_3 receptors and subsequently ER cisternas in cultured hippocampal neurons (Sala et al., 2002, 2005).

In contrast with the influence of genotype on spine morphology, we did not observe an effect of chronic intermittent IP injection of ethanol on spine morphology or an interaction between genotype and ethanol treatment on long spine density. Although speculative, the intermittent IP ethanol exposure paradigm used in the present study could explain this lack of effect. Several rodent models of ethanol exposure have been shown to induce morphological adaptations in dendrites and dendritic spines in the NAc. In a free-choice drinking model, alcohol-preferring rats given either continuous access or repeated deprivation of ethanol resulted in fewer spines with larger heads in MSNs of the NAc (Zhou et al., 2007). In a separate study, alcohol-dependent rats on a liquid diet exhibited decreased overall spine density in the NAc shell, with long spines being selectively decreased (Spiga et al., 2014). In addition, we have previously shown that chronic intermittent ethanol exposure by vapor inhalation resulted in an overall increase in dendritic spine density in the NAc core that was attributed to an increase in long spine density (Uys et al., in press). Unfortunately, we were unable to test the *Homer2* KO mice in this dependence model because our preliminary experiments showed reduced survival rates during the withdrawal phase (unpublished observation), suggesting that *Homer2* KO mice are more sensitive to withdrawal-induced hyperexcitability than WT mice. Though the intermittent IP ethanol exposure paradigm used in the current study has been previously reported to induce biochemical adaptations in mice (Szumlinski et al., 2005; Goulding et al., 2011), in our hands this model did not result in changes in dendritic spine morphological plasticity in the NAc core. However, we did observe that *Homer2* KO mice have alterations in the density of long spines similar to the morphological phenotype in this region reported in other models of chronic ethanol exposure. As other abused substances (e.g., cocaine, heroin, and methamphetamine) produce morphological adaptations in dendritic spines, future studies should determine a role for Homer2 in regulation of spine density and morphology in other models of chronic drug exposure.

Increased expression of NMDA receptor subunits is associated with repeated ethanol exposure (Carpenter-Hyland and Chandler, 2007; Nagy, 2008). In particular, there is a marked increase in the expression and function of NMDA receptors in the NAc in continuous access and repeated withdrawal models of ethanol exposure (Zhou et al., 2007; Spiga et al., 2014). It is thought that this may represent a compensatory adaptation

to counterbalance the inhibitory effect on neuronal excitability (Carpenter-Hyland and Chandler, 2006). Using western blot analysis we examined the expression of GluA1, NMDA receptor subunits and the scaffolding protein PSD95 1 h after the final injection. Consistent with previous findings, WT ethanol-exposed mice exhibited increased expression of the GluN2B subunit compared to saline controls, validating the ability of the intermittent IP injection paradigm of exposure to induce biochemical adaptations in NMDA receptors. We also observed that *Homer2* KO mice exhibited the same ethanol-induced increase in GluN2B expression. This finding is particularly interesting given that Homer2 clusters with NMDA receptors containing the GluN2B subunit (Shiraishi-Yamaguchi and Furuichi, 2007). These data therefore suggest that while Homer2 may play a role in PSD plasticity, increased GluN2B expression by ethanol is mediated through an additional process that is independent of Homer2.

In contrast to previous work showing a reduction in GluN2A expression in the accumbens of Homer2 KO mice (Szumlinski et al., 2005), we did not observe an effect of genotype on the expression of GluN2A. This discrepancy may be explained by the fractionation method used in preparation of the western blot samples. In the present study, a synaptosomal fraction was obtained and treated with Triton X-100 to isolate PSD-enriched proteins, yet in the previous studies western blot analysis was carried out using a total synaptosomal fraction (i.e., one containing proteins from both the synaptic and extrasynaptic membrane; Toda et al., 2003). Our data may therefore indicate that the KO-induced decrease in GluN2A might be restricted to the extrasynaptic pool of receptors.

Our studies attempted to link ethanol-induced behavior and *Homer2* KO by measuring latency to right in both WT and KO mice to confirm that the previously reported behavioral phenotypes were maintained in the current cohorts of mice (Szumlinski et al., 2005). Unexpectedly, we did not observe an effect of genotype on righting behavior after a sedating dose of acute ethanol. This is in direct contrast to previously published work (Szumlinski et al., 2005), which showed that the *Homer2* KO mice are more sensitive to the sedative effects of ethanol compared to their WT littermates. One potential explanation for this discrepancy could be genetic drift, which describes the tendency of genes to continuously evolve without selective pressure (Silver, 1995). As an example of this phenomenon, extensive homozygotic inbreeding of 5-HT_{1B} KO mice resulted in the loss of a unique drinking phenotype (Bouwknecht et al., 2000). To avoid genetic drift, mutant and control (C57BL/6J) populations should occasionally be interbred and the resulting heterozygotic offspring should be used to breed KO animals (Phillips et al., 1999). We implemented this breeding schema, suggesting that it is unlikely that genetic drift can account for the lack of effect of genotype on ethanol sedation in the present study. It is also possible that the progenitor strain (129Xi/SvJ; Shin et al., 2003), from which the embryonic stem cells (ES) were derived to generate the *Homer2* KOs, is a phenotypic match for the predicted ethanol-induced behavioral adaptations associated with *Homer2* deletion. This was the case in a dopamine receptor 2 deficient mouse where the ES progenitor strain (129/SvEv) used to generate the mutant mouse strain endogenously exhibited the same locomotor deficits as was predicted for the mutant. As a result, the mutant mouse performed the same as the inbred 129/SvEv mouse strain despite backcrossing the mutant with C57BL/6 mice (Kelly et al., 1998). In the case of the *Homer2* KO mice, it is possible that the effect of ethanol on loss of righting originally seen by Szumlinski et al. (2005) might have been a phenotype endogenous to 129Xi/SvJ mice that dissipated in our colony as the *Homer2* KO mice were continually crossbred with C57BL/6J mice over several more generations.

In summary, the results of the present study show that while chronic intermittent IP injections of ethanol induce increases in GluN2B expression in NAc of both WT and *Homer2* KO mice, deletion of *Homer2* does not effect this ethanol-induced adaptation. A lack of effect of intermittent IP ethanol exposure on dendritic spine morphology in both WT and KO mice indicate that the IP ethanol injection model of exposure does not appear to be sufficient to induce morphological adaptations. Finally, these studies show that deletion of *Homer2* leads to an increase in long thin spines of MSNs in the NAc core, providing the first evidence that *Homer2* directly affects dendritic spine morphology *in vivo*.

ACKNOWLEDGMENTS

This work was supported by NIH grants AA020930, AA010983 and AA010761, and AA021618. The authors would also like to thank Andrew McGuier and Vincent Pistelli for writing the Python scripts to collate the dendritic spine data exported from the ImarisXT imaging program.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 December 2014; accepted: 03 February 2015; published online: 23 February 2015.

*Citation: McGuier NS, Padula AE, Mulholland PJ and Chandler LJ (2015) Homer2 deletion alters dendritic spine morphology but not alcohol-associated adaptations in GluN2B-containing N-methyl-D-aspartate receptors in the nucleus accumbens. Front. Pharmacol. **6**:28. doi: 10.3389/fphar.2015.00028*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 25 November 2014
doi: 10.3389/fphar.2014.00256

$GABA_A$ receptor drugs and neuronal plasticity in reward and aversion: focus on the ventral tegmental area

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$GABA_A$ receptors are the main fast inhibitory neurotransmitter receptors in the mammalian brain, and targets for many clinically important drugs widely used in the treatment of anxiety disorders, insomnia and in anesthesia. Nonetheless, there are significant risks associated with the long-term use of these drugs particularly related to development of tolerance and addiction. Addictive mechanisms of $GABA_A$ receptor drugs are poorly known, but recent findings suggest that those drugs may induce aberrant neuroadaptations in the brain reward circuitry. Recently, benzodiazepines, acting on synaptic $GABA_A$ receptors, and modulators of extrasynaptic $GABA_A$ receptors (THIP and neurosteroids) have been found to induce plasticity in the ventral tegmental area (VTA) dopamine neurons and their main target projections. Furthermore, depending whether synaptic or extrasynaptic $GABA_A$ receptor populations are activated, the behavioral outcome of repeated administration seems to correlate with rewarding or aversive behavioral responses, respectively. The VTA dopamine neurons project to forebrain centers such as the nucleus accumbens and medial prefrontal cortex, and receive afferent projections from these brain regions and especially from the extended amygdala and lateral habenula, forming the major part of the reward and aversion circuitry. Both synaptic and extrasynaptic $GABA_A$ drugs inhibit the VTA GABAergic interneurons, thus activating the VTA DA neurons by disinhibition and this way inducing glutamatergic synaptic plasticity. However, the $GABA_A$ drugs failed to alter synaptic spine numbers as studied from Golgi-Cox-stained VTA dendrites. Since the GABAergic drugs are known to depress the brain metabolism and gene expression, their likely way of inducing neuroplasticity in mature neurons is by disinhibiting the principal neurons, which remains to be rigorously tested for a number of clinically important anxiolytics, sedatives and anesthetics in different parts of the circuitry.

Keywords: $GABA_A$ receptor, benzodiazepines, THIP, neurosteroids, dopamine neurons, neuroadaptation, dendritic spines

INTRODUCTION

$GABA_A$ receptor agonists generally depress brain activity. Benzodiazepines (BZs) and other GABAmimetic drugs depress gene expression in the brain, including the neuroplasticity-related genes such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and Fos-genes (Zafra et al., 1991; Huopaniemi et al., 2004). BZs and volatile anesthetic isoflurane also have been reported to reduce long-term potentiation (LTP) in several brain regions (Higashima et al., 1998; Kulisch et al., 2011; Piao et al., 2013). Moreover, BZs and general anesthetics are able to impair neurogenesis in both young and adult animals (Wu and Castren, 2009; Erasso et al., 2013; Thal et al., 2014). All these facts suggest that $GABA_A$ receptor agonists have little potential for neuroplasticity outside the critical periods of brain development, when the role of GABAergic interneurons is obligatory (Hensch and Stryker, 2004). On the other hand, by suppressing the local inhibitory regulation, $GABA_A$ receptors on GABAergic interneurons can indirectly cause activation of principal neuron populations, a network effect called disinhibition. For example, GABAergic neurons in the lateral part of the central nucleus of amygdala are excited by low acute doses of BZs through disinhibition and this is associated with anxiolytic effects (Beck and Fibiger, 1995; Salminen et al., 1996; Panhelainen and Korpi, 2012). Furthermore, $GABA_A$ receptor agonists can activate the dopaminergic (DAergic) neurons in the ventral tegmental area (VTA) by disinhibition (Heikkinen et al., 2009; Tan et al., 2010; Vashchinkina et al., 2012).

In this review we will discuss recent advances in understanding how $GABA_A$ receptor modulators affect both GABAergic and glutamatergic synapses and also how their acute or repeated treatments can modulate plasticity of the reward system. Given the widespread application of $GABA_A$ receptor drugs, understanding more fully how they modulate brain reward system may help to develop new strategies for designing novel compounds to overcome their therapeutic limitations.

GABA$_A$ RECEPTORS AND THEIR MODULATORS

Benzodiazepines, inhalational and intravenous anesthetics, barbiturates, neurosteroids, and other GABAmimetic drugs – all share the interaction with the GABA$_A$ receptor and facilitation of receptor function to produce strong pharmacological and behavioral actions (Sieghart, 1995; Korpi et al., 2002). They act on distinct sites on the GABA$_A$ receptor and increase membrane anion (Cl^- and bicarbonate) conductance, thereby in most cases inducing hyperpolarization, which has an inhibitory effect on the firing of the postsynaptic neurons.

The GABA$_A$ receptors belong to the "cys-loop" superfamily of ligand-gated ion channels (Alexander et al., 2013). They exist as heteropentameric structures, commonly composed of two α subunits, two β subunits, and one γ or δ subunit (McKernan and Whiting, 1996). The BZ-sensitive GABA$_A$ receptors contain either α1, α2, α3, and/or α5 subunits and as a result of containing the γ2 subunit, they are preferentially located at synaptic sites (with the exception of α5), whereas the α4 or α6 subunit-containing GABA$_A$ receptors are highly sensitive to neurosteroids, GABAmimetic drugs such as muscimol and THIP (gaboxadol; 4,5,6,7-tetrahydroisoxazolol[4,5-c]pyridine-3-ol), general anesthetics such as isoflurane and etomidate. Since they often possess the δ subunit instead of γ2, they are located at peri- or extrasynaptic sites (Olsen and Sieghart, 2009).

GABA$_A$ receptor modulators are effective in the wide range of indications (anxiety disorders, panic, insomnia, muscle spams, seizure control in epilepsy and alcohol withdrawal, sedation of aggressive patients, calming down anxious patients before operations, and induction of anesthesia) by acting on distinct GABA$_A$ receptor subtypes which demonstrate a unique heterogeneity in terms of function, kinetics, pharmacological profile, and distribution in brain (Uusi-Oukari and Korpi, 2010). Combination of pharmacological and genetic approaches has revealed that 1) α1 subunit-containing GABA$_A$ receptors mainly mediate the sedative and addictive effects of BZs, 2) α2 or α3 subunit-containing receptors mediate the anxiolytic and muscle-relaxant effects, and 3) α5 subunit-containing receptors mediate the memory-impairing effects of BZs (Crestani et al., 1999; Rudolph and Mohler, 2004). Furthermore, there is growing evidence that hippocampal extrasynaptic α5 subunit-containing receptors contribute to amnestic effects by general anesthetics (Grasshoff et al., 2005), while the stimulation of δ subunit-containing receptors mediate anxiolytic, anticonvulsive, anesthetic, and aversive effects of neurosteroids in mice (Mihalek et al., 1999; Vashchinkina et al., 2014).

Despite the usefulness of GABA$_A$ receptor modulators, their use may lead to side-effects that limit their efficacy. General anesthetics may induce postoperative cognitive dysfunction for weeks or months in elderly patients (Canet et al., 2003; Newman et al., 2007), and their use in prolonged operations at neonatal period, although necessary, have raised serious thoughts about possible neuronal damage and long-term cognitive effects as seen in preclinical models (Jevtovic-Todorovic et al., 2013). Beside the short-term undesirable effects of BZs such as dizziness and motor impairment, their long-term effects include disruption of sleep architecture, confusion and memory impairment, tolerance and dependence (Licata and Rowlett, 2008). Furthermore, chronic increased exposure to neurosteroids appears to accelerate development of Alzheimer's disease symptoms in various mouse models (Bengtsson et al., 2012, 2013). Thus, the use of these drugs causes persistent neuroadaptation in the brain that also may contribute to adverse effects.

GABA$_A$ DRUGS IN THE ANIMAL MODELS OF ADDICTION

Addiction is increasingly seen as a disease of aberrant neuroadaptation in the brain reward system (Volkow and Baler, 2014). The VTA dopamine neurons are considered as an essential hub at least for the early phases of addiction. VTA has reciprocal connections with many forebrain centers such as the nucleus accumbens (NAc, ventral striatum), dorsal striatum, the medial prefrontal cortex (mPFC), the extended amygdala (particularly including the basolateral (BLA) and central nuclei of amygdala and the bed nuclei of stria terminalis) and lateral habenula, which circuitries form the major part of reward and aversion pathways.

The risk of developing addiction is one of the main challenges restricting the clinical use of BZs and other GABA$_A$ modulators (O'Brien, 2005). Addiction-related behaviors can also be studied in experimental animal models. Drugs of abuse activate the reward system and produce reinforcing effects in drug self-administration, in potentiation of the intracranial electrical self-stimulation reward, and in drug-induced learning and conditioning. Various non-selective BZ agonists, such as diazepam and midazolam, are self-administered (Tan et al., 2010). They are rewarding in conditioned place preference paradigm (Spyraki et al., 1985) and able to increase the rate of responding for and to decrease the threshold of the intracranial self-stimulation reward (Straub et al., 2010). The α1 subunit-preferring agonist zolpidem is self-administered in baboons (Ator, 2002), but produces no place preference in rats (Meririnne et al., 1999), showing thus somewhat unclear effects. This might be due to zolpidem's strong dose-dependent sedative effect. The α2 subunit-containing GABA$_A$ receptors in the NAc were necessary for the midazolam preference in a test giving the mice a choice between sucrose solution and sucrose/midazolam solution (Engin et al., 2014). The endogenous neurosteroid GABA$_A$ agonist allopregnanolone displays variable effects in animal models of addiction. It can maintain oral self-administration, although not in operant setting (Sinnott et al., 2002). It decreases the threshold of the intracranial self-stimulation reward (Fish et al., 2014), and when injected intraperitoneally (ip), it has been shown to induce either rewarding (Finn et al., 1997) or aversive conditioning effects (Beauchamp et al., 2000). Propofol is rewarding in conditioned place preference (Pain et al., 1996), while isoflurane and GABA$_A$ β2/3 subunit-selective etomidate have not been tested so far.

In attempts to further localize the reward-related effects of GABA$_A$ modulators in brain circuitry, intracerebral drug infusions have been used. GABA$_A$ agonist muscimol and antagonist bicuculline show interesting effects as they both are reinforcing and rewarding in drug-naïve animals when infused into the VTA (Ikemoto et al., 1997, 1998; Laviolette and van der Kooy, 2001). These results have been explained as effects arising from two reward systems, one being DAergic and the other non-DAergic. Muscimol is believed to inhibit VTA GABA neurons and thus disinhibit

VTA DA neurons leading to DA antagonist-sensitive reward, while bicuculline also acts on VTA GABA neurons but activates them to target a poorly defined non-DAergic reward pathway insensitive to DA receptor antagonists (Laviolette and van der Kooy, 2001). It remains to be studied, e.g., whether bicuculline acts on VTA GABA neurons projecting to NAc cholinergic interneurons, thereby potentiating appetitive associative learning (Brown et al., 2012). These intracerebral infusion studies should be replicated by regionally and neuronally more specific methods such as chemogenetics or optogenetics to avoid possible confounding infusion-site diffusion to other regions and to various neuronal populations. Furthermore, the brain circuitry for reward and aversion goes well beyond the VTA, stressing the importance of systemic drug experiments for translational relevance.

PLASTICITY OF GLUTAMATERGIC AND GABAergic SYNAPSES

Growing evidence indicates that neuroadaptation (plasticity) in the brain appears in both glutamatergic and GABAergic synapses (for review, see Castillo et al., 2011; Luscher and Malenka, 2011; Kullmann et al., 2012). Synaptic connections are highly plastic and constantly modified by environmental factors and learning tasks. It should be noted that generation of new glutamatergic and GABAergic synapses proceeds under distinct mechanisms with different factors regulating the processes (described below).

The major sites of contact for glutamatergic presynaptic terminals are dendritic spines (Gray, 1959; Dailey and Smith, 1996; Hering and Sheng, 2001), and dynamic changes in the morphology of dendritic spines have been associated with changes in synaptic strength (Segal, 2005). Spine morphology is subject to rapid alteration by patterns of neuronal activity and by activation of postsynaptic glutamate receptors (Lang et al., 2004; Matsuzaki et al., 2004). Thus, synaptic efficacy can be regulated by multiple mechanisms.

Conversely, the predominant sites of contact for GABAergic presynaptic terminals are not located on spines, but directly on dendritic shafts (Freund and Buzsaki, 1996; Somogyi et al., 1998). However, GABAergic system also interacts with synapses on spines. Recent work has indicated that the activity of GABA$_A$ receptors affects spine maturation (Heinen et al., 2003; Jacob et al., 2009). In rat hippocampal cultures, reduced GABA$_A$ receptor endocytosis (and increased activity) is associated with reduced spine maturation and reduced levels of postsynaptic density protein-95 (PSD-95; Jacob et al., 2009), and in the visual cortex of adult GABA$_A$ receptor α1 subunit knockout mice (resulting in compensatory increased inhibition), the spine density is reduced with a corresponding reduction in PSD-95 expression (Heinen et al., 2003), results of both studies being in agreement with GABA$_A$ receptor activation reducing LTP (Higashima et al., 1998). Another study (Shen et al., 2010) showed that development-dependent expression of α4 subunit-containing GABA$_A$ receptors in dendritic spines of CA1 hippocampal neurons plays a role in LTP induction. Notably, activation of these receptors reduces depolarization that is needed to remove Mg^{2+} block of NMDA receptors, thus leading to reduced LTP. Furthermore, a small population of GABAergic interneurons that express somatostatin makes synapses targeting directly on dendritic spine heads in the mouse mPFC (Chiu et al., 2013), being able to control local postsynaptic Ca^{2+} fluxes within the spines. All these different mechanisms indicate that the GABA$_A$ receptor activity may regulate rapid dynamics and long-term structural events in glutamate synapses.

The strength of GABAergic synapses is determined by the size of releasable pool of presynaptic GABA and number and/or diversity of GABA$_A$ receptors sitting on postsynaptic membrane, which, in turn, is largely determined by receptor trafficking to and from the plasma membrane, including receptor insertion, lateral diffusion within membrane, removal, recycling and degradation (Vithlani et al., 2011). Interestingly, high-resolution two-photon imaging in organotypic hippocampal cultures has revealed that new GABAergic synapses are formed by the appearance of new boutons at pre-existing axon-dendrite crossings (Wierenga et al., 2008). Hence, in that model, plasticity in GABAergic synapses depends on the number of available axon-dendrite crossings, which makes it more restricted than plasticity sites in glutamatergic connections. However, it is not known yet whether these *in vitro* results can be generalized to all GABAergic synapses, and, therefore, further *in vivo* studies are needed.

DRUG-INDUCED PLASTICITY OF GABAergic SYNAPSES

In addition to fast modulation of channel gating, both BZs and neurosteroids have longer lasting effects by controlling the number and subtypes of GABA$_A$ receptors on the plasma membrane (Vithlani et al., 2011; Deeb et al., 2012; Abramian et al., 2014). Notably, treatment of hippocampal cultures with BZ agonist flurazepam modulates GABA$_A$ receptor trafficking by promoting selective degradation of α2 subunit-containing GABA$_A$ receptors after their removal from the postsynaptic membrane, leading to a reduction in synapse size and number, and finally to depression of synaptic inhibition (Jacob et al., 2012). The development of tolerance to the sedative effects of diazepam is associated with a decrease in [^{3}H]L-655,708 binding to the hippocampal dentate gyrus α5 subunit-containing receptors (dependent on α1 subunit-containing receptors), an effect which was extended by the absence of tolerance in α5 subunit point-mutant mice (van Rijnsoever et al., 2004). Treatment of *C. elegans* with the GABA-site agonist muscimol also results in selective removal of GABA$_A$ receptors from synapses (Davis et al., 2010). In contrast, neurosteroids can selectively enhance the trafficking of extrasynaptic GABA$_A$ receptors by insertion of new α4 subunit-containing GABA$_A$ receptors into the membrane, resulting in an enhancement of tonic inhibition in mice (Abramian et al., 2014). Thus, the neuronal adaptations to GABA$_A$ drugs via modulation of receptor trafficking produce long-lasting changes in the efficacy of GABAergic inhibition. However, it should be noted that the effects of GABA$_A$ ligands on receptor subunits are very much dependent on the experimental model, and the effects seen in cell culture models have often been difficult to reproduce *in vivo* (Uusi-Oukari and Korpi, 2010).

The most obvious impediment to understanding neuroadaptation induced by GABA$_A$ receptor modulators is the diversity of their target neurons. In fact, each neuron controlled by inhibitory terminals expresses its unique combination of GABA$_A$ receptors (Luddens et al., 1995; Olsen and Sieghart, 2009). A further obstacle

to the studying of neuronal and structural drug-induced plasticity is made by the fact that interneurons, as a major target for GABA$_A$ receptor modulators, themselves are innervated by both glutamatergic and GABAergic synapses. In addition, endogenous GABA$_A$ receptor modulators, such as neurosteroids, whose levels constantly fluctuate, e.g., during stress, menstrual cycle and development, can directly affect memory and learning processing (Maguire et al., 2005; Shen et al., 2010), as well as influence the action of other GABA$_A$ receptor modulators.

KCC2-MEDIATED SPINE MORPHOGENESIS

Potassium-chloride co-transporter 2 (KCC2) is expressed in neurons to create the driving force for chloride ions to travel into the cell through the GABA$_A$ receptor anion channel, which then leads to hyperpolarizing GABA actions (Rivera et al., 1999). Independently of its Cl^- transport function KCC2 has also gained attention due to its structural role in both glutamatergic and GABAergic synapses (Li et al., 2007; Horn et al., 2010; Sun et al., 2013). In glutamatergic synapses, KCC2 located in the neck and head of dendritic spines binds to actin cytoskeleton via the linker protein 4.1 N (Li et al., 2007). While the exact molecular mechanisms still remain elusive, the interaction of KCC2 with the cytoskeleton is crucial for the maturation of spines and for the stability of AMPA receptor clusters (Li et al., 2007; Gauvain et al., 2011). In contrast to glutamatergic synapses, GABAergic synapses are usually located directly on the dendritic shaft (Freund and Buzsaki, 1996; Somogyi et al., 1998). KCC2 expression is regulated by a cell adhesion molecule neuroligin-2 which is mostly localized at GABAergic synapses. Knockdown of neuroligin-2 down-regulates expression of KCC2 and reduces GABAergic synaptogenesis, and interestingly, by affecting the KCC2 levels it also down-regulates the number of glutamatergic synapses (Sun et al., 2013).

Although there is no direct evidence yet on drug-induced structural changes through KCC2-dependent mechanisms, several studies demonstrated changes in expression levels of KCC2. Chronic treatment with the BZ agonist zolpidem up-regulated the KCC2 expression in mouse limbic forebrain (Shibasaki et al., 2013). Also the neurosteroid allopregnanolone transiently modifies KCC2 expression and protein levels during brain maturation in male rats (Modol et al., 2014). However, treatment with general anesthetics, midazolam, propofol, and ketamine, does not alter the expression of KCC2 in rats during the first two postnatal weeks when developmental maturation of KCC2 expression is going on (Lacoh et al., 2013). Future studies should be directed to clarify whether KCC2-mediated mechanisms play a role in neuronal plasticity induced by GABAergic drugs (Kang et al., 2006), and which particular isoforms of KCC2 play role in structural changes, since a recent study suggests that KCC2a and KCC2b isoforms have different brain regional distributions and likely different roles in neuronal functions (Markkanen et al., 2014).

DRUG-INDUCED SYNAPTIC PLASTICITY IN THE VTA

The VTA has been widely studied given its fundamental role in motivation and reward (for review, see Luscher and Malenka, 2011). VTA DA neurons project mainly to the NAc and mPFC and less extensively to the hippocampus and amygdala. They receive glutamatergic inputs from many brain regions, including the mPFC, lateral hypothalamus, lateral habenula and hippocampus, and in addition to local inhibitory control from the VTA GABAergic interneurons, GABAergic inputs to DA neurons arise from the NAc, ventral pallidum, nuclei of the extended amygdala, and rostromedial tegmental nucleus (Jhou et al., 2009; Omelchenko and Sesack, 2009; Watabe-Uchida et al., 2012).

Glutamatergic transmission in the VTA is critical to the reinforcing effects of drugs of abuse: suppressing the glutamatergic transmission in the VTA attenuates cocaine and heroin reward (Xi and Stein, 2002; You et al., 2007) and prevents the reinstatement of cocaine- or heroin seeking (Bossert et al., 2004; Sun et al., 2005). Glutamatergic synapses on VTA DA neurons can undergo both NMDAR-dependent LTP and NMDAR-independent long-term depression (LTD; Jones et al., 2000; Thomas and Malenka, 2003). Synaptic plasticity in the mesolimbic DA system was early on hypothesized to play a role in the process of drug reinforcement and addiction. During the last decade, evidence from electrophysiological studies have accumulated showing that in addition to acute activation of VTA DA neurons, drugs of abuse also induce long-lasting plasticity in the synapses of these neurons. Several classical drugs of abuse such as cocaine, amphetamine, morphine, nicotine, and ethanol share the ability to induce an NMDAR-dependent LTP at glutamatergic synapses of VTA DA neurons via insertion of new GluA2 subunit-lacking AMPARs (Ungless et al., 2001; Saal et al., 2003; Luscher and Malenka, 2011).

In addition to the potentiation of glutamatergic transmission, different addictive drugs such as morphine, nicotine, cocaine, and ethanol have been found to impair GABAergic transmission in the VTA DA neurons by preventing the LTP of GABAergic synapses (LTP$_{GABA}$; Nugent et al., 2007; Guan and Ye, 2010). In the VTA, LTP$_{GABA}$ is triggered by NMDA receptor activation at glutamate synapses and requires nitric oxide-cGMP signaling (Nugent et al., 2007; Nugent and Kauer, 2008). Blockade of LTP$_{GABA}$ could additionally increase release of DA by silencing local GABA neurons (Liu et al., 2000; Nugent and Kauer, 2008; Niehaus et al., 2010). Which particular GABAergic inputs are involved in LTP$_{GABA}$ and whether the GABA$_A$ receptor modulators induce LTP$_{GABA}$ have not been investigated so far.

GABA$_A$ RECEPTOR BENZODIAZEPINE-SITE DRUGS INDUCE NEURONAL PLASTICITY IN THE VTA

Since the positive modulators of GABA$_A$ receptor benzodiazepine site have well-known abuse potential and act as positive and/or negative (Panlilio et al., 2005) reinforcers in different animal models of addiction, their effects on synaptic plasticity in VTA DA neurons have been recently studied. Indeed, diazepam, and zolpidem, similarly to the other drugs of abuse, were shown to induce plasticity in the glutamatergic synapses contacting VTA DA neurons (Heikkinen et al., 2009). Particularly, BZs induced an LTP that was prevented by co-administration of the BZ antagonist flumazenil and by the NMDA receptor antagonist dizocilpine (MK-801; Heikkinen et al., 2009). BZ-induced LTP in VTA DA neurons was associated with insertion of new GluA2-lacking AMPA receptors, and intra-VTA local network was sufficient for the LTP induction via inhibition of VTA GABAergic interneurons (Tan et al., 2010).

Furthermore, Tan et al. (2010) examined a mutant mouse with BZ-insensitive α1 subunits, and found that in these mice midazolam was not able to inhibit the firing of VTA GABAergic interneurons, to disinhibit the DA neurons, to induce plasticity at glutamatergic synapses or to support drug-reinforcement behavior. This suggests that the BZ-induced disinhibition of DA neurons is a key mechanism involved in BZ-induced plasticity in VTA DA neurons as well as in BZ reinforcement.

DRUGS TARGETING THE EXTRASYNAPTIC GABA$_A$ RECEPTORS INDUCE VTA DA NEURON PLASTICITY BUT ARE AVERSIVE

There is a need for new anxiolytic/sedative drugs with no abuse potential. One approach has been to target a GABAergic system separate from the benzodiazepine-sensitive GABA$_A$ receptors, i.e., the extrasynaptic GABA$_A$ receptors containing δ-subunit (Olsen and Sieghart, 2009). However, δ-subunit is expressed along the reward pathway in the VTA, NAc, mPFC, and hippocampus (Pirker et al., 2000; Hortnagl et al., 2013). THIP and muscimol, which act on GABA$_A$ receptor agonist sites with preferential activation of the high-affinity extrasynaptic receptors (Chandra et al., 2010), have been shown to increase firing rates of DA neurons (Waszczak and Walters, 1980). Thus, it was necessary to study the effects of modulators of the extrasynaptic GABA$_A$ system on plasticity in reward pathway and the possible reinforcing potential of these drugs.

A single dose of THIP and another extrasynaptic GABA$_A$ receptor modulator neurosteroid ganaxolone dose-dependently induced similar AMPA receptor-mediated LTP in VTA DA neurons via the primary action of increased tonic inhibition of VTA GABAergic interneurons (Vashchinkina et al., 2012, 2014). Importantly, both THIP- and ganaxolone-induced plasticity lasted at least for six days, while BZs cause this effect for three days only (Heikkinen et al., 2009). The effects of THIP and ganaxolone were absent in δ-GABA$_A$ receptor knockout mice, and both treatments enhanced AMPA current rectification, indicating reduced targeting of GluA2 subunits.

Surprisingly, despite of aforementioned similar effects on neuroadaptations in VTA DA neurons, modulators of synaptic, and extrasynaptic GABA$_A$ receptors induce distinct behavior in the drug self-administration and place conditioning paradigms which have been previously associated with activity of VTA DA neurons (for review, see Luscher and Malenka, 2011). While activation of the synaptic α1 subunit-containing GABA$_A$ receptors in the VTA by oral midazolam is reinforcing in the self-administration, the activation of extrasynaptic receptors by ip THIP or ganaxolone leads to avoidance behavior as seen in conditioned place aversion, and THIP is not self-administered either by mice or baboons (Tan et al., 2010; Vashchinkina et al., 2012, 2014). These aversive effects were abolished in GABA$_A$ receptor δ subunit-deficient mice (Vashchinkina et al., 2014), suggesting a specific role of this receptor population in the VTA. These behavioral findings support the hypothesis that activation of synaptic and extrasynaptic GABA$_A$ receptors is rewarding and aversive, respectively. This may depend on the primary brain areas targeted, as illustrated by intracerebral injections of muscimol (see below) that also preferentially targets extrasynaptic receptors (Chandra et al., 2010). By using place-conditioning system, muscimol infusion into the NAc shell provoked conditioned place preference when infused anteriorly and conditioned aversion when infused posteriorly (Reynolds and Berridge, 2002). Intra-BLA infusions of muscimol and bicuculline had no effect on reward (Zarrindast et al., 2004; Macedo et al., 2006).

Furthermore, it is now becoming clear that aversive drugs or experiences can acutely activate certain DA neurons in the VTA and also induce a long-lasting potentiation at their glutamatergic synapses. This was actually reported already in the early paper of Saal et al. (2003) where the authors showed that in mice a 5-min swimming stress at 6°C water bath induces similar plasticity in the VTA DA neurons as the classical drugs of abuse.

DO GABA$_A$ RECEPTOR DRUGS TARGET THE SAME POPULATIONS OF VTA DA NEURONS?

DA neurons are divergent in many respects, e.g., in their electrophysiological features, vulnerability to neurodegeneration and regulation by neuropeptides (Korotkova et al., 2004; Lammel et al., 2011). In particular, Lammel et al. (2011) have shown that DA neurons in the mouse VTA are organized into anatomical and electrophysiological subpopulations inside the DAergic nuclei depending on their projection terminal fields. However, in rats, the VTA neurons might be more heterogeneously organized (Margolis et al., 2006, 2012). The inhibitory control from VTA GABA interneurons is an important regulator of VTA DA neuron activity and the following behavioral outcome. Aversive stimuli have been shown to increase the firing of VTA GABA neurons (Creed et al., 2014). However, while GABA neurons in the VTA seem to quite faithfully respond to aversion by excitation, the responses in VTA DA neurons are more heterogeneous: activation, no response or inhibition have been observed in monkeys (Matsumoto and Hikosaka, 2009). Rewarding or aversive stimuli might modulate the activity of DA neurons differently depending on the brain area to which these neurons project. In mice, a cocaine experience selectively affected DA cells projecting to the NAc medial shell, while an aversive stimulus influenced DA cells projecting to the PFC, and the DA neurons projecting to the NAc lateral shell were modified by both rewarding and aversive stimuli, suggesting that the mesocorticolimbic DA system is comprised of anatomically distinct circuits, modified by different motivational relevance (Lammel et al., 2011). In anesthetized rats, foot shock inhibited DA neurons in the dorsal VTA, whereas the DA neurons in the ventral VTA became phasically excited (Brischoux et al., 2009). In mice, the majority of the VTA DA neurons decreased firing under fearful events, but a small group of DA neurons were activated (Wang and Tsien, 2011). Another study reported that a similar number of DA neurons were activated, inhibited or unaltered by tail pinch, and it also showed that in mice with an impaired NMDA receptor-mediated control of DA neurons the DAergic activation in response to an aversive stimulus was attenuated, leading to impaired aversive conditioning (Zweifel et al., 2011). These findings suggest that increases in DA signaling can be evoked by stimuli with motivational relevance to either rewarding or aversive direction, and they point toward putative multiple populations of VTA DA neurons with different afferent and efferent connections.

Mouse DA neurons with pronounced hyperpolarization-activated cation current I_h-current are found in the lateral VTA and they project to the lateral NAc shell, while the DA neurons of the medial posterior VTA project to the mPFC and medial NAc shell, and have no or very small I_h-currents (Lammel et al., 2011). It should be noted that the study revealing BZ-induced glutamatergic plasticity in VTA DA neurons used a large I_h-current as a marker for DA neurons (Heikkinen et al., 2009). Thus, mostly a subpopulation of VTA DA neurons that projected to the lateral NAc shell was studied. In the studies with THIP and ganaxolone, a genetically modified mouse line with a fluorescent protein marker expressed in tyrosine hydroxylase-positive neurons was used (Vashchinkina et al., 2012, 2014). This allowed recording also from the DA neurons in more medial areas of the VTA with small or no I_h-currents and projecting to the mPFC. It is possible that BZs are positively reinforcing due to mainly activating and modifying the DA neurons detecting reward whereas the drugs activating the extrasynaptic GABA$_A$ receptors lead to conditioned aversion because they activate and induce LTP in the DA neurons involved in negative motivation. This hypothesis remains to be carefully tested, although the post-study examination of the recording sites for DA neuron plasticity by THIP failed to indicate any anatomical localization within the VTA (Vashchinkina et al., 2012).

Both BZs and extrasynaptic GABA$_A$ modulators produce strong inhibition of the VTA GABA interneurons (Heikkinen et al., 2009; Tan et al., 2010; Vashchinkina et al., 2012, 2014). Thus, a similar disinhibitory mechanism is believed to induce the activation and persistent modulation of VTA DA neurons by both classes of GABA$_A$ drugs, which in turn suggests that they might be targeting different populations of VTA GABA interneurons enriched with synaptic and/or extrasynaptic GABA$_A$ receptors. Interestingly, a recent study shows that the volatile solvent toluene, that also is a positive modulator of GABA$_A$ receptors, induces LTP at the glutamatergic synapses of VTA DA neurons that project to NAc core and shell, whereas it failed to affect the synapses of mPFC-projecting neurons (Beckley et al., 2013). Importantly, toluene is abused by humans, and in rodent models of addiction it increases firing of VTA DA neurons and DA release in the NAc and exhibits positive reinforcement (Lee et al., 2006; Riegel et al., 2007; Lubman et al., 2008). Effects of toluene on GABA interneurons have not been studied so far.

In summary, the possible VTA heterogeneity of both the principal DA neurons and the GABA interneurons and projection neurons in responding to and mediating stimuli and affecting behaviors is an important subject for future research. This information would be needed to further develop rational treatment ideas for addiction.

DRUG-INDUCED STRUCTURAL PLASTICITY IN THE VTA AND ITS EFFERENTS

Midbrain (VTA and substantia nigra) and striatal (NAc and the dorsomedial and dorsolateral striatae) components of the basal ganglia and mesolimbic pathway are connected in a spiraling manner so that ventral striatal regions project to medial parts of the midbrain DAergic area, which subsequently sends projections to more dorsal striatal regions which in turn project to the more lateral midbrain and so on (Haber et al., 2000). An appealing emerging hypothesis is that the initial drug-induced plasticity in the DAergic midbrain and subsequently in the ventral striatum would recruit more and more dorsal striatal regions during chronic drug use and reinforce the connectivity within these spiral projections, thus leading to compulsive drug-seeking manifesting at the late stages of addiction (Grueter et al., 2012). The dorsomedial and the dorsolateral parts of striatum regulate goal-directed and stimulus–response habitual movements, respectively (Yin and Knowlton, 2006; Redgrave et al., 2010). The majority of the neurons of ventral and dorsal striatum are projecting medium spiny neurons (MSNs), whose activity depends to a great extent on excitatory inputs from cortical and limbic regions (Sesack and Grace, 2010). Plasticity at excitatory synapses of the striatum would thus change the output of striatal circuits, i.e., the motivated as well as compulsive behaviors. Addiction seems to involve exceptionally intense drug experience-driven synaptic and structural plasticity at different levels of the mesolimbic DA system (Robinson and Kolb, 1999).

GABA$_A$ receptor modulators are abused, but to date there are no data available whether they are able to induce changes in neuronal spine density in the reward system. Earlier work by Sarti et al. (2007) demonstrated a correlation of cocaine-induced plasticity of glutamatergic synapses with an increase in spine density of the rat VTA neurons. In that work, the traditional Golgi-Cox impregnation was used to study dendritic spines and cell morphology was used to identify and subtype VTA DA neurons. Since GABA$_A$ receptor modulators also induce similar glutamate receptor neuroplasticity in VTA DA neurons, we were puzzled with a question whether diazepam and/or THIP alter morphology and spine density. At 24 h after single injections of the doses of diazepam and THIP, which induce LTP in the VTA (Heikkinen et al., 2009; Vashchinkina et al., 2012), the brains of mice were subjected to Golgi staining and spine counting (**Figure 1**). In addition to the VTA, we analyzed the hippocampal CA1 and CA3 regions, since BZs are known to induce persistent synaptic plasticity particularly in the pyramidal neurons of the CA1 and CA3 regions (**Table 1**).

We found that basal spine density counts in both hippocampal and VTA neurons are in line with the results of (Sarti et al., 2007). Treatment with THIP increased the spine density on the basal secondary dendrites of CA1 pyramidal cells (**Figure 1**), but not in CA3 or VTA. In contrast, diazepam treatment did not show any changes in spine density of the basal dendritic spines in these areas, nor in the apical ones (data not shown). This is generally consistent with a previous study, in which acute and chronic treatment with BZs was not associated with up-regulation of BDNF or c-Fos protein levels in the hippocampus (Licata et al., 2013). Since Sarti et al. (2007) found clear increase in VTA spines after neuroplasticity-inducing dose of cocaine, our results suggest different mechanisms or different VTA DA neuron populations might have been involved in the effects of various GABA$_A$ ligands. Additional studies using retrograde neurotracers to clarify the targets and novel immunofluorescent co-staining methods for precise phenotyping of the neurons (Spiga et al., 2011) are needed to resolve the affected VTA neurons. In spite of the comparable VTA

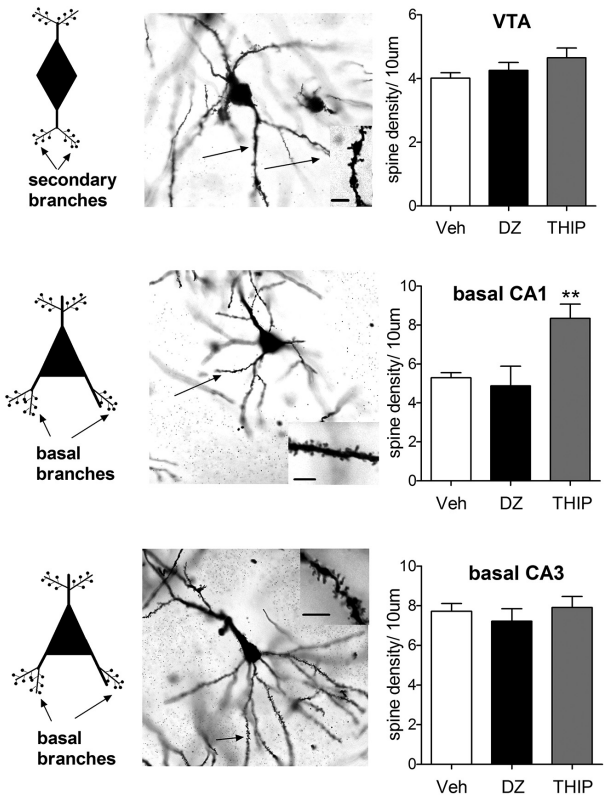

FIGURE 1 | Pyramidal cells of hippocampal CA1 region exhibit distinct morphological responses to diazepam and THIP treatment. THIP increased the spine density on the basal secondary dendrites of CA1 pyramidal cells ($F_{2,15} = 9.69$, $p < 0.01$), but not in CA3 or VTA ($p > 0.05$). In contrast, diazepam (DZ) did not show any changes in spine density of the basal nor apical dendritic spines in these areas ($p > 0.05$). *On the left,* schematic neurons depict the spines either secondary or basal dendritic branches that were assessed here. *In the middle*, representative images of Golgi-stained neurons on horizontal sections. Insets are dendrites showing spines. Scale bar is 10 μm. *On the right*, plots showing the spine densities 24 h after a single injection of DZ or THIP. Mean ± SEM for $n = 10$, 5, and 5 mice vehicle (Veh), DZ, and THIP groups, respectively. $^{**}p < 0.01$, (one-way ANOVA followed by Bonferroni *post hoc* test). C57BL/6J mice (21–26 days old) were decapitated at 24 h after treatment with DZ (5 mg/kg, ip), THIP (6 mg/kg, ip) or vehicle (Heikkinen et al., 2009; Vashchinkina et al., 2012; All animal procedures were approved by the Southern Finland Provincial Government). Brains were rapidly dissected and immediately placed into Golgi-Cox fixative and processed using the FD Rapid GolgiStain Kit (FD Neuro Technologies). Impregnated brains were then cut in serial horizontal 80-μm-thick sections using a cryostat (Leica Microsystems, Nussloch, Germany) and stained using the kit protocol. For each brain area, spine density per 10 μm was assessed in five cells. Notably, in VTA cells spines were determined on secondary dendrites, in CA1 and CA3 pyramidal cells on basal dendritic branches, using the 100× objective of Zeiss Axioplan microscope (Carl Zeiss MicroImaging GmbH, NA = 1.3, Ph3, DIC, oil), and later analyzed by National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij). Spine counting was performed blind concerning the treatments. Only protrusions that show continuity with the dendritic shaft were considered as spines.

neuroplasticity-inducing doses used, diazepam and THIP produced distinct effects on spine remodeling. These results show that the acute effects of GABA-drugs are not consistently accompanied by changes in spine densities of the dendrites in hippocampal or VTA neurons.

Repeated exposure to cocaine has been shown to increase the number of silent synapses and the density of dendritic spines in NAc shell (Robinson and Kolb, 1999; Huang et al., 2009; Dobi et al., 2011; Kim et al., 2011). Also dorsal striatum MSNs exhibit an increased dendritic spine density following chronic cocaine exposure (Ren et al., 2010). Months after repeated exposure to methamphetamine the spine density increased in MSNs of the dorsolateral striatum, a structure that supports habitual behaviors but decreased in dorsomedial striatum which is important in goal-directed movements (Jedynak et al., 2007). A primate study of chronic ethanol drinking reported increased spine density in the putamen (the primate analog of dorsolateral striatum in rodents) as well as enhanced glutamatergic transmission and increased intrinsic excitability of MSNs in this area (Cuzon Carlson et al., 2011). This pattern of structural plasticity in the dorsal striatum supports the concept that during the progression of addiction the behavior is driven toward habitual drug taking and seeking.

Withdrawal from chronic treatment with addictive drugs leads to hypofunction of VTA DA neurons. For example, withdrawal from cannabinoids or morphine profoundly affects the morphological characteristics of VTA DA neurons and spine density of MSNs of the NAc shell (Spiga et al., 2003, 2010). Whether chronic treatment with GABA$_A$ receptor modulators alters morphology of VTA DA neurons or NAc and dorsal striatal MSNs, with respect to addiction and/or withdrawal, remains to be studied.

Broad distribution of GABA$_A$ receptors throughout the brain suggests more widespread neuroplasticity effects of GABA$_A$ receptor modulators in other brain regions such as the NAc, mPFC, hippocampus, and amygdala (Licata and Rowlett, 2008). In fact, several reports showed blockade of hippocampal LTP after acute *in vitro* effects of BZs and isoflurane (summarized in **Table 1**). This is consistent with the known effect of cognitive dysfunction induced by general anesthetics, and memory impairment by BZs in humans (Canet et al., 2003; Newman et al., 2007). On the other hand, withdrawal symptoms after repeated administration of these drugs are associated with synthesis of new glutamatergic receptors and potentiation of LTP in the hippocampus and cortex (**Table 1**). Thus, the glutamatergic receptors appear to be regulated differently depending on the specific phase of the drug effect.

CONCLUSION

GABAergic neurotransmission is known to participate in neuronal plasticity processes during the critical periods in development, and drugs acting on GABA$_A$ receptors, such as BZs, have been considered more as blunting neuroplasticity than inducing it. However, recent experiments on the midbrain dopamine systems have revealed that GABA$_A$ drugs acting on different receptor subtypes induce persistent neuroplasticity in glutamate receptors of the VTA DA neurons, but so far there is little evidence in support of widespread structural changes caused by these drugs. The main mechanism of how the GABA$_A$ drugs induce plasticity involves disinhibition via primary inhibition of GABAergic interneurons. It is likely that more dynamic methods, such as *in vivo* microscopy on dendritic spines, will be needed

Table 1 | Summary of neuroplasticity induced by GABA$_A$ receptor drugs.

Brain area, cell type (species)	Drug treatment	Method	Main findings	Reference
Ventral tegmental area				
Dopamine neurons (mouse)	24–120 h after DZ (5 mg/kg), THIP (6 mg/kg), ganaxolone (30 mg/kg), zolpidem (5 mg/kg), midazolam (0.5 mg/kg)	Electrophysiology	Insertion of new GluA2-lacking AMPA-Rs	Heikkinen et al. (2009), Tan et al. (2010), Vashchinkina et al. (2012, 2014)
Nucleus accumbens				
			No data available	
Cortex				
Layer V pyramidal neurons (rat)	96 h after diazepam for 14 days at increasing doses (17.6–70.4 μmol/kg)	IHC with gold-immunolabeling	Up-regulation of GluA1 subunit of AMPA-R's mRNA and cognate protein	Izzo et al. (2001)
Amygdala				
Neurons of lateral nucleus (mouse)	Short-term deep isoflurane anesthesia	Electrophysiology	Enhancement of capsaicin-induced LTP via TRPV1-mediated mechanism	Zschenderlein et al. (2011)
Hippocampus				
CA1 pyramidal neurons (rat)	Withdrawal 96 h after DZ for 14 days at increasing doses (17.6–70.4 μmol/kg)	IHC with gold-immunolabeling	Up-regulation of GluA1 subunit of AMPA-R's mRNA and cognate protein	Izzo et al. (2001)
CA1 pyramidal neurons (rat)	Flurazepam-induced DZ withdrawal	Electrophysiology	Up-regulation of AMPA-R function	Van Sickle et al. (2004), Song et al. (2007), Xiang and Tietz (2007)
CA1, CA3 pyramidal neurons, granule layer of DG (rat)	Acute *in vitro* (5 μM) and *in vivo* DZ and triazolam	Electrophysiology	Inhibition of LTP	del Cerro et al. (1992), Mori et al. (2001), Maubach et al. (2004), Xu and Sastry (2005)
CA1 pyramidal neurons (mouse)	*In vitro* isoflurane (0.125-0.5 mM)	Electrophysiology	Inhibition of LTD and LTP in GABA$_A$R- dependent manner, nAChR α4β2 subtype-mediated mechanism	Simon et al. (2001), Haseneder et al. (2009)
CA1 pyramidal neurons (mouse)	24 h–7 day after 2 h – isoflurane anesthesia	Electrophysiology, Western blotting	Enhancement of LTP via up-regulation of GluN2B subunit of NMDA-Rs	Rammes et al. (2009)

AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CA, Cornu Ammonis, hippocampal areas; DG, dentate gyrus; DZ, diazepam; IHC, immunohistochemistry; nAChR, nicotinic acetylcholine receptor; NMDA-R, N-methyl-D-aspartate receptor; THIP, gaboxadol.

to better understand the structural neuroplasticity/neurotoxicity effects of general anesthetics, BZs and other types of anxiolytics and sedatives. Based on the present data, the selection of an anesthetic agent will be important for these future experiments!

AUTHOR CONTRIBUTIONS

Elena Vashchinkina, Anne Panhelainen, Teemu Aitta-aho, and Esa R. Korpi conceived and designed the experiments, Elena Vashchinkina performed the experiments and data analyses, and Elena Vashchinkina, Anne Panhelainen, Teemu Aitta-aho, and Esa R. Korpi wrote the paper.

ACKNOWLEDGMENTS

The work was supported by the Academy of Finland (Esa R. Korpi), the Sigrid Juselius Foundation (Esa R. Korpi, Teemu Aitta-aho), the Finnish Foundation for Alcohol Studies (Elena Vashchinkina, Anne Panhelainen, Teemu Aitta-aho), the Orion-Farmos Research Foundation (Esa R. Korpi, Teemu Aitta-aho), the Jane and Aatos Erkko Foundation (Anne Panhelainen, Esa

R. Korpi), and the Finnish Cultural Foundation (Anne Panhelainen).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 August 2014; accepted: 03 November 2014; published online: 25 November 2014.

*Citation: Vashchinkina E, Panhelainen A, Aitta-aho T and Korpi ER (2014) GABA$_A$ receptor drugs and neuronal plasticity in reward and aversion: focus on the ventral tegmental area. Front. Pharmacol. **5**:256. doi: 10.3389/fphar.2014.00256*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

REVIEW ARTICLE
published: 19 December 2014
doi: 10.3389/fphar.2014.00279

Role of NG2 expressing cells in addiction: a new approach for an old problem

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Neuron-glial antigen 2 (NG2) is a proteoglycan expressed predominantly in oligodendrocyte progenitor cells (OPCs). NG2-expressing OPCs (NG2-OPCs) are self-renewing cells that are widely distributed in the gray and white matter areas of the central nervous system. NG2-OPCs can mature into premyelinating oligodendrocytes and myelinating oligodendroglia which serve as the primary source of myelin in the brain. This review characterizes NG2-OPCs in brain structure and function, conceptualizes the role of NG2-OPCs in brain regions associated with negative reinforcement and relapse to drug seeking and discusses how NG2-OPCs are regulated by neuromodulators linked to motivational withdrawal. We hope to provide the readers with an overview of the role of NG2-OPCs in brain structure and function in the context of negative affect state in substance abuse disorders and to integrate our current understanding of the physiological significance of the NG2-OPCs in the adult brain.

Keywords: prefrontal cortex, amygdala, hippocampus, progenitors, NG2, BrdU

INTRODUCTION

For centuries, the cellular composition of the healthy, intact mammalian brain was thought to be maintained in a static state, in the absence of cellular turnover. The relatively recent discovery of neural stem cells in all mammalian species, including humans, has forced researchers to adapt their understanding of the basal cortical structure and function under physiologic and pathologic conditions within the framework of this novel cellular phenomenon (Eriksson et al., 1998; Curtis et al., 2007). Nearly every major disorder which perturbs typical functioning of the brain, including addiction to illicit drugs, has been shown to influence or be influenced by rates of neural stem cell proliferation, differentiation, and/or survival (Canales, 2010; Mandyam and Koob, 2012; Drew et al., 2013). While much focus has centered on the role of generation of new neurons, or neurogenesis, it is critical also to evaluate the role of generation of new glia, or gliogenesis in typical and atypical cortical function. This review aims to highlight the role of a particular glial cell type, the neuron-glial 2 (NG2) cell in the adult mammalian cortex and speculate on the potential role of this specialized cell type in addiction to drugs of abuse.

CHARACTERIZATION OF NG2 CELLS IN THE ADULT MAMMALIAN BRAIN

NG2-GLIA: WHAT'S IN A NAME?

Glia in the brain are mainly classified into three subtypes: astroglia, microglia, and oligodendroglia. A fourth kind of proliferative glial cells termed NG2 cells that express their namesake characteristic marker, chondroitin sulfate proteoglycan, are found to be widely distributed throughout the gray and white matter of the adult rodent brain (Dawson et al., 2000, 2003; Levine et al., 2001). The notion that NG2 cells are oligodendrocyte progenitor cells (OPCs) was confirmed with *in vitro* studies demonstrating that NG2 labeled cells differentiated into oligodendrocytes in a differentiating culture preparation (Stallcup and Beasley, 1987); thus, these NG2 cells have been referred to as NG2-OPCs. Other names have been suggested for the NG2 cells, such as polydendrocytes to describe their multiple projections (Nishiyama et al., 2002), and synantocytes to describe their contact with neurons and astroglia (Butt et al., 2002). An in depth review on the biology and function of NG2 cells has been published elsewhere (Hill and Nishiyama, 2014; Tomassy and Fossati, 2014) and the current review will briefly discuss the phenotypic fate of NG2 cells and their role in the mammalian brain in the context of addictive disorders.

PHENOTYPIC FATE OF NG2-GLIA

Following the initial identification of NG2 cells, one line of investigation pursued the phenotypic fate of these unique cells. NG2 cells isolated by immunopanning for A2B5 (an antibody that tags the ganglioside moiety expressed in pre-oligodendrocytes) revealed that NG2 cells *in vitro* mature into A2B5+ pre-oligodendrocytes (Abney et al., 1983; Baracskay et al., 2007), confirming that NG2 cells are directed into an oligodendrocyte phenotype. *Ex vivo* studies demonstrate that NG2 cells express two distinct markers of early oligodendroglial lineage, namely, platelet-derived growth factor α receptor (PDGFαR) and O-antigen 4 (O4), further supporting that NG2-cells are directed into an oligodendrocyte lineage in the adult brain (Reynolds and Hardy, 1997; Nishiyama et al., 1999; Dawson et al., 2003). Additional support for the differentiation of NG2-cells into oligodendrocyte phenotype comes from various genetic models and such studies confirm the direction of NG2 cells into oligodendrocytes and premyelinating oligodendrocytes *in vivo*. For example, using the NG2-CreERT2 transgenic

line, it has been recently demonstrated that a large proportion of NG2 cells in various regions of the brain express oligodendroglial markers Olig2, Sox10 and adenomatous polyposis coli CC1 at various stages of development; embryonic brain [(E)17.5], postnatal brain (P3-P4), adolescent brain (P30-P34) and aged brain [10 month old; (Huang et al., 2014)]. Taken together, it is evident that NG2 cells are capable of generating oligodendrocytes all through the life-span of rodents.

Beyond NG2 cells developing into oligodendrocytes, several studies suggest that NG2 cells are mulitpotent cells that are capable of generating neuronal progenitor cells in addition to oligodendrocytes. For example, 50% of glioblastoma cells isolated from human subjects were co-labeled with NG2 and nestin, a marker expressed by neuronal progenitors (Svendsen et al., 2011). The *ex vivo* evidence is further supported by *in vivo* findings, where a significant proportion of NG2 cells expressed GFP in the transgenic nestin-GFP reporter mice (Ehninger et al., 2011). Furthermore, using transgenic lines Plp-CreERT2 and PDGFRα-CreERT2, postnatal NG2 cells were found to generate new neurons in the piriform cortex, albeit significantly lower in number when compared with oligodendrocytes (Doerflinger et al., 2003; Rivers et al., 2008; Guo et al., 2009). Taken together, these findings suggest that a small proportion of NG2 cells may have the capacity to generate neural progenitor cells and neurons in the adult brain.

While there is consistent evidence that small populations of NG2 cells can develop into neurons during adulthood, controversy remains over whether NG2 cells can similarly generate neurons during development. For example, using transgenic lines NG2-Cre and CreER BAC transgenic mice, it was demonstrated that embryonic NG2 cells did not mediate neurogenesis (Zhu et al., 2008b, 2011). These discrepancies in the neuronal phenotype of NG2 cells may be attributable either to variability in cell-specific expression of transgene due to non-homologous recombination strategies or to the developmental profile of NG2 cells in the central nervous system (Nishiyama et al., 2009, Richardson et al., 2011). To this end, a recent study attempted to overcome one limitation by using NG2-CreERT2 transgenic line using homologous recombination, thus enabling the transgene to be under the regulatory control of the endogenous regulators of the NG2 locus (Huang et al., 2014). In this study, inducing Cre activity in NG2 cells in the second postnatal week (P14) resulted in reporter gene colocalizing with neuronal markers such as NeuN and Tuj1 in the ventral cortex, suggesting that NG2 cells can differentiate into neurons in this brain region. Interestingly, inducing Cre activity during young adulthood (P30), did not reveal colocalization with neuronal markers in the previously demonstrated ventral cortex or regions of established adult neurogenesis (the hippocampal dentate gyrus and subventricular zone). Instead, the reporter-positive cells in the non-neurogenic regions in the adult brain (ventral cerebral cortex and hippocampal CA3 regions) were found to co-express neuronal markers NeuN and HuC/D. Given that NG2 cells generated adult born neurons in the non-neurogenic regions in the adult brain demonstrated morphology of interneurons and normal electrophysiological properties, it is tempting to speculate that NG2 cells indeed have neurogenic potential, but to a limited capacity and with brain region specificity compared with their more prolific oligodendrocyte potential (Huang et al., 2014). Alternatively, the NG2 cells with neurogenic potential may be a different population compared with the NG2 cells that differentiate into oligodendrocytes, and future studies are needed to confirm the multipotency of the NG2 cell population.

With respect to NG2 cells generating astroglial cells and microglial cells, there is evidence to both support and to reject the supposition. For example, the *in vitro* finding that NG2 cells differentiate into type-2 astrocyte led to the conceptualization of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells; this was, however, considered to be an *in vitro* artifact based on *ex vivo* and *in vivo* findings (Raff et al., 1983, Espinosa de los Monteros et al., 1993, Nishiyama et al., 2009). *Ex vivo* studies show that there is little or no evidence for co-labeling of astroglial specific markers [glial fibrillary acidic protein (GFAP)] and microglial specific markers OX-42 with NG2, however, the NG2 cells were reported in close apposition with astrocytes and microglia creating points of apparent overlap (Dawson et al., 2003, Mandyam et al., 2007). *In vivo* studies using transgenic mice have also been equivocal about the astroglial lineage of NG2 cells. Studies using the NG2-Cre and CreER BAC transgenic mice revealed that 40% of the protoplasmic astrocytes in the gray matter were generated from NG2 cells. In contrast, the radial glial cells in the gray matter and all the astroglial cells in the white matter were shown to arise from a non-NG2 lineage (Zhu et al., 2008a,b, 2011). Furthermore, in the Plp-CreERT2 transgenic mice, postnatal NG2 cells were found to generate astroglia in the ventral gray matter (Doerflinger et al., 2003; Guo et al., 2009). Additionally, using NG2-CreERT2 mice, NG2-CreER BAC transgenic lines as well as PDGFRα-CreER PAC and PDGFRα-CreERT2 BAC transgenic lines, no evidence was found to suggest that NG2 cells in mice (age ranging from P8 to adulthood) were capable of generating new astroglia (Rivers et al., 2008; Kang et al., 2010; Huang et al., 2014). Taken together, it appears that the astrogenic potential of NG2 cells may be restricted to certain brain regions and certain developmental periods; therefore more work is needed to confirm the astrogenic potential of NG2 cells in the adult brain.

From the evidence presented, it is clear that NG2-glia have the potential to develop into both neurons and astrocytes in adult animals, but develop primarily into oligodendrocytes; therefore, NG2 cells with oligodendrocyte potential will be the developmental lineage of focus for the remainder of the review and these cells will henceforth be called NG2-OPCs.

ROLE OF NG2-OPCs IN THE ADULT MAMMALIAN BRAIN

Beyond the phenotypic fate of NG2-OPCs, it is critical to understand the role of NG2-OPCs in typical cortical function. While neurons are thought to be the most prominent postmitotic cells in the brain that are uniquely capable of generating action potentials as a means of communicating with other cell types and maintaining plasticity, this notion has been challenged by studies showing that NG2-OPCs exhibit several neuron-like properties. For example, NG2-OPCs juxtapose with pre- and post-synaptic neurons, and share direct synaptic connections with glutamatergic neurons, suggesting a potentially significant contribution to ongoing brain plasticity (Ong and Levine, 1999; Bergles et al., 2000;

Paukert and Bergles, 2006). NG2-OPCs express glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and regulate extrasynaptic glutamate, suggesting that these cells may be involved in glutamatergic signaling (Dawson et al., 2003; Stegmuller et al., 2003; Karram et al., 2005; Paukert and Bergles, 2006). Even more interesting was the finding that some (but not all) NG2 cells, in both the gray and white matter regions of the brain, are capable of generating action potentials (Chittajallu et al., 2004; Ge et al., 2006; Karadottir et al., 2008) and display activity-dependent cellular plasticity (Ge et al., 2006). Therefore, it can be hypothesized that there are two distinct populations of NG2-glia based on the expression of (or lack of expression of) voltage-gated Na^+ channels and formation of glutamatergic synapses; alternatively, expression of voltage-gated Na^+ channels and the associated cellular characteristics are typical of a few but not all stages of maturation of NG2-OPCs. Another open–ended question is whether the two populations of NG2-OPCs, if present, are functionally distinct, or have distinct capacities for differentiating into oligodendrocytes. It is tempting to speculate that the NG2-glia expressing voltage-gated Na^+ channels are either terminally differentiated or are designated to a neuronal fate, and hence are not capable of generating oligodendrocytes *in vivo*. Recent studies using NG2-reporter transgenic mice have empirically evaluated these questions (De Biase et al., 2010). The study demonstrated that NG2-OPCs received synaptic inputs from glutamatergic neurons during early postnatal development. However, all NG2-OPCs did not respond similarly to the glutamatergic inputs, where some NG2-OPCs exhibited depolarization but none generated action potential. Furthermore, differentiation of NG2-OPCs into oligodendrocytes resulted in downregulation of both voltage-gated Na^+ channels and glutamatergic receptors (De Biase et al., 2010). These findings suggest that NG2-OPCs have distinct physiological roles during development and during adulthood. Thus, these studies confirm that NG2-OPCs can contribute significantly to ongoing synaptic plasticity, predominantly during the critical stages of neural development.

Neuron-glial antigen 2 is the hallmark protein of the NG2-OPCs and is expressed in the cell body as well as the processes radiating from the soma (Dawson et al., 2003). Several physiological roles have been suggested for the NG2-OPCs. For example, the proteoglycan shares structural features with neural cell adhesion molecules, and mediates the physical and synaptic connections between NG2 cell processes and the cell membrane of pre- and post-synaptic neurons (Bergles et al., 2000; Stegmuller et al., 2003). NG2-OPCs function as precursors to oligodendrocytes and closely interact with axons in various brain regions including the corpus callosum, cortex, and hippocampus [further reviewed in section **role of NG2-OPCs in addiction;** (Kukley et al., 2007; Ziskin et al., 2007; Karadottir et al., 2008; Ge et al., 2009; Etxeberria et al., 2010)]. Electrophysiological studies revealed glutamatergic synapses transmitting AMPA receptor-mediated currents between axons and NG2-OPCs, indicating functional synapses (Ziskin et al., 2007; Etxeberria et al., 2010). Furthermore, it has been demonstrated that the NG2-axon synapses could help to promote growth and myelination after nerve injury (Yang et al., 2006). Such studies allow us to speculate that the NG2-OPC – axon synapses may convey or receive information about the microenvironment, which could assist with triggering trophic signaling pathways that can aid in proliferation and differentiation of NG2-OPCs. Nevertheless, it can be hypothesized that the NG2-OPC – axon synapses allow for quick responses to injury, particularly those requiring remyelination processes.

Indeed, NG2-OPCs respond to axon injury by increasing their capacity to proliferate and differentiate into myelinating oligodendrocytes (Ong and Levine, 1999; Chida et al., 2011; Xiong et al., 2013). Such responses have been demonstrated in multiple types of demyelinating models of axon injury including hypothermia (Xiong et al., 2013), kainate (Ong and Levine, 1999), ischemia (Chida et al., 2011), and cuprizone (Kumar et al., 2007), where NG2-OPC density was enhanced at the site of injury concurrently with increased expression of myelin basic protein (MBP; a marker for myelinating oligodendrocytes). Notably, the NG2-OPC response to demyelinating lesions has been associated with up regulation of the trophic factor brain-derived neurotrophic factor (BDNF; VonDran et al., 2011), suggesting a cell-intrinsic type mechanism. These results demonstrate a functional role of NG2-OPC up regulation in maintaining myelin plasticity in the adult brain (Patel et al., 2010; Chida et al., 2011; Xiong et al., 2013).

DEVELOPMENTAL STAGES OF NG2-OPCs IN THE ADULT MAMMALIAN BRAIN

PROLIFERATION OF NG2-OPCs

Neuron-glial antigen 2-OPCs are predominantly found in the corpus callosum and in the gray matter regions of the brain (Ong and Levine, 1999), where they continue to proliferate during adulthood. Experiments using mitotic markers of cellular proliferation such as 5-bromo-2′-deoxyuridine (BrdU) in mice have shown that the cell cycle dynamics of NG2-OPCs are affected by age (**Figure 1**). Cell cycle time increases with age, where NG2-OPCs cycle through one cell cycle of ∼70 days beyond postnatal day (P)240 compared with a cell cycle length of 2 days at P6 (Psachoulia et al., 2009). These changes in cell cycle dynamics could support the decline in density of NG2-OPCs to approximately 75% of the initial cell mass in older animal subjects compared with newborn and young adults (He et al., 2009). These studies allow us to hypothesize that the cell cycle dynamics (such as length of time a cell spends in the cell cycle) of NG2-OPCs during development regulates the differentiation of the cell, such that reducing the length of the cell cycle assists with maintenance of the undifferentiated state (Lange et al., 2009; Salomoni and Calegari, 2010).

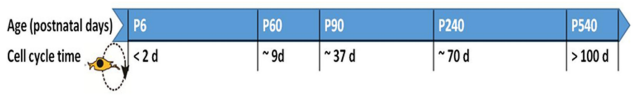

FIGURE 1 | Cell cycle time for NG2-OPCs in the rodent brain at different postnatal (P) ages. The cell cycle has been found to slow down with age, and this effect has been reported in the cortex as well as corpus callosum (Psachoulia et al., 2009; Simon et al., 2011). Age in days post-partum; cell cycle time in days (d).

DIFFERENTIATION OF NG2-OPCs

Neuron-glial antigen 2-OPCs in the adult brain primarily differentiate into premyelinating oligodendrocytes and myelinating oligodendrocytes, which produce myelin to maintain white matter tracts (**Figure 2**; Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2008a,b, 2011; Geha et al., 2010; Kang et al., 2010; Clarke et al., 2012). The oligodendrocyte lineage of NG2 cells is dependent upon expression of basic helix-loop-helix transcription factors OLIG1 and OLIG2 (Zhu et al., 2012). For example, Zhu et al. (2012) used conditional knockout mice to cause constitutive and inducible deletions of *Olig2* specifically in NG2 cells to show that when *Olig2* is knocked down, NG2 cell fate switch to astrocytes rather than oligodendrocytes (Zhu et al., 2010). Such loss-of-function genetic studies confirm the importance of OLIG2 expression in directing the NG2-OPCs to a myelinating oligodendrocyte lineage. Recent research supports the generation of myelinating oligodendrocytes from the proliferating pool of NG2-OPCs during adulthood, and these cells eventually express markers associated with myelin, such as myelin basic protein (Rivers et al., 2008; Geha et al., 2010; Clarke et al., 2012). However, the functional significance of adult-generated myelinating oligodendrocytes remains to be explained. Below we will briefly discuss an important marker that is key to the process of differentiation of NG2-OPCs into myelinating oligodendrocytes.

OLIG1 and OLIG2

Oligodendrocyte transcription factor 1 and 2 (OLIG1 and OLIG2) are transcription factors that are predominantly expressed in OPCs and in myelinating oligodendrocytes (Takebayashi et al., 2000; Zhou et al., 2000). Structurally, these transcription factors dimerize at the basic helix-loop-helix region and bind to their DNA targets to regulate transcription of other genes associated with ongoing biological functions such as neurogenesis and reactive gliogenesis (Fancy et al., 2004; Buffo et al., 2005; Menn et al., 2006). Detailed descriptions of the structural and functional differences between OLIG1 and OLIG2 have been recently published elsewhere and the readers are referred to the review by Meijer et al. (2012). Briefly, OLIG1 regulates the expression of several genes involved in oligodendroglial maturation, including MBP, myelin oligodendrocytic glycoprotein, myelin proteolipid protein, and zinc finger protein 488 (Arnett et al., 2004; Xin et al., 2005; Wang et al., 2006; Guo et al., 2010). Additionally, OLIG2 has been found to play several critical roles in oligodendrocyte differentiation including enhancing the expression of Sox10 and Sip 1, proteins that enhance oligodendrogial activity and maturation of NG2-OPCs (Wang et al., 2006; Kuspert et al., 2011; Weng et al., 2012; Yu et al., 2013). However, OLIG2 also has been identified as a transcription repressor for several targets and consequently has been implicated in human glioma (Lee et al., 2005; Ligon et al., 2007; Mehta et al., 2011). For example, OLIG2 is also implicated in modulating the response of non-oligodendrocytic glial cells, such as in reactive gliogenesis (proliferation of astrocytes and microglia) following a demyelinating injury in the cortex (Arnett et al., 2004; Chen et al., 2008b). These findings are in apparent contradiction to studies that reveal an absence of lineage relationship between OLIG 1/2 expressing cells and astrocytes, as well as studies showing that NG2-OPCs are not progenitors of reactive astroglia (Dimou et al., 2008; Komitova et al., 2011). These contradictions may be explained by the transient, non-lineage-dependent expression pattern of OLIG2 in the reactive astroglia (Magnus et al., 2007; Zhao et al., 2009).

ROLE OF NG2-OPCs IN ADDICTION

Addiction to illicit drugs has taken emotional and financial tolls on society, cutting across ages, races, ethnicities, and genders. Eventual dependence on any illicit drug increases mortality, morbidity, and economic costs. Despite the increase in the prevalence of addiction to illicit substances and the market for novel therapeutics, the research into understanding the neurobiological basis of addiction and relapse has progressed less well. Broadly defined, addiction is one of the many disorders that involves impulsivity and compulsivity (Heilig and Koob, 2007; Koob and Volkow, 2010), in which the impulsive phase involves the pleasurable effects of the drug and upon abstinence produces reward-induced craving

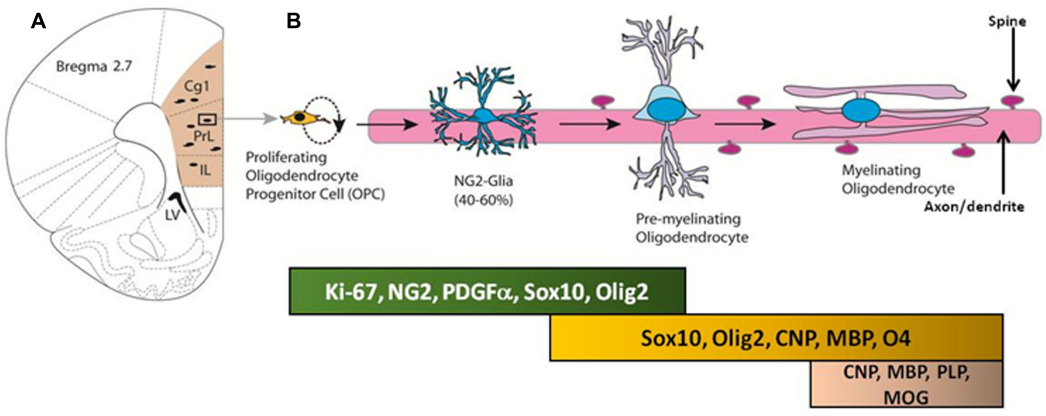

FIGURE 2 | (A) Coronal section of the adult rat brain through the mPFC; the mPFC is shaded in peach. Proliferating cells in the mPFC differentiate into oligodendrocyte progenitor cells (OPC) and premyelinating oligodendrocytes. **(B)** Developmental stages of oligodendrocyte progenitors in the mPFC; proteins and transcription factors that are expressed in distinct stages are indicated below.

and the compulsive phase develops after prolonged use of the drug when the individual seeks avoidance of the negative effects associated with drug withdrawal. The impulsive and compulsive phases of addiction can be characterized into three stages: (1) binge/prolonged intoxication, (2) withdrawal neutral/negative affect, and (3) preoccupation/anticipation (craving). The last stage of the addiction cycle describes a key element of relapse in humans and therefore defines addiction as a chronic relapsing disorder. Relapse to drug-seeking behavior is one of the least studied aspects of addiction, which has been a challenging area for neuroscientists.

Utilizing intravenous self-administration models of drug exposure, the research on addiction indicates dysregulation of the 'hedonic set point' and alteration in allostasis of the brain reward system which underlies the relapse to drug seeking and consequently addiction to the drug of abuse (Koob and Nestler, 1997; Koob and Le Moal, 2001, 2005). The relapse circuitry in adult mammalian brain is carved out based on multiple groundbreaking studies performed in rodent models of reinstatement (Shaham et al., 2003). The key brain regions implicated in the reinstatement of drug-seeking behavior include, but are not limited to, the prefrontal cortex (PFC), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), amygdala, hippocampus and the ventral tegmental area (VTA; Shaham et al., 2003; Koob and Volkow, 2010). The mesocorticolimbic dopamine system possesses neural connections from the VTA to the PFC and NAc and was crowned as the key circuit for reward and reinstatement of drug-seeking behaviors (Schultz, 1998; Horvitz, 2000; Robinson and Berridge, 2000; Comoli et al., 2003; Steketee, 2005). Most importantly, it is believed that the release of the neurotransmitters such as dopamine, glutamate, and corticotrophin-releasing factor in the key brain regions associated with relapse are essential for the behavioral outcomes of the drug (Wise, 1998; Di Chiara, 1999; Koob, 1999; Lu et al., 2003; Knackstedt and Kalivas, 2009). Furthermore, recent evidence supports the hypothesis that elevated anxiety, low mood, and increased sensitivity to stress (collectively labeled as negative affect) is the driving force behind the transition to addiction (George et al., 2014). Particularly interesting is the accumulating evidence that pathological neuroadaptations in the mPFC, extended amygdala and hippocampus may contribute to the negative affect state (Deschaux et al., 2012; George et al., 2012; Vendruscolo et al., 2012; Cohen et al., 2014). The following topics will characterize the NG2-OPCs in the brain regions associated with the negative affect state and hope to provide promising insights into translating the science of NG2-OPCs to future novel therapeutic approaches to target the relapse stage of the addiction cycle.

CHARACTERIZATION OF NG2-OPCs IN THE PREFRONTAL CORTEX

The PFC regulates executive functions such as decision making, impulse control and working memory (Bechara et al., 1994, 2001). Decreased PFC function has been associated with addiction, such that preexisting impairments in PFC function (as seen in attention deficit hyperactivity disorder and schizophrenia) serve as predictors for enhanced vulnerability for addiction (Crews and Boettiger, 2009; de Wit, 2009; Groman et al., 2009; Wing et al., 2012). Furthermore, chronic use of addictive drugs has been shown to enhance functional deficits in the PFC, particularly those related to decision making and impulse control, as a mechanism to perpetuate the recurrent relapsing drug-addicted phenotype (Franklin et al., 2002; Johnson et al., 2008; Xiao et al., 2008; Crews and Boettiger, 2009; de Wit, 2009; Koob and Volkow, 2010).

Studies have shown that within the mammalian cortex there is an abundant NG2 cell population that forms an evenly distributed and dense network (**Figure 3**; Dawson et al., 2003; Girolamo et al., 2010), however, the explicit role of NG2-OPCs in the mammalian cortex is still unclear. Recent studies in rodents have been directed at determining the role of these unique cells. While functional implications of NG2-OPCs are of critical interest, most recent studies of such cells in the cortex, and more specifically the PFC, are targeted at understanding proliferation and differentiation of NG2-OPCs.

Due to the important role the PFC plays in mood disorders such as depression, research with regard to this clinical condition has added information to further the understanding of this issue. Studies focusing on electroconvulsive seizure (ECS) treatment have explored phenotypic analysis and proliferation of cells in the PFC. Studies showed that in addition to an increase in proliferation of cells following ECS treatment, there is a larger proportion of NG2-OPCs when compared with other cell types such as astrocytes, neurons, or endothelial cells. While some studies show only a modest increase in the number of NG2-OPCs, others demonstrate that 40–50% of newly born cells are co-labeled as NG2 positive cells (Madsen et al., 2005; Czeh et al., 2007; Ongur et al., 2007). Other cell types, such as endothelial cells, were detectable but at a much lower proportion than NG2-OPCs. Astrocytes and neurons were consistently absent from such phenotypic analysis in these ECS studies. Additional inquiry into the role of NG2-OPCs in the cerebral cortex has revealed that stab wound injuries to the cortex also produce a large proportion of NG2-OPCs in the injured area, in this case as much as five times as many NG2 cells when compared to non-injured controls (Simon et al., 2011). This evidence is very similar to the previous findings in the PFC region.

Depression and affective disorders are not the only realm where NG2 cells in the PFC play an important part. Drug abuse research considers the PFC function to be an important factor in the negative reinforcement associated with motivational withdrawal and research in this field has demonstrated increased levels of NG2-OPCs in the PFC when animals are exposed to intermittent exposure to powerful psychostimulants like methamphetamine (Mandyam and Koob, 2012). Chronic exposure to methamphetamine or to sedative-hypnotics, such as alcohol, proves to be too toxic for these NG2-OPCs to survive (Mandyam et al., 2007; Richardson et al., 2009; Kim et al., 2014). Therefore, these studies support the hypothesis that the glial disturbance observed with chronic drug exposure may serve as an indirect mechanism to promote neurotoxicity.

The only deviation with regards to cellular differentiation from previously discussed studies is discovered when examining the effect of other proliferation enhancing treatments such as physical activity. Animals were given voluntary access to running wheels and were examined postmortem to determine cellular phenotype

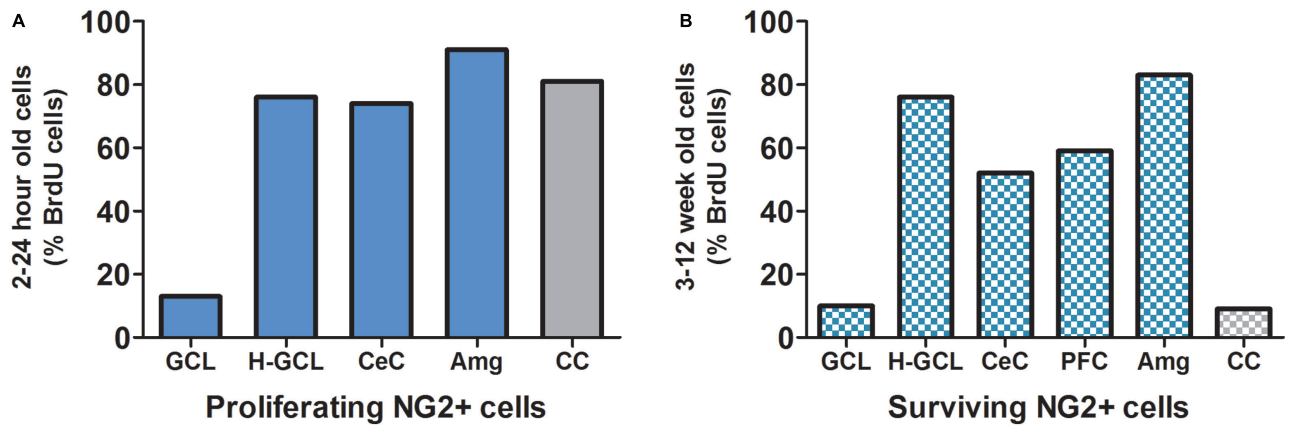

FIGURE 3 | (A) NG2 positive (NG2+) cells are the predominant proliferating cell type in most brain regions except in the granule cell layer (GCL) of the hippocampus. Data derived from previous publications (Dawson et al., 2003; Wennstrom et al., 2004; Orre et al., 2009; Hattiangady and Shetty, 2010; Ehninger et al., 2011). **(B)** Survival/persistence of NG2+ cells is low in the GCL of the hippocampus. In contrast more than 50% of the surviving BrdU cells are NG2+ in the hippocampal non-GCL areas, cerebral cortex and prefrontal cortex (PFC) as well as the amygdala. Data derived from previous publications (Wennstrom et al., 2004; Komitova et al., 2006; Czeh et al., 2007; Mandyam et al., 2007; Orre et al., 2009; Hattiangady and Shetty, 2010; Ehninger et al., 2011). GCL, granule cell layer; H-GCL, hippocampus without GCL; CeC, cerebral cortex; PFC, prefrontal cortex; Amg, amygdala; CC, corpus callosum.

in the PFC (Mandyam et al., 2007). In contrast to studies observing the effects of harmful processes on cell differentiation in the PFC, the wheel running data showed that newly born cells in the PFC are still mostly labeled as NG2-OPCs, but there were higher amounts of other kinds of cells observed such as neurons and astrocytes than have been seen in other studies. Therefore, there may be a relationship between the overall environment of the PFC and the capability of cells to differentiate into different cell types. Another study supports this hypothesis in the cerebral cortex by observing that when voluntary physical exercise was provided to animals, there was a reduced population of NG2-OPCs and increased levels of premature differentiation into oligodendrocytes (Simon et al., 2011).

Collectively, the data suggests that when there is an insult to the brain, say for example, by depression, stab wound or drug abuse, a large population of NG2-OPCs is needed to protect against these harmful effects and does not allow these cells to mature into different types of cells. Whereas if there is already a protective mechanism in place, like exercise, NG2-OPCs are still abundant in the PFC but allow for a wider range of maturation within the cell population. One last piece of compelling evidence was seen using *in vivo* recordings via cranial windows to observe NG2-OPCs. Following focal laser lesions in the cortex, NG2 cells were seen migrating to the injured sites and eventually surrounding the site and contributed to forming a glial scar (Hughes et al., 2013).

In summation, studies focusing on NG2 in the cortex and PFC have shown that NG2-OPCs are decreased along with the entire cell population in pathology. Additionally, NG2-OPCs are increased with treatments like pharmacological and physiological interventions. However, exercise supported the proliferation and differentiation into astrocytes and neurons. Taken together, this evidence provides support that NG2-OPCs in the PFC are involved in repair processes following insult.

CHARACTERIZATION OF NG2-OPCs IN THE HIPPOCAMPUS

The hippocampus is involved in learning and memory, cognitive function as well as mood regulation (Li et al., 2009; Jun et al., 2012). While the dorsal hippocampus has been implicated in modulating declarative and contextual memory, the ventral hippocampus via its reciprocal connections with the amygdala has been implicated in regulation of mood; both of these systems undergo maladaptive changes following chronic exposure to drugs of abuse (White, 1996; Volkow et al., 2004; Koob and Volkow, 2010). Adult neurogenesis in the hippocampal subgranular zone is particularly sensitive to chronic drug induced disruption leading to aberrant adaptions in memory (Canales, 2007, 2010; Jun et al., 2012). Neuroadaptions in the hippocampus are particularly important as they contribute to enhanced sensitivity to context as well as stress induced relapse (Kilts et al., 2001; Crombag et al., 2008; Koob, 2008; Koob and Volkow, 2010; Belujon and Grace, 2011).

The existence of NG2-OPCs in the hippocampus is well established. In the normal adult, NG2-OPCs are distributed throughout all layers of the hippocampus (Levine and Nishiyama, 1996; Ong and Levine, 1999; Xu et al., 2014) with higher numbers in the stratum lucidum and dentate hilus (**Figure 3**; Bu et al., 2001). The functional role of NG2-OPCs in the hippocampus, however, remains to be found. Possible functions can be discussed based on what is known about NG2 cell development, NG2 cell interactions with other cell types, and NG2 cell reactions to changes in the hippocampus.

Neuron-glial antigen 2-OPCs are present and proliferate both during development and into adulthood. The density of NG2-OPCs in the adult hippocampus changes with age (Levine and Nishiyama, 1996; Chen et al., 2008a, Hughes et al., 2013). Initially, the number of NG2-OPCs increased from P0–P7, and then decreased when measured at P21, P50, and P450 as evidenced by NG2 immunostaining and NG2 protein level expression (Chen

et al., 2008a). NG2 cell morphology also changes with age, with neonatal rats expressing NG2 in cells with 'simpler' morphology, and 'complex' morphology appearing by P7. Most of the NG2-OPCs in the adult hippocampus have a stellate morphology with several processes radiating from the soma (Ong and Levine, 1999; Chen et al., 2008a; Xu et al., 2014). NG2-OPCs are motile and extend processes to survey the surrounding environment. NG2-OPCs maintain homeostatic control of cell density through a balance of differentiation, proliferation, cell-death, and self-repulsion (Hughes et al., 2013).

The proliferation and differentiation of NG2-OPCs in the hippocampus has been described extensively [for review see (Richardson et al., 2011)]. NG2 cells are considered to be NG2-OPCs that eventually differentiate into myelinating oligodendrocytes (Butt et al., 1999; Nishiyama et al., 1999, 2009; Wigley et al., 2007; Kang et al., 2010); however, there are studies that investigated the multipotency of NG2 cells in the CNS (Kondo and Raff, 2000; Sypecka et al., 2009). There is evidence that hippocampal cell cultures provide a neuronal microenvironment that can induce NG2-derived neurogenesis (Sypecka et al., 2009). However, several fate-mapping studies in transgenic mice show that in the hippocampus, NG2-OPCs primarily develop into myelinating oligodendrocytes (Zhu et al., 2008a,b, 2011).

The spatial organization of NG2-OPCs led researchers to investigate communication between NG2-OPCs and other cell types. NG2-OPCs are in close contact with astrocytes and neurons (Nishiyama et al., 1999; Ong and Levine, 1999; Xu et al., 2014). There is now surmounting evidence of NG2-OPCs making synaptic contact with neurons [for review see (Karram et al., 2005)]. Neuron-NG2 cell synapses have been identified by electrophysiological techniques (Bergles et al., 2000; Lin and Bergles, 2004; Jabs et al., 2005; Ge et al., 2006; Kukley et al., 2008; Mangin et al., 2008). Kukley et al. (2010) used a NG2cre:Z/EG double-transgenic mouse line to identify developmental stages of NG2-OPCs and see at what stages they make synaptic contact with neurons in CA1 of hippocampus. Whole-cell patch clamp recordings revealed that synaptic input is restricted to NG2-OPCs, and is lost when they differentiate into premyelinating and myelinating oligodendrocytes (Kukley et al., 2010).

AMPA receptors that are calcium permeable have been found on hippocampal NG2-OPCs (Seifert and Steinhauser, 1995; Bergles et al., 2000; Lin and Bergles, 2002). NG2-OPCs can respond to neuronal stimulation and neurotransmitter release because they harbor *N*-methyl-D-aspartate (NMDA) receptors, AMPA receptors and γ-aminobutytic acid (GABA) receptor subtype A (GABAA; Bergles et al., 2000; Lin and Bergles, 2002, 2004; Chittajallu et al., 2004; Jabs et al., 2005; Karadottir et al., 2005; Salter and Fern, 2005; Ge et al., 2006; Paukert and Bergles, 2006; Kukley et al., 2007). Neuron-NG2 synapses can have activity-dependent changes analogous to long-term potentiation (LTP) at glutamatergic neuronal synapses. LTP expression in neuron-NG2 synapses is mediated by calcium-permeable AMPA receptors located on NG2-OPCs (Ge et al., 2006).

Connections between neurons and NG2-OPCs allow NG2 cells to be highly responsive to injuries in the hippocampus, inducing different morphological and proliferative changes over time (Nishiyama et al., 1999; Bu et al., 2001). After kainic acid-induced excitotoxic lesions in the hippocampus, NG2-OPCs showed two types of reactive changes: the early and persistent change 24 h to 3 months after lesion and the late, transient change 2 weeks after lesion (Bu et al., 2001). The first early change was characterized as an increase in NG2 immunoreactivity and an increase in processes extending from the cell body. NG2-OPCs during the late change had large round cell bodies, had short processes and they also expressed OX42 and ED1, markers for microglia/macrophages. There was also a corresponding change in the distribution of GFAP+ astrocytes in CA3. At 3 days post lesion, NG2 reactivity was high while GFAP cells were low, and at 2 weeks post lesion, NG2 reactivity was reduced while astrocytes filled in (Bu et al., 2001). Increases in NG2 reactivity is similar for multiple injury types, including inflammation (Nishiyama et al., 1997), viral infection (Levine et al., 1998), mechanical wound (Levine, 1994), ECSs (Jansson et al., 2009) and excitotoxic lesion (Ong and Levine, 1999). Similar changes in morphology and antigen expression in NG2-OPCs occurred in parallel with activation of microglia using a model of selective neurodegeneration in the mouse dentate gyrus with trimethyltin (Fiedorowicz et al., 2008). The monocyte properties of NG2-OPCs suggest that the NG2 cells may serve a function in phagocytosis.

The response of increasing NG2-OPC proliferation can be the first stages of a myelination response. Lesions of the entorhinohippocampal perforant pathway induces formation of axonal sprouts, which recruits NG2-OPCs to divide and become oligodendrocytes as evidenced by NG2 cells and oligodendrocytes incorporating BrdU 9 weeks post lesion (Drojdahl et al., 2010). One way NG2-OPCs respond to the environment is through acid chemosensors, specifically the ASIC1a channel found in the cell membrane. Activation of these channels can induce membrane depolarization and Ca^{2+}, which would serve as a quick response to injury following ischemia (Lin et al., 2010). It appears that NG2-OPCs in the hippocampus function to maintain glial homeostasis. Their responses to mechanical and cellular injury suggest that NG2-OPCs in the hippocampus may assist with maintaining hippocampal myelin plasticity.

CHARACTERIZATION OF NG2-OPCs IN THE AMYGDALA

The amygdala is involved in modulating emotional memory and affective behavior, and is particularly important for fear learning and adverse reactions; these behavioral maladaptations in the amygdala are associated with negative reinforcement which triggers relapse to drug seeking (Grant et al., 1996; Maren, 1999; Blair et al., 2001; Kilts et al., 2001; Funk et al., 2006; Koob, 2008). Structural abnormalities in the amygdala are associated with the pathophysiology of addiction and several neurological disorders, including but not limited to depression, schizophrenia and temporal lobe epilepsy (Sheline et al., 1998; Mervaala et al., 2000; Wright et al., 2000; Drevets et al., 2002; Faber-Zuschratter et al., 2009; Chen et al., 2014). Several studies have uncovered neuronal aspects of amygdalar plasticity (for example synaptic plasticity and neurotropic mechanisms) in emotional behaviors [for review, (Maren, 2005)], however few studies have evaluated gliogenesis in amygdalar plasticity.

Amygdalar NG2 cells share several similarities with NG2-OPCs in the cortex and hippocampus. Approximately, 88–94% of the proliferating cells in the adult rodent amygdala were NG2-OPCs (**Figure 3**), and this pool of NG2 cells appear to exhibit limited differentiation over time (Wennstrom et al., 2004; Ehninger et al., 2011). These cells exhibit electrophysiological properties that were similar to the NG2-OPCs from corpus callosum (Ehninger et al., 2011). Similar to hippocampus and PFC, ECSs [established therapeutic strategy for depression (Clarke et al., 1989)], increased proliferation of NG2-OPCs (NG2+/BrdU+), increased expression of NG2 protein (Jansson et al., 2009), and subsequent differentiation into mature oligodendrocytes 3 weeks after cell division (RIP:oligodendrocyte marker and BrdU+) in the amygdala (Wennstrom et al., 2004; Jansson et al., 2009). Furthermore, oral administration of lithium chloride (established treatment for bipolar disorder) was reported to increase proliferation of NG2-OPCs in the amygdala (a similar response compared with the hippocampus and PFC), but enhanced proliferation was not associated with an increase in differentiation of NG2 cells into mature oligodendrocytes (Orre et al., 2009). However, physical activity via wheel running and environmental enrichment in rats did not alter the proliferation or the survival of newly born NG2-OPCs in the amygdala (an opposite response compared with the hippocampus and PFC), but decreased formation of new astroglia (S100β: astroglial marker and BrdU+) in the amygdala (Ehninger et al., 2011). The differences in responses to the wheel running stimulus could be due to variability in experimental design as the study in the amygdala used control rats that were not maintained under impoverished environmental conditions [for description impoverished environment, (Bardo et al., 1997)]. Taken together, research thus far suggests that amygdalar NG2-OPCs proliferation is a neuroplastic response restricted to certain pharmacological or mechanical type injuries.

In the above context, amygdalar gliosis and aberrant NG2 cell expression is associated with temporal lobe epilepsy (Faber-Zuschratter et al., 2009). Structural abnormalities in the amygdala are associated with the pathophysiology of mood disorders like depression (Sheline et al., 1998; Mervaala et al., 2000; Drevets et al., 2002) and of psychiatric disorders such as schizophrenia (Wright et al., 2000; Chen et al., 2014). In fact, postmortem studies suggest that schizophrenia patients exhibit decreased oligodendrocyte density in the amygdala compared with age-matched controls (Uranova et al., 2004; Williams et al., 2013). It appears that much more data is available in the amygdala to demonstrate that NG2 glial disturbance occurs in response to mood disorders and neurodegenerative diseases. However, further studies are required to uncover the role of NG2-OPCs in the amygdala in addiction and relapse to drug seeking.

NEUROMODULATORS OF STRESS RESPONSES AFFECT NG2-OPCs

Addiction and eventual dependence to illicit drugs and alcohol induces attenuated (opposing) basal stress hormone levels [adrenocorticotropic hormone (ACTH) and corticosterone (CORT) an agonist at the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR)] compared with non-dependent subjects (enhanced stress hormone levels), and it has been demonstrated that the blunted stress response is a consequence of chronic drug or alcohol exposure (Zorrilla et al., 2001; Mandyam et al., 2008; Richardson et al., 2008). Importantly, the findings from the animal studies are consistent with clinical studies that link maladaptive hypothalamic-pituitary-adrenal (HPA) axis function with drug dependence and alcoholism, including a reduced ability to cope with stress and negative correlations between cortisol and craving and relapse in dependent subjects (Lovallo et al., 2000; Winhusen and Somoza, 2001; O'Malley et al., 2002; Nava et al., 2006). Although the precise mechanism underlying the attenuated stress response is unknown, several studies have implicated activation of pro-stress hormones [via enhanced expression of corticotrophin releasing factor (CRF) and altered expression of the CRF receptors] in the extended amygdala to contribute to the dysregulated stress system associated with dependence (Wand, 2005; Koob, 2008). Furthermore, enhanced GR levels in the extended amygdala during protracted abstinence has been demonstrated in dependent animals and such associated changes in GR system could play a mechanistic role in sensitivity to stress/reward and relapse associated with dependence (Vendruscolo et al., 2012). However, the functional significance of altered GR system in mediating blunted stress responses in drug dependence is unknown.

The few select studies that have attempted to ascertain a relationship between stress and NG2-OPCs have established a role for CORT in the proliferation, differentiation, and generation of myelin of these developmentally derived cells that appears to be dose and duration dependent (Chari, 2014). Oligodendroglia are sensitive to activation of the HPA axis and subsequent release of stress hormones as they express MRs and GRs (Jenkins et al., 2014). Prolonged exposure to glucocorticoids suppresses NG2-OPC proliferation, and synthetic glucocorticoids inhibit oligodendrocyte death. Stress hormones, or their analogs, can have protective effects when the exposure is acute. For example, treatment with methylprednisolone, a GR agonist, resulted in typical rates of cell death of cells expressing neuronal markers but preferential survival of cells expressing oligodendrocyte markers (Lee et al., 2008). Similarly, exposure to physiological (physical restraint) or pharmacological (CORT injection) stress resulted in a skewed ratio of neuron to glia in terms of neurogenic development (Chetty et al., 2014). For example, while under typical conditions, the newly born cells in the subgranular zone of the hippocampus develop primarily into neurons but following stress exposure and stress hormone exposure, there was a significant increase in the number of oligodendrocytes and a reduced number of neurons (Chetty et al., 2014). Additionally, exposure to dexamethasone on oligodendrocytes in culture results in increased rates of myelin formation and a subsequent increase in the overall quantity of myelin (Chan et al., 1998). However, excessive exposure to CORT has been shown to suppress proliferation of NG2-OPCs (Alonso, 2000; Wennstrom et al., 2006) and this effect does not appear to be age dependent, as decreases in both MBP expression and the number of oligodendrocytes are reported in fetal animals subjected to a GR agonist, betamethasone (Kumar et al., 2007).

These findings are of further importance as they shed light into the cellular pathology of human depression and affective disorders. Studies of chronically depressed human subjects have reported significant reductions in the number of glia in frontal cortical areas (Ongur et al., 1998; Rajkowska et al., 1999; Uranova et al., 2004; Rajkowska and Miguel-Hidalgo, 2007), findings which are paralleled in animal models of chronic stress. Rodents which were subject to chronic social defeat, a model of chronic stress (Rygula et al., 2005, 2006a,b; Krishnan et al., 2008), had a significantly depressed rate of cell proliferation in the mPFC, however, the percentage of the proliferating cells which were NG2-OPCs was comparable to non-stressed controls (Czeh et al., 2007). As human patients diagnosed with chronic depression are reported to have dysfunction of the HPA axis resulting in systemically elevated CORT levels (Stokes, 1995; Pariante and Miller, 2001; Burke et al., 2005; Cieslik et al., 2007), the reduced number of NG2-OPCs in these patients corresponds to the preclinical findings with chronic stress or artificial stress hormone administration. Further supporting this damaging relationship of stress hormones and NG2 proliferation is the work with antidepressant therapies, both pharmacological (i.e., fluoxetine) and physical (i.e, electroconvulsive therapy), which produced measureable increases in the number of NG2-OPCs (Kodama et al., 2004; Wennstrom et al., 2004; Czeh et al., 2007). Taken together, there is a clear link between exposure to chronic stress hormones and the dysregulation of NG2-glia proliferation and differentiation.

Oligodendroglia express dopaminergic receptors, and their activation can drive and influence development of premyelinating oligodendrocytes and myelin; the activation of D2 and D3 receptors on oligodendrocytes following oxidative stress injury can reduce glial loss. For example, NG2-OPCs have been shown to express dopamine receptors, specifically the D3 receptor (Bongarzone et al., 1998), and there is evidence that analogs of these glial precursors in human embryonic stem cell cultures express serotonergic receptors ($5HT_{2A}$; Schaumburg et al., 2008). However, there has been no clear establishment of the function of dopamine or serotonergic receptors in NG2-OPCs with regard to disease pathology. However, a link has been established associating the diagnosis of mood disorders and NG2 proliferation, and while a likely biochemical mediator of these effects is the dysregulation of the monoamiergic system, it is not clear in which order the events occur; does deleterious behavior precede biochemistry implying NG2 proliferation as a pathologic response, or does a change in cortical function at a cellular level lead to pathological cortical function, implying that NG2 dysfunction is potentially driving the disease state? Future studies directed at specifically answering this question, as well as the precise involvement and function of monoaminergic receptors will be required to elucidate these processes.

CONCLUDING THOUGHTS

In conclusion, there is substantial evidence that NG2-OPCs cells are critical for homeostatic control of oligodendrocytes in the adult brain. There is also evidence demonstrating that under pathologic conditions, NG2-OPCs can play a role in potential repair processes. Similarly, further studies are required in the future to confirm the neurogenic potential of NG2-OPCs particularly during adulthood, findings which could aid in the understanding of the role in NG2-OPCs in addiction. The prospective role of NG2-OPCs in the cortical pathology associated with drug addiction is a promising and underexplored area of research for investigating both pathophysiological mechanisms and potential strategies for recovery. NG2-OPCs are therefore a novel cell type requiring critical investigation into their function and role in the mammalian cortex.

ACKNOWLEDGMENTS

Preparation of this review was supported by funds from the National Institute on Drug Abuse (DA022473 and DA034140 to Chitra D. Mandyam), National Institute on Alcoholism and Alcohol Abuse (AA020098 and AA06420 to Chitra D. Mandyam, and T32AA00747 to Miranda C. Staples), Alcohol Beverage Medical Research Foundation (to Chitra D. Mandyam), and National Science Foundation (DGE-1144086 to Melissa H. Galinato). This is manuscript number 28046 from The Scripps Research Institute.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 September 2014; accepted: 30 November 2014; published online: 19 December 2014.

*Citation: Somkuwar SS, Staples MC, Galinato MH, Fannon MJ and Mandyam CD (2014) Role of NG2 expressing cells in addiction: a new approach for an old problem. Front. Pharmacol. **5**:279. doi: 10.3389/fphar.2014.00279*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

ORIGINAL RESEARCH ARTICLE
published: 23 February 2015
doi: 10.3389/fphar.2015.00027

Repeated cycles of chronic intermittent ethanol exposure increases basal glutamate in the nucleus accumbens of mice without affecting glutamate transport

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Repeated cycles of chronic intermittent ethanol (CIE) exposure increase voluntary consumption of ethanol in mice. Previous work has shown that extracellular glutamate in the nucleus accumbens (NAc) is significantly elevated in ethanol-dependent mice and that pharmacologically manipulating glutamate concentrations in the NAc will alter ethanol drinking, indicating that glutamate homeostasis plays a crucial role in ethanol drinking in this model. The present studies were designed to measure extracellular glutamate at a time point in which mice would ordinarily be allowed voluntary access to ethanol in the CIE model and, additionally, to measure glutamate transport capacity in the NAc at the same time point. Extracellular glutamate was measured using quantitative microdialysis procedures. Glutamate transport capacity was measured under Na^+-dependent and Na^+-independent conditions to determine whether the function of excitatory amino acid transporters (also known as system X_{AG}) or of system X_c^- (glial cysteine–glutamate exchanger) was influenced by CIE exposure. The results of the quantitative microdialysis experiment confirm increased extracellular glutamate (approximately twofold) in the NAc of CIE exposed mice (i.e., ethanol-dependent) compared to non-dependent mice in the NAc, consistent with earlier work. However, the increase in extracellular glutamate was not due to altered transporter function in the NAc of ethanol-dependent mice, because neither Na^+-dependent nor Na^+-independent glutamate transport was significantly altered by CIE exposure. These findings point to the possibility that hyperexcitability of cortical–striatal pathways underlies the increases in extracellular glutamate found in the ethanol-dependent mice.

Keywords: alcohol, mouse, microdialysis, uptake, transport

INTRODUCTION

Prolonged excessive alcohol (ethanol) consumption can lead to dependence, a condition characterized by many neuroadaptive changes in brain reward and stress systems (Hansson et al., 2008; Koob and Le Moal, 2008; Spanagel, 2009). These neuroadaptive changes trigger withdrawal symptoms when drinking is terminated, increase vulnerability to relapse, and facilitate a shift from regulated drinking to less controlled and more excessive ethanol consumption (Becker, 2008; Vengeliene et al., 2009; Heilig et al., 2010). In particular, an adaptive up-regulation of glutamatergic activity following chronic ethanol treatment is well-documented in animal studies (Gass and Olive, 2008). For example, in rodents, microdialysis studies have revealed elevated extracellular levels of glutamate following chronic ethanol treatment in several brain regions including dorsal striatum, nucleus accumbens (NAc), hippocampus, and the ventral tegmental area (Dahchour et al., 2000; Baker et al., 2002; Dahchour and De Witte, 2003; Melendez et al., 2005; Ding et al., 2012, 2013; Griffin et al., 2014). Similarly, using magnetic resonance spectroscopy (MRS) techniques in rodents, increased glutamate activity has been reported in prefrontal cortex (Hermann et al., 2012) and basal ganglia (Zahr et al., 2009; Gu et al., 2014). Emerging evidence indicates similar findings in human alcoholics (Hermann et al., 2012; Bauer et al., 2013). Thus, across different model systems and procedures, chronic exposure to ethanol produces increased glutamatergic activity.

We have developed a mouse model of ethanol dependence and relapse drinking that involves chronic intermittent ethanol (CIE) exposure and produces significant escalation of voluntary ethanol consumption in dependent compared to non-dependent mice (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009b, 2014; Griffin, 2014). Recently, using *in vivo* microdialysis techniques, we reported that ethanol-dependent mice have increased extracellular glutamate concentrations in the NAc (Griffin et al., 2014). Importantly, this effect was shown to be

sustained beyond acute withdrawal (at least 6–7 days following CIE exposure). Further, pharmacologically increasing or decreasing glutamatergic concentrations in the accumbens increased or decreased, respectively, ethanol drinking in the model (Griffin et al., 2014). These findings indicate an important role for accumbal glutamatergic transmission in regulating ethanol drinking, and increased glutamate activity in the NAc following chronic ethanol exposure may contribute to escalated drinking associated with dependence. Further, our findings are consistent with other reports demonstrating a relationship between glutamate activity and the regulation of ethanol consumption in mice (Kapasova and Szumlinski, 2008; Szumlinski et al., 2008). Together, these results provide evidence for a significant role for glutamate in the addiction process (Kalivas and O'Brien, 2008).

Although it is known that extracellular glutamate levels are tightly regulated by numerous neuronal and glial functions (Danbolt, 2001), the mechanism underlying elevated glutamatergic activity in ethanol dependence is unknown. Active transporter mechanisms in neurons and glia play a critical role in maintaining glutamate homeostasis in the synapse. Glutamate transporters (excitatory amino acid transporters, EAATs) operate to remove glutamate from the extracellular space (synapse) in a sodium (Na^+)-dependent manner (Danbolt, 2001). The glia-based system X_c^- is Na^+-independent and exchanges extracellular cysteine for intracellular glutamate, which contributes significantly to the extrasynaptic pool of glutamate (Baker et al., 2002). Interestingly, while previous work using rats indicates that non-contingent ethanol exposure increases glutamate concentrations in the accumbens, this was not associated with significant alterations in Na^+-dependent glutamate transporter expression (Melendez et al., 2005). However, recent studies using voluntarily drinking P rats have found increases in glutamate in the NAc to be associated with decreases in Na^+-dependent transporters, specifically EAAT1, but not EAAT2 or system X_c^- expression (Ding et al., 2013; Alhaddad et al., 2014a). The present study was conducted to confirm our earlier findings of increased extracellular glutamate levels in the following CIE exposure using quantitative microdialysis procedures, as well as to investigate whether CIE exposure produces alterations in glutamate transporter function that contributes to the observed increase in basal glutamate in ethanol-dependent mice.

MATERIALS AND METHODS

SUBJECTS

Male C57BL/6J mice (10–14 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in a temperature and humidity controlled AAALAC accredited facility under a 12 h light cycle (lights on 0200 h). Mice were initially group housed during a 2–4 week period of acclimation to the vivarium, and then individually housed for the remainder of the experiments. Food and water were available *ad libitum* at all times. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and were consistent with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. Separate cohorts of mice were utilized for the microdialysis and glutamate transport experiments.

CHRONIC INTERMITTENT ETHANOL EXPOSURE

Chronic intermittent ethanol exposure was administered via inhalation using a well-established ethanol dependence model in mice (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin, 2014; Griffin et al., 2014). Briefly, one group of mice (ethanol-dependent; EtOH group) received chronic intermittent exposure to ethanol vapor in inhalation chambers (16 h/day for 4 days) while the remaining mice (non-dependent; CTL group) were similarly handled, but maintained in control (air) inhalation chambers. This pattern of CIE (or air) vapor exposure was repeated over 3 weekly cycles for the quantitative microdialysis study and 4 weekly cycles for the studies involving glutamate transport assays. In both studies, the weekly inhalation exposure cycles were alternated with intervening weeks when animals were left undisturbed in the colony room. Ethanol (95%) was volatilized, mixed with fresh air, and delivered to Plexiglas inhalation chambers at a rate set to yield blood ethanol levels in the range of 175–225 mg/dl. These values were verified by measuring chamber ethanol concentrations (daily) and blood ethanol concentrations (weekly) as previously described (Griffin et al., 2009a). Prior to being placed in the ethanol vapor chambers, EtOH mice were administered a loading dose of ethanol (1.6 g/kg; 8% w/v) and the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) by intraperitoneal injection. CTL mice received injections of saline and pyrazole before being placed in control chambers. During the inhalation treatment, the housing conditions were identical to those in the colony room.

MICRODIALYSIS GUIDE IMPLANT SURGERY

Surgical procedures were performed as previously described (Griffin et al., 2007, 2009b; Griffin, 2014). Briefly, mice were anesthetized with isoflurane gas (4% induction, 1.5% maintenance) and placed in a Kopf stereotaxic instrument with digital display (Model 942). Guide cannulae (10 mm long) were implanted just above the left (NAc) (coordinates relative to Bregma: AP +1.7, ML +0.8 and DV −3.5) and secured to the skull using a light-cured resin system. Guide obdurators remained in place until microdialysis procedures were initiated (see below). After surgery, mice were allowed at least 1 full week of recovery before experimental procedures commenced.

QUANTITATIVE (NO NET FLUX) MICRODIALYSIS PROCEDURES

Microdialysis was conducted in EtOH and CTL groups at 72 h after the final CIE (or air) exposure cycle. On the day before microdialysis, mice were lightly restrained and microdialysis probes (CMA/7; CMA Microdialysis, Sweden) were inserted at least 16 h prior to sample collection (probes extended 1 mm beyond the guide cannulae). The aCSF consisted of: 140 mM NaCl; 7.4 mM Glucose; 3 mM KCl; 0.5 mM MgCl2; 1.2 mM CaCl2; 1.2 mM Na_2HPO_4; 0.3 mM NaH2PO$_4$; pH = 7.4 and was filtered (0.22 μm) before use. aCSF was perfused through the probe at an overnight flow-rate of 0.2 μL/min, as previously described (Griffin et al., 2014). The flow rate was increased to 1.0 μL/min on the following day and sample collection commenced 2 h later. Dialysates were collected every 15 min and an aliquot (10 μL) of each sample was immediately frozen on dry ice and stored at −80°C until analysis. During the collection

procedure, increasing concentrations of glutamate were added to the aCSF and perfused through the probes (0, 0.2, 1, 2.5, and 5 μM). Four samples were collected at each concentration and the last three samples from each series were averaged and analyzed for glutamate content. Glutamate concentrations were determined using high pressure liquid chromatography (HPLC) with fluorescence detection, as previously described (Griffin et al., 2014).

HISTOLOGY

At the end of the quantitative dialysis experiment, mice were overdosed with urethane (1.5 g/kg i.p.), transcardially perfused, and brains were then extracted and stored in 10% formalin for a period of 3–5 days as previously described (Griffin et al., 2009b). The brains were sectioned at 50 μm and stained with cresyl violet to evaluate probe placement using a mouse brain atlas as a guide (Paxinos and Franklin, 2001).

GLUTAMATE TRANSPORT ASSAY

At 72 h following the final CIE (or air) exposure cycle, mice were sacrificed via rapid decapitation, the brains were rapidly extracted, and 1 mm bilateral tissue punches of the NAc core were dissected on ice. To ensure an adequate amount of tissue was available for analysis, accumbal tissue samples were pooled from two mice in each condition. Using a McIllwan tissue chopper (St. Louis, MO, USA), the tissue was cut into 250 × 250 μm slices which then underwent three 10 min washes at 37Â°C using an oxygenated Krebs–Ringer's solution (140 mM NaCl, 1.3 mM CaCl2, 1.2 mM KH_2PO_4, 5 mM HEPES, 10 mM glucose and 1 mM $MgCl_2$; final pH 7.4). To measure glutamate uptake, L-[^{3}H] glutamate (250 nM, 51 Ci/mM; Perkin-Elmer, Boston, MA, USA) was added to aliquots of tissue-slice samples in the presence of 0.1, 1, 10 100 and 1000 μM unlabeled L-glutamate in a final volume of 250 μL of oxygenated buffer. Na^+-independent uptake was measured by conducting the final wash and slice incubation in buffer where NaCl was replaced with 140 mM choline chloride. All incubations were conducted in triplicate. After incubation at 37°C for 15 min, the reaction was terminated by washing the slices in ice-cold, Na^+-free buffer. Slices were then solubilized using 1% sodium dodecyl sulfate at room temperature for 12 h and radioactive counts subsequently determined using a liquid scintillation counter (Packard 1900 TR). Protein content in the slices was determined using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Counts per minute were converted to uptake/mg protein/15 min.

To confirm Na^+-dependent and -independent transport in this assay, a separate cohort of ethanol naïve animals ($n = 3$) were sacrificed and measurements conducted in the presence of the non-selective EAAT inhibitor TBOA (0, 10, or 50 μM) or the System X_c^- inhibitor CPG (0, 5, or 25 μM), respectively. These experiments were conducted at the 10 μM glutamate concentration prepared in both Na^+-containing and Na^+-free buffers.

CHEMICALS

DL-threo-β-benzyloxyaspartate (TBOA) and (S)-4-Carboxyphenylglycine (CPG) were obtained from Tocris Bioscience, Inc., (Bristol, UK). The drugs were frozen in 2.5 mM and 50 mM stocks, respectively, in 1× phosphate buffered saline (PBS) and diluted as needed. All other chemicals were purchased from Sigma-Aldrich, Inc (St Louis, MO, USA).

DATA ANALYSES

The data from the quantitative microdialysis experiment were analyzed by first subtracting the known amount of glutamate added to the perfusate from the amount measured in the dialysate by HPLC analysis ($[Glu]_{in} - [Glu]_{out}$) which is taken as the net flux of glutamate across the dialysis membrane. For individual mice, slopes of the linear function provide a measure of glutamate clearance and the X intercepts are taken to reflect basal glutamate concentration (Parsons and Justice, 1994). Therefore, these values were calculated for individual mice using the linear function ($y = mx + b$) and Student's t-test was used to compare these measures between EtOH and CTL groups.

Glutamate transport analyses were conducted separately for the Na^+-dependent and Na^+-independent experiments. Non-linear curves, as shown in **Figure 2**, were generated using GraphPad, Prism 4 software (La Jolla, CA, USA). However, the data were analyzed using a mixed model procedure (SPSS® version 18), with group and concentration as between-subject factors. Finally, for the TBOA and CPG uptake experiments, data were analyzed using analysis of variance with *post hoc* analysis using Bonferroni's corrected t-test. For all analyses, alpha was set to 0.05.

RESULTS

QUANTITATIVE MICRODIALYSIS

A summary of results from the quantitative dialysis experiment (i.e., no net flux dialysis) for ethanol-dependent and non-dependent mice is shown in **Figure 1A**. As can be seen, the calculated X-intercept was greater for dependent (EtOH group) compared to non-dependent (CTL group) mice, indicative of higher basal glutamate concentrations in CIE-exposed mice. In contrast, the slopes of the linear functions for the two groups were parallel, suggesting that rate of glutamate uptake was similar for both groups. Individual x-intercepts, representing basal extracellular glutamate concentrations, and slopes of the linear function were calculated for mice in both groups and these data are summarized in **Figures 1B,C**, respectively. Ethanol-dependent mice exhibited significantly greater basal extracellular glutamate concentrations ($t(10) = 3.362$, $p < 0.01$) but the slopes of the non-net-flux function were not different ($t(10) = 0.059$, $p > 0.05$). Finally, **Figure 1D** shows the placements of the microdialysis probes.

GLUTAMATE TRANSPORT

Because regulation of extracellular glutamate concentrations is regulated by multiple processes (Winder and Conn, 1996; Danbolt, 2001), tritiated-glutamate uptake assays were used to further examine the influence of ethanol dependence on transporter function. Results of the glutamate transport assays are summarized in **Figure 2**. As can be seen, ethanol-dependent and non-dependent mice did not differ in either Na^+-dependent (**Figure 2A**) or Na^+-independent (**Figure 2B**) glutamate transport. These observations were supported by a mixed model analysis of the data. As expected, for the Na^+-dependent transport assay, ANOVA indicated a significant main effect of glutamate Concentration [$F(4,165) = 25.15$, $p < 0.0001$], but there was no

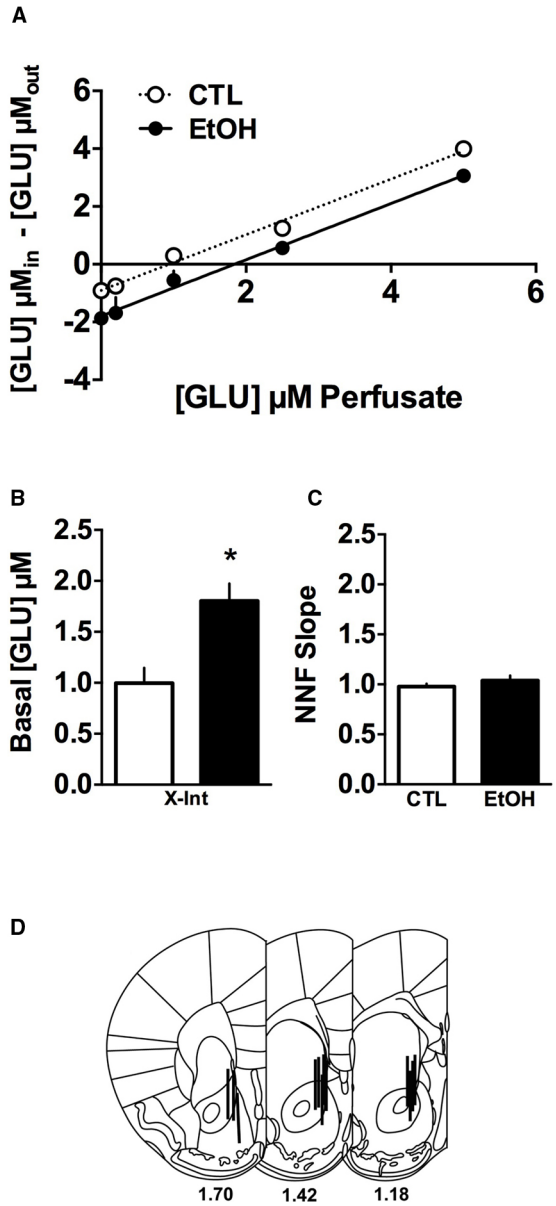

FIGURE 1 | Glutamate quantitative microdialysis in the nucleus accumbens (NAc) of ethanol-dependent (EtOH) and non-dependent (CTL) mice ($n = 5$–7/group). (A) The group means and linear regressions on the dialysate glutamate levels are plotted for the no net flux function and indicate that the functions are parallel and there is a right shift for the ethanol-dependent mice, indicating higher basal levels of glutamate in the NAc of these mice. **(B)** After calculating the *x*-intercept for each mouse to determine basal glutamate concentrations use and comparison of the group means indicate that the basal glutamate concentrations were twofold higher (*$p < 0.05$). **(C)** The slopes of the no net function were calculated and the statistical comparison indicated no difference, consistent with the observation that the functions are parallel. **(D)** Probe placements for the mice included in the analyses. Data are means ± SEM.

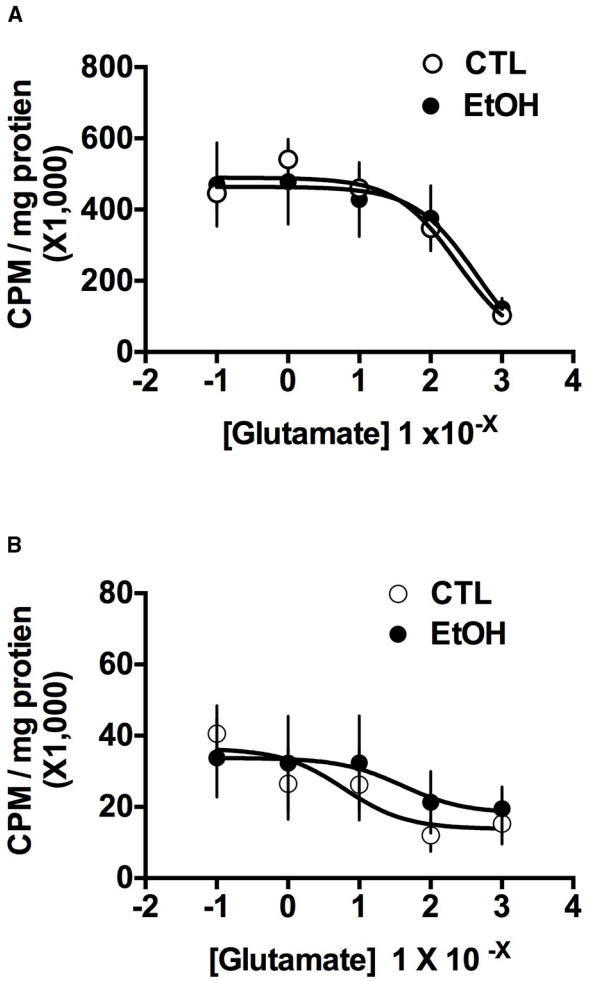

FIGURE 2 | Glutamate transport in NAc tissue from ethanol-dependent (EtOH) and non-dependent (CTL) mice ($n = 6$–7/group). (A) Na^+-dependent glutamate transport, which reflects the function of excitatory amino acid transporters in the cell membrane, was not influenced by ethanol dependence. **(B)** Na^+-independent glutamate transport, which reflects the function of System X_c^- in glial membranes, also was not affected by ethanol dependence. Data are means ± SEM.

main effect of group [$F(1,165) < 1$] or an interaction of the group and concentration factors [$F(1,165) < 1$]. Similarly, for the Na^+-independent transport experiment, ANOVA revealed a significant main effect of glutamate concentration [$F(4,164) = 7.274$, $p < 0.0001$], but no effect of group [$F(1,164) = 1.160$, $p = 0.283$] or the group × concentration interaction [$F(4,164) = 2.079$, $p = 0.086$]. These analyses indicate that increasing concentrations of glutamate significantly influenced glutamate transport (i.e., reaching saturation of the transport proteins) under both Na^+-dependent and -independent conditions, but a history of ethanol dependence did not affect glutamate transport.

To confirm the specificity of the glutamate transport assay in mouse tissue, the ability of TBOA to decrease Na^+-dependent transport and CPG to decrease Na^+-independent transport were tested (**Table 1**). In both cases, data were expressed as cpm/mg protein/15 min and normalized to the zero concentration condition. As expected, TBOA reduced glutamate transport by ∼50% at the low concentration and ∼65% at the higher concentration, relative to the control condition. This was confirmed by one-way ANOVA ($F(2,21) = 12.752$, $p < 0.001$). *Post hoc* analysis indicated

Table 1 | Effects of TBOA and CPG on glutamate uptake.

Condition/inhibitor	Inhibitor (μM)	% of control
Na^+-dependent/TBOA	10	*55 ± 10
	50	*37.8 ± 11.5
Na^+-independent/CPG	5	*32.6 ± 4
	25	*30.3 ± 7.3

*n = 3; data are means ± SEM. *p < 0.05 versus control condition, normalized to 100%.*

significant differences between transport at 0 μM TBOA and 10 μM TBOA and 50 μM TBOA (both $p < 0.05$). Similarly, in the Na^+-independent condition, CPG significantly inhibited glutamate transport to ∼35% of control levels at both concentrations tested. One-way ANOVA on these data indicated a significant effect of dose ($F(2,22) = 16.574$, $p < 0.001$) and *post hoc* tests revealed differences between 0 μM CPG and the 5 μM CPG and 25 μM CPG conditions (both $p < 0.05$). These data provide confirmation that the glutamate transport assay is sensitive to changes in both Na^+-dependent and Na^+-independent glutamate transport functions.

DISCUSSION

Results from the *in vivo* quantitative dialysis experiment indicated that extracellular concentrations of glutamate in the NAc were increased approximately twofold in ethanol-dependent compared to non-dependent mice. This finding is consistent with our earlier work (Griffin et al., 2014) that reported a twofold increase in baseline glutamate concentrations in CIE-exposed mice using conventional dialysis procedures. The data presented here are also consistent with several other studies showing ethanol exposure increased basal glutamate levels in the NAc (Melendez et al., 2005; Kapasova and Szumlinski, 2008; Ding et al., 2013), as well as other brain regions including hippocampus (Moghaddam and Bolinao, 1994; Dahchour and De Witte, 1999; Chefer et al., 2011) and the amygdala (Roberto et al., 2004). While there are some studies using have not reported increases in glutamate concentrations following ethanol exposure (Szumlinski et al., 2005; Goulding et al., 2011), in general it appears that ethanol exposure consistently increases glutamatergic activity in several brain regions including the NAc.

Because we did not find differences between ethanol-dependent and non-dependent mice in the slopes of the linear function calculated in the microdialysis experiment, these results suggested that the difference in basal glutamate levels was not due to alterations in glutamate transport mechanisms as the slope has been suggested to be a measure of the extraction fraction (E_d), which is potentially an estimate of neurotransmitter clearance (Bungay et al., 2003). But the slope has only been empirically demonstrated to be an indication of clearance rate for the neurotransmitters dopamine (Smith and Justice, 1994) and acetylcholine (Vinson and Justice, 1997); a study of this sort has not yet been conducted for glutamate. It is likely that this relationship between slope and uptake is different for glutamate, the levels of which are not only regulated by re-uptake via EAATs, but also by export via system X_c^- (for further discussion, see Trantham-Davidson et al., 2012). Therefore, to directly examine the possibility that chronic ethanol may alter glutamate transporter function, we used an *ex vivo* preparation to measure the transport capacity for glutamate in ventral striatal tissue harvested from ethanol-dependent and non-dependent mice. Glutamate transporter function was assessed under Na^+-dependent and -independent conditions to determine whether the excitatory amino acid transporter (EAATs) systems and system X_c^-, respectively, were influenced by ethanol dependence. The membrane bound EAATs are Na^+-dependent and their role is to remove glutamate from the extracellular space, keeping extracellular glutamate low (Danbolt, 2001). On the other hand, system X_c^- is Na^+-independent and exchanges extracellular cysteine for intracellular glutamate, and contributes significantly to the extrasynaptic pool of glutamate (Baker et al., 2002). It is clear from our results that Na^+-dependent transport constituted a much larger fraction of glutamate transport than did Na^+-independent transport, a finding consistent with reports in rats (Melendez et al., 2005). Further, the specificity of the assay system was confirmed using the inhibitors TBOA and CPG to pharmacologically probe Na^+-dependent and -independent transport function, respectively. Overall, our results indicate that function of these two important glutamate transport systems are not altered in our CIE model, at least in the NAc. This finding is in contrast to previous work (Melendez et al., 2005), showing that increased extracellular glutamate was associated with reduced transporter function. The reason for the discrepancy in results between the earlier study and the present one is unclear, but in addition to the use of different species, the differences could also be related to the amount of ethanol exposure and timing of the experimental measurements. Melendez et al. (2005) administered daily injections of 1 g/kg ethanol for 1 week whereas the CIE exposure procedure produced blood ethanol concentrations of ∼200 mg/kg that were sustained for 16 h, four times per week continuing for 3–4 weeks in the present study. Additionally, whereas the previous study conducted measurements 24 h after discontinuing ethanol exposure (Melendez et al., 2005), the current study used a 72 h time point because it coincides with a time when mice are allowed to resume voluntary drinking and signs of physical withdrawal from ethanol have abated (Becker and Hale, 1993). In fact, a very recent study found differences in the timing of specific physical withdrawal symptoms between two strains of rats exposed to high doses of ethanol (Abulseoud et al., 2014), suggesting that different ethanol exposure models may result in temporally distinct patterns of functional and/or expression changes in relevant proteins. Therefore, the amount of ethanol exposure as well as the timing of measurements relative to the initiation of withdrawal may be important variables to examine in future studies.

Taken together, results from the quantitative microdialysis and transport assay experiments indicate that glutamate transport function in the NAc is not altered by CIE exposure in this model of ethanol dependence. In the present study these measurements were determined at 3 days (72 h) following final CIE exposure. While we have previously reported that repeated cycles of CIE exposure produced elevated extracellular glutamate levels in NAc at least 6–7 days following the chronic ethanol treatment, it is not known whether alterations in glutamate transporter function contribute to this protracted effect. Future studies will need

to determine the durability of this hyperglutamatergic state in NAc following CIE exposure. In addition, there is a possibility that expression of one or more of the transporter systems is altered. Despite large increases in extracellular glutamate following ethanol exposure, other reports indicate that EAAT2 (Melendez et al., 2005; Ding et al., 2013) and xCT (e.g., System X_c^-; Ding et al., 2013; Alhaddad et al., 2014a) expression in the NAc of rats was not significantly affected by ethanol exposure, although significant reductions in EAAT1 were reported (Ding et al., 2013). Other reports have found that EAAT2 expression in the NAc is significantly reduced in continuously drinking P rats (Sari and Sreemantula, 2012; Sari et al., 2013; Alhaddad et al., 2014a,b). Together, these findings underscore the complexity of the systems regulating glutamate homeostasis and suggest that alterations in one or more these proteins can be crucial to the outcome.

The mechanisms underlying elevated basal accumbal glutamate activity following CIE exposure are unknown. While action potential-dependent glutamate release does not ordinarily contribute to basal glutamate levels as measured by microdialysis (Baker et al., 2002), one possibility is that vesicular glutamate release may be increased after CIE exposure and this contributes to higher basal glutamate levels. In this situation, the increased extracellular glutamate in the would likely come from excitatory projections arising in the prefrontal cortex, amgydala, or hippocampus (e.g., Britt et al., 2012) that become hyperexcitable as a result of ethanol dependence. Another possibility is that expression or function of group II metabotropic receptors is altered. These receptors, mGluR2 and mGluR3, are found in the NAc (Ohishi et al., 1998; Tamaru et al., 2001). In addition, group II receptors play a crucial role in regulating extracellular glutamate concentrations because activating mGluR2/3 receptors reduces pre-synaptic glutamate release (Lovinger and McCool, 1995; Cartmell and Schoepp, 2000) and also reduces release of glutamate via system X_c^- (Winder and Conn, 1996; Baker et al., 2002). Further, other reports have specifically implicated mGluR2 receptors in ethanol drinking by non-dependent (Zhou et al., 2013) and ethanol-dependent (Meinhardt et al., 2013) rats, making these receptors an important target for future work.

In summary, these data indicate that repeated cycles of CIE exposure disrupt glutamate homeostasis in the NAc, an important brain region implicated in motivated behaviors such as ethanol drinking. Specifically, in a model of ethanol dependence that produces escalated drinking, significant (twofold) elevation in basal extracellular glutamate concentrations in NAc was observed. Using an *ex vivo* assay to evaluate glutamate transporter function, CIE exposure did not alter Na^+-dependent and -independent transporter functions. That is, CIE-induced elevation of basal glutamate levels in the NAc does not appear related to alterations in transport mechanisms responsible for bringing glutamate back into cellular compartments (i.e., Na^+-dependent processes; EAATs) or mechanisms that release glutamate into the extracellular space (i.e., Na^+-independent processes; system X_c^-). Given the importance of group II receptors in regulating extracellular glutamate concentrations, including pre-synaptic release, ongoing work is investigating the role of group II receptors on regulating extracellular glutamate as well as ethanol drinking in the CIE model.

ACKNOWLEDGMENTS

NIAAA P50 AA010761, T32 AA007474, F32 AA021321, and VA Medical Research.

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Conflict of Interest Statement: The Guest Associate Editor Justin Gass declares that, despite being affiliated to the same institution as authors William C. Griffin, Vorani S. Ramachandra, and Howard C. Becker, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 October 2014; accepted: 02 February 2015; published online: 23 February 2015.

Citation: Griffin WC, Ramachandra VS, Knackstedt LA and Becker HC (2015) Repeated cycles of chronic intermittent ethanol exposure increases basal glutamate in the nucleus accumbens of mice without affecting glutamate transport. Front. Pharmacol. 6:27. doi: 10.3389/fphar.2015.00027

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 March 2015
doi: 10.3389/fphar.2015.00044

Ceftriaxone and cefazolin attenuate the cue-primed reinstatement of alcohol-seeking

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Alcohol consumption and the reinstatement of alcohol-seeking rely on glutamate and GABA transmission. Modulating these neurotransmitters may be a viable treatment strategy to prevent alcohol relapse. *N*-acetylcysteine (NAC) and the antibiotic ceftriaxone (CEF) alter the glial reuptake and release of glutamate while the antibiotic cefazolin (CEFAZ) modulates GABA signaling without affecting glutamate. Here, we used the extinction-reinstatement model of relapse to test the ability of these compounds to attenuate the reinstatement of alcohol-seeking. Male Sprague-Dawley rats were trained to self-administer 20% (v/v) alcohol in the home cage using an intermittent schedule (24 h on, 24 h off) for 12 sessions. Subsequently, animals self-administered alcohol during daily 45-min operant sessions for 26 sessions, followed by extinction training. We tested whether chronic administration of NAC, CEF, or CEFAZ attenuated the cue-primed reinstatement of alcohol-seeking. CEF and CEFAZ attenuated cue-primed reinstatement of alcohol-seeking while NAC had no effect. We subsequently investigated whether CEF and CEFAZ alter the self-administration of sucrose and chow pellets and if CEFAZ attenuates the reinstatement of cocaine-seeking. The operant self-administration of regular chow and sucrose was not altered by either CEF or CEFAZ. CEFAZ had no effect on cocaine reinstatement, a behavior that has been strongly tied to altered glutamate homeostasis in the nucleus accumbens. Thus the ability of CEFAZ to attenuate alcohol reinstatement likely does not involve the glial modulation of glutamate levels. The dampening of GABA transmission may be a common mechanism of action of cefazolin and ceftriaxone.

Keywords: glutamate, alcohol, GABA, cocaine, addiction, relapse, *N*-acetylcysteine, ceftriaxone

OPEN ACCESS

Edited by:
M. Foster Olive, Arizona State University, USA

Reviewed by:
George V. Rebec, Indiana University, USA
Jamie Peters, Medical University of South Carolina, USA

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 January 2015*
Accepted: *19 February 2015*
Published: *10 March 2015*

Citation:
Weiland A, Garcia S and Knackstedt LA (2015) Ceftriaxone and cefazolin attenuate the cue-primed reinstatement of alcohol-seeking. Front. Pharmacol. 6:44. doi: 10.3389/fphar.2015.00044

Introduction

Alcoholism or alcohol dependence is a chronic, progressive disease which results from an inability to regulate drug-seeking behavior. Approximately 17 million Americans are dependent on alcohol (NIH, 2006), resulting in significant legal and medical costs to society totaling an estimated $184 billion per year (Kenna et al., 2004). At present, only disulfiram, naltrexone, and acamprosate are approved by the Food and Drug Administration to treat alcoholism. However, the ability of these medications to reduce the risk of relapse is modest (Heilig and Egli, 2006; Soyka and Roesner, 2006) and thus there is a need to develop more targeted pharmacological treatments for alcohol addiction.

Animal models of relapse permit the identification of the underlying neurobiology of alcohol relapse in humans and thus the targeted development of pharmacotherapies. Relapse is modeled in animals with the reinstatement paradigm, where animals are trained to self-administer drug in an operant chamber. The drug-seeking response is extinguished and reinstated with one of the stimuli known to cause relapse in humans such as stress, cues associated with drug delivery, or the drug itself (Epstein et al., 2006). It has proven challenging to get laboratory rats to voluntarily consume doses of alcohol relevant to human alcohol addiction. Various procedures have been attempted to increase oral consumption of alcohol in rodents, such as sucrose fading (Samson, 1986), food and water-deprivation (Meisch and Thompson, 1972), alcohol vapor exposure (Walker and Koob, 2007; Walker et al., 2008), and the use of rat strains selectively bred for high preference to alcohol (Samson et al., 1998; Vacca et al., 2002). Since its introduction in the 1980s, sucrose fading was largely adopted as the chief method of inducing rodents to self-administer alcohol in an operant setting (Samson, 1986). While sucrose fading yields high alcohol consumption in the presence of sucrose, drinking drops sharply when the sweetener is removed (Samson, 1986; Koob and Weiss, 1990; Samson et al., 1999; Carrillo et al., 2008). Additionally, sucrose has addictive properties itself (Colantuoni et al., 2002; Avena et al., 2008), possibly contributing to the motivation to consume sweetened alcohol. One method of inducing high amounts of unsweetened alcohol consumption in rodents is the intermittent access to alcohol (IAA) procedure in which rodents are provided 24 h access to alcohol followed by a 24 h period with no access (Wayner and Greenberg, 1972; Wise, 1973; Pinel and Huang, 1976). Simms et al. (2008) recently resurrected this model and induced levels of drinking (20% alcohol v/v) in the 5–8 g/kg/day range in Wistar and Long-Evans strains. Furthermore, Simms et al. (2010) adapted the IAA paradigm to an operant self-administration model and engendered high levels of alcohol-seeking in the operant chamber after using 14 h operant sessions on intermittent days (MWF), followed by shorter (30–45 min) daily sessions in the operant chamber. Here, we modified this method to eliminate the use of 14 h operant sessions and trained animals in the operant chamber only during daily 45-min sessions for 26 sessions (MTWRF).

We then sought to test the ability of translational compounds which modulate glutamate and/or GABA transmission to attenuate the cue-primed reinstatement of alcohol-seeking. Glutamate transmission in general and specifically in the nucleus accumbens (NAs) and amygdala is strongly implicated in cue-induced alcohol reinstatement. During the cue-primed reinstatement of extinguished operant alcohol-seeking, synaptic glutamate release increases in the NA and the amygdala (Gass et al., 2011). Thus, it follows that both systemic and intra-NA or intra-amygdala infusion of antagonists to post-synaptic glutamate receptors attenuate alcohol reinstatement (e.g., Backstrom and Hyytia, 2004; Backstrom et al., 2004; Sanchis-Segura et al., 2006; Sinclair et al., 2012).

The pharmacological manipulations which prevent relapse to alcohol-seeking also prevent the reinstatement of cocaine-seeking (for review see Kalivas et al., 2009; Knackstedt and Kalivas, 2009). Following cocaine self-administration and withdrawal, evidence exists for altered glutamate homeostasis, defined as the homeostatic regulation of synaptic and extrasynaptic glutamate levels and signaling. Glutamate homeostasis in the accumbens is complex and regulated by multiple mechanisms, including by the major glutamate transporter GLT-1 and by system x_c-. System x_c- exchanges extracellular cystine for intracellular glutamate, thereby contributing the majority of basal extrasynaptic glutamate in the accumbens (Baker et al., 2002). The catalytic subunit of system x_c- is the protein xCT and the hallmarks of altered glutamate homeostasis after cocaine include a reduction in expression and function of both GLT-1 and xCT/system x_c- (Knackstedt et al., 2010; Trantham-Davidson et al., 2012). Based on the widespread involvement of various types of glutamate receptors in alcohol relapse, it is likely that glutamate homeostasis is altered following alcohol consumption as well.

Modulation of GABA transmission also impacts alcohol consumption and reinstatement. $GABA_A$ antagonists and inverse agonists decrease alcohol consumption (see review by McBride and Li, 1998) and operant self-administration of alcohol (Samson et al., 1987).The $GABA_B$ (autoreceptor) agonist baclofen decreases self-administration in non-dependent rats (Janak and Michael Gill, 2003). Conversely, allopregnanolone, an endogenous neurosteroid which has also been shown to be a potent positive modulator of $GABA_A$ receptors, dose-dependently induces the reinstatement of alcohol-seeking (Nie and Janak, 2003; Finn et al., 2008).

We hypothesized that glutamate homeostasis is altered post-alcohol administration, and restoring homeostasis would reverse alcohol-induced plasticity and thereby attenuate the reinstatement of alcohol-seeking. It is possible to restore glutamate homeostasis and reverse cocaine-induced pathologies in synaptic plasticity following with *N*-acetylcysteine (NAC; Moussawi et al., 2009, 2011) or ceftriaxone (CEF; Trantham-Davidson et al., 2012) and here we tested their ability to attenuate cue-primed reinstatement of alcohol-seeking. NAC serves as a cystine prodrug that drives system x_c- to export more glutamate while CEF is an antibiotic that upregulates the expression of both GLT-1 (Lee et al., 2008) and xCT (Lewerenz et al., 2009) at the transcriptional level. We also hypothesized the antibiotic cefazolin (CEFAZ), which has been demonstrated to inhibit $GABA_A$ transmission (Yamazaki et al., 2002; Sugimoto et al., 2003), would attenuate cue-primed alcohol reinstatement. In order to investigate a possible effect of CEFAZ on glutamate homeostasis, we tested its ability to attenuate the reinstatement of cocaine-seeking, a behavior that is dependent on altered glutamate release, clearance and basal levels (for review see Knackstedt and Kalivas, 2009).

Materials and Methods

Animals and Housing

Adult male Sprague-Dawley rats ($n = 84$), weighing 250–275 g upon arrival were individually housed in ventilated Plexiglas

cages in a climate-controlled room on a 12-h reverse light/dark cycle (lights off at 9 AM) and given at least 1 week to acclimate to the individual housing conditions and handling. Rats were provided ad libitum water throughout the experiment and were food-deprived to ∼85% of free-feeding weight with a daily allowance of 20 g of food. Food-restriction generally increases responding without altering reinstatement relative to non-restricted controls (Bongiovanni and See, 2008). Experiment 1 was conducted at the Medical University of South Carolina and Experiments 2–4 were conducted at the University of Florida. All procedures were pre-approved by the Medical University of South Carolina and University of Florida Institutional Animal Care and Use Committees and were in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Operant Chambers and Training

Prior to beginning operant self-administration (Experiments 1–4), animals underwent a single, overnight food-training session in the operant chambers where presses on the active lever resulted in the delivery of food pellets. Operant chambers (Med Associates) were equipped with two retractable levers, stimulus lights, and tone generators. On the day following food training, animals were trained to self-administer alcohol, cocaine, or food pellets in the operant chamber. For the food self-administration study (Experiment 3), presses on the active lever delivered food pellets. For drug self-administration studies, presses on the active lever activated a pump which dispensed drug. For alcohol studies (Experiments 1 and 2), 0.1 mL alcohol (20% v/v) was dispensed into a dipper tray; infrared sensors verified head entry into the trough area to consume the alcohol. For the cocaine study (Experiment 4), 0.25 mg cocaine HCl (kindly provided by NIDA) in 0.1 mL 0.9% saline was delivered per infusion. For both cocaine and alcohol studies, drug delivery was accompanied by the presentation of discrete cues (a light and 2900 Hz tone) and for cocaine self-administration, was followed by a timeout (20 s) during which time presses on the active lever did not yield drug. Inactive lever presses had no consequences, but were recorded. For alcohol self-administration, the trough was blotted with a tissue at the end of the session to verify that all alcohol was consumed. Rats who habitually did not consume alcohol despite lever presses were eliminated from the experiment ($n = 3$ for Experiment 1; $n = 2$ for Experiment 2). Additionally, three rats that were in ill-health were removed from Experiments 1 and 2. For both alcohol and cocaine self-administration studies, animals self-administered drug for a pre-determined number of days in the operant boxes (26 for alcohol; 12 for cocaine) regardless of whether drug intake was stable or not. This method was chosen because we wanted to prevent animals from self-administering drug for varying amounts of time which could lead to a wide range of drug intake. The amount of cocaine consumed during self-administration has been correlated with later drug-seeking during reinstatement tests (e.g., Deroche et al., 1999; Baker et al., 2001).

Alcohol Self-Administration, Extinction Training, and Reinstatement Testing

All rats that later self-administered alcohol in the operant chambers were first trained to drink with the IAA (e.g., Simms et al., 2008). This paradigm provides rats with 24-h access to unsweetened 20% alcohol on alternating days (3 days/weeks) without water-deprivation. Animals experienced 12 sessions (over 4 weeks) of the intermittent-access drinking paradigm.

Following exposure to alcohol using the IAA, animals were trained to self-administer 20% alcohol (v/v) using daily 45 min training sessions on an FR-1 schedule of reinforcement for 6 days followed by an FR-3 schedule for 20 daily sessions. This procedure followed that of Simms et al. (2010), with the exception of using 45 min FR-3 operant sessions in place of 30 min sessions and replacing the 12 overnight operant sessions with 12 IAA sessions. Extinction training began following the 20th FR-3 session. During extinction training, presses on the previously active lever no longer provided drug and cue presentation. Extinction training lasted a minimum of 10 sessions and until animals reached the criteria of less than 20 lever responses per session. Once this criteria was reached, animals underwent a 45 min cue-primed reinstatement test during which presses on the previously active lever once again yielded presentation of discrete cues that had been paired with alcohol delivery (stimulus light, tone and the sound of the syringe pump activating). In addition, 2 mL of 20% EtOH was sprinkled on the bedding of the operant chamber to provide an olfactory cue.

Experiment 1

Here, we tested the ability of chronically administered NAC to attenuate the cue-primed reinstatement of alcohol-seeking. *N*-acetylcysteine (Sigma, 30 or 60 mg/kg IP) or vehicle (VEH; saline 0.3 mL IP) was administered 2 h prior to the last 6–8 extinction sessions and prior to the reinstatement test (see **Figure 1A**). The dose and timing of injections was based on previous studies demonstrating the ability of 60 mg/kg NAC to restore basal glutamate levels after cocaine and attenuate cocaine reinstatement when given both acutely prior to testing (Baker et al., 2003) and chronically during extinction training (Amen et al., 2011) while a lower dose (33 mg/kg) only partially inhibits cocaine-seeking (Moussawi et al., 2009). NAC works acutely, within 2 h of administration, to increase basal glutamate by serving as an exogenous source of cysteine, which drives system x_c- to export more glutamate (Baker et al., 2003), but also alters glutamate homeostasis when administered daily for 5 or more days (Knackstedt et al., 2010; Amen et al., 2011). After reaching extinction criteria, the responding of the NAC-treated animals during a cue- primed reinstatement trial was compared with VEH-treated animals. Animals were assigned to receive NAC or VEH treatment in a counterbalanced manner to ensure no group differences in drug self-administration. One animal was excluded from reinstatement testing (and all data analysis) due to a failure to reach extinction criteria.

Experiment 2

This experiment was carried out in an identical manner to Experiment 1, with the exception that CEF (Hospira, 200 mg/kg

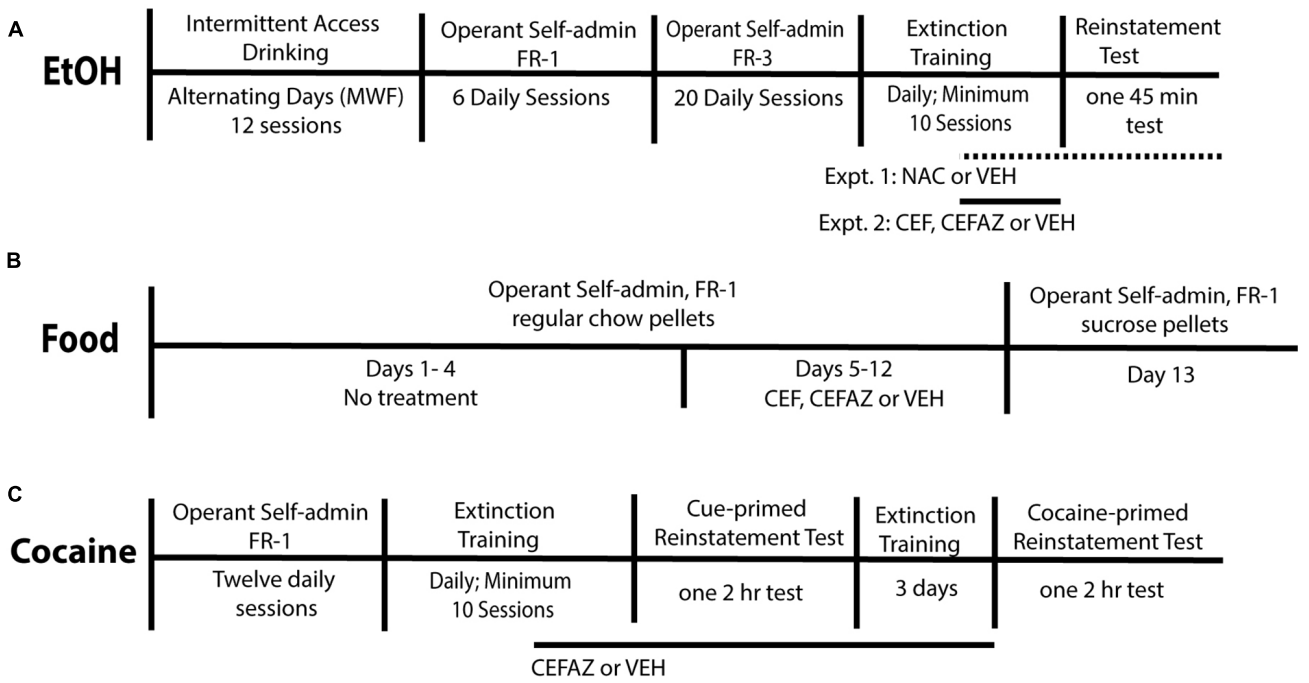

FIGURE 1 | Experimental Methods. (A) Timeline of the experimental procedure for Experiments 1 and 2. All animals were trained to self-administer food pellets immediately prior to beginning operant alcohol self-administration. VEH, NAC, CEF, and CEFAZ treatment commenced on the last 6–8 days of extinction training. For the NAC experiment, animals were also injected 2 h prior to the test. **(B)** Timeline of the experimental procedure for Experiment 3. All animals were trained to self-administer food pellets immediately prior to beginning operant alcohol self-administration. VEH, CEF, and CEFAZ treatment commenced on Day 5 of food self-administration and continued through Day 12. **(C)** Timeline of the procedure for Experiment 4. All animals were trained to self-administer food pellets immediately prior to beginning operant cocaine self-administration. VEH and CEFAZ was administered for the last 6–8 extinction sessions and continued after the cue-primed reinstatement test.

IP), CEFAZ (Sigma, 100 mg/kg IP), or VEH (0.9% saline) were administered immediately following the last 6–8 extinction sessions, but not on test day (see **Figure 1A**). After reaching extinction criteria, the responding of the CEF and CEFAZ-treated animals during a cue- primed reinstatement trial was compared with VEH -treated animals. The dose and timing of CEF injections was based on previously published reports showing that 200 mg/kg effectively attenuates cocaine reinstatement and restores glutamate homeostasis when given chronically *after* extinction sessions for at least 5 days (Knackstedt et al., 2010; Trantham-Davidson et al., 2012). The dose of CEFAZ was based on allometric calculations from the maximum human dose recommended by the manufacturer to the equivalent rat dose of 100 mg/kg maximum and was given after extinction sessions akin to the administration of CEF.

Food and Sucrose Self-Administration (Experiment 3)

Animals self-administered standard chow pellets (45 mg; Bio-serv) in the operant chamber using an FR-1 schedule of reinforcement for 2 h/day for 12 days. On the 13th day, sucrose pellets (45 mg; Bio-serv) replaced the standard chow pellets. Animals received CEF, CEFAZ, or VEH in an identical manner as described for Experiment 2, with the exception that injections began after the operant session on Day 5 of food self-administration (see **Figure 1B**).

Cocaine Self-Administration, Extinction, and Reinstatement (Experiment 4)

For the implantation of catheters, rats were anesthetized with ketamine HCl (87.5 mg/kg, IM) and xylazine (5 mg/kg, IM). Ketorolac (3 mg/kg, IP) was administered before surgery to provide analgesia. Catheter construction and surgical implantation is described in detail elsewhere (Knackstedt et al., 2014). Rats recovered 6 days after surgery prior to beginning self-administration training. Cocaine self-administration was conducted using an FR-1 schedule; sessions lasted 2 h/day and are described in more detail above. Self-administration continued until subjects had attained 12 days with a minimum of 10 cocaine infusions and was followed by extinction training. Animals were either treated with CEFAZ or VEH in manner identical to that in Experiment 2, immediately following the last 6–8 extinction sessions (see **Figure 1C**). Once extinction criteria was attained, animals were tested for cue- and cocaine-primed reinstatement (each test lasting 2 h), with tests separated by 3 days of extinction training. During cue-primed reinstatement, presses on the previously active lever once again yielded the discrete cues paired with cocaine delivery but no drug was delivered. During cocaine-primed reinstatement, animals were injected with 10 mg/kg cocaine (IP) immediately prior to being placed into the operant chamber; presses on the previously active lever had no consequences during this test.

Data Analysis

The alpha level was set at $p < 0.05$ for all statistical tests, which were conducted using SPSS software. Active lever presses during self-administration and extinction components of the experiment were analyzed with mixed-factorial 2-way analysis of variances (ANOVAs) test, with time as the repeated measure. Reinstatement tests were planned comparisons (extinction vs. test day) and thus were analyzed with paired-sample *t*-tests. Independent-sample *t*-tests were used to compare active lever presses during reinstatement tests between treatment groups. Group differences in food self-administration were examined using one-way ANOVAs. Outliers were defined as values more than two standard deviation +/– the mean and were excluded from further analysis. Groups with outlier values are mentioned in the Results section.

Results

The Effects of NAC on Cue-Primed Reinstatement of Alcohol-Seeking (Experiment 1)

VEH- and NAC-treated groups did not differ in the amount of alcohol self-administered (**Figure 2A**). A two-way ANOVA with repeated measures on Day was computed on the active lever press data during self-administration and found no significant effect of Day [$F(19,475) = 1.480$, n.s.], or Group [$F(2,25) = 0.080$, n.s.]. There was a significant Day × Group interaction [$F(19,475) = 2.020$, $p < 0.01$]. Similarly, a two-way ANOVA was computed on the lever presses during extinction training (**Figure 2B**) and revealed a significant effect of Day [$F(9,225) = 6.977$, $p < 0.001$], but not Group [$F(2,25) = 0.240$, n.s.]. There was not a significant Day × Group interaction [$F(18,225) = 0.809$, n.s.]. We used paired-sample *t*-tests to analyze the data presented in **Figure 2C** to compare active lever presses during cue-primed reinstatement to those during the average of the last 3 days of extinction. Significant differences were found between extinction and cue-primed reinstatement for all treatment groups [Veh: $t(1,7) = 4.036$, $p < 0.01$; NAC60: $t(1,8) = 4.302$, $p < 0.01$; NAC30: $t(1,9) = 3.651$, $p < 0.05$], indicating that neither dose of NAC was able to prevent cue-primed reinstatement of alcohol-seeking.

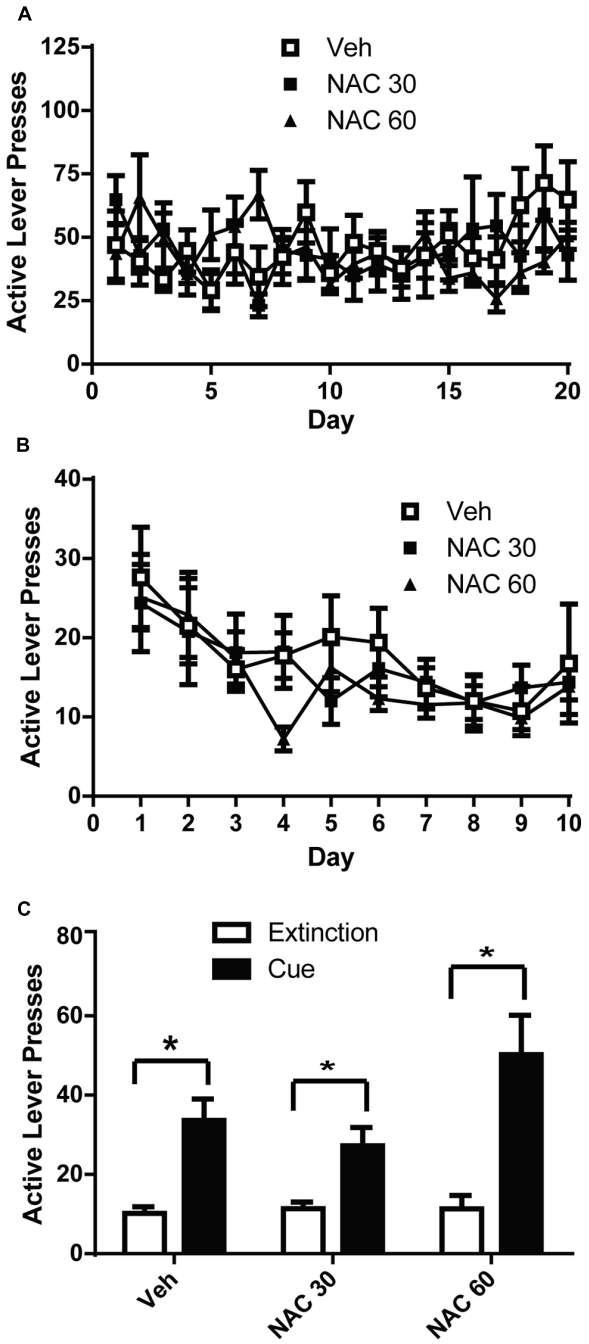

FIGURE 2 | *N*-acetylcysteine did not attenuate cue-primed reinstatement of alcohol-seeking. (A) Mean active lever presses during the FR3 portion of the experiment did not differ between groups. **(B)** Mean active lever presses during extinction training did not differ between groups. **(C)** *N*-acetylcysteine did not attenuate cue-primed reinstatement of alcohol-seeking. *$p < 0.05$ comparing extinction to test.

The Effects of CEF and CEFAZ on Cue-Primed Reinstatement of Alcohol-Seeking (Experiment 2)

Vehicle, CEF, and CEFAZ groups did not differ in the amount of active lever presses during self-administration (**Figure 3A**). A two-way ANOVA with repeated measures on Day was computed on the active lever presses during alcohol self-administration (**Figure 3A**) and revealed a significant effect of Day [$F(19,380) = 2.612$, $p < 0.01$], but not Group [$F(2,20) = 0.370$, n.s.]. There was not a significant Day × Group interaction [$F(19,380) = 1.277$, n.s.]. Similarly, a two-way ANOVA was computed on the number of lever presses during extinction training (**Figure 3B**) and revealed a significant effect of Day [$F(9,180) = 8.050$, $p < 0.001$], but not Group [$F(2,20) = 0.989$, n.s.]. There was not a significant Day × Group interaction [$F(2,180) = 1.249$, n.s.]. We used paired-sample *t*-tests to compare active lever presses during cue-primed reinstatement to those during the

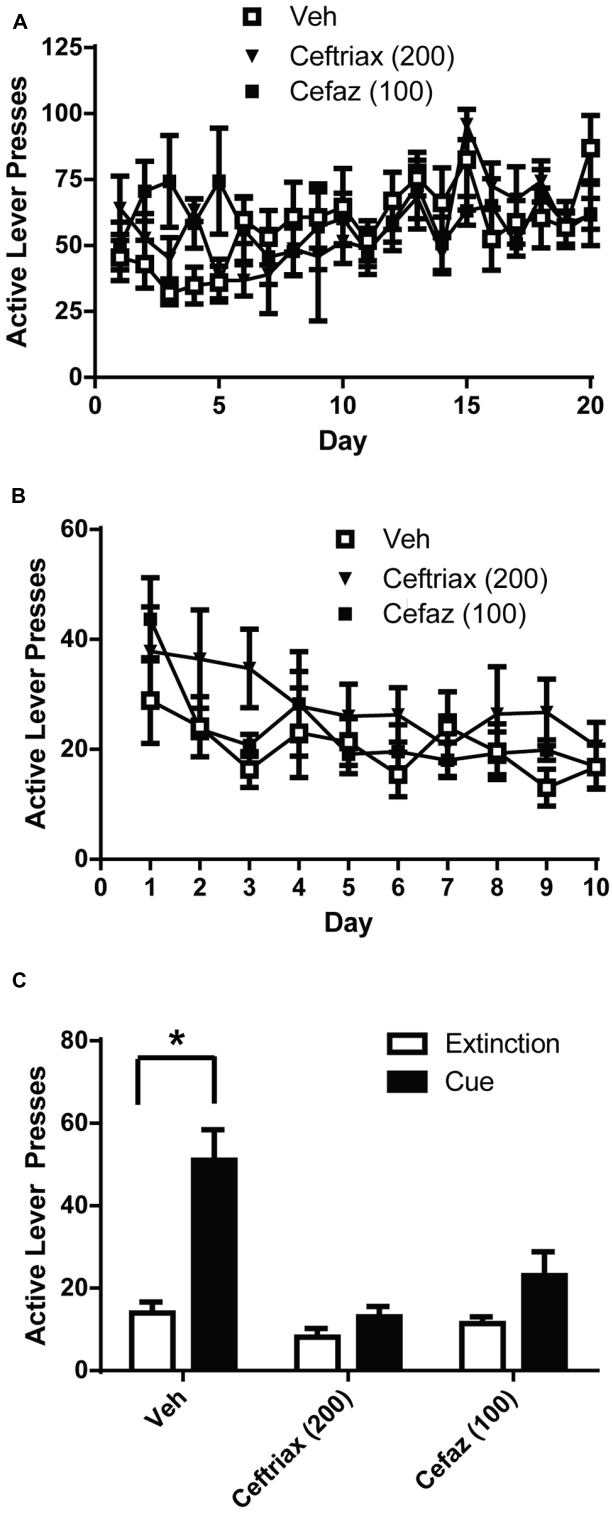

FIGURE 3 | Ceftriaxone and cefazolin attenuated the reinstatement alcohol-seeking. (A) Mean active lever presses during the FR3 portion of the experiment did not differ between groups. **(B)** Mean active lever presses during extinction training did not differ between groups. **(C)** Only vehicle-treated animals reinstated lever-pressing during a cue-primed reinstatement test. *$p < 0.05$ comparing extinction to test.

last 3 days of extinction (**Figure 3C**). A significant difference between extinction and cue-primed reinstatement was only observed for vehicle-treated animals [$t(1,8) = 2.892$, $p < 0.05$], indicating that CEF [$t(1,6) = 1.3998$, n.s.] and CEFAZ [$t(1,6) = 2.090$, n.s.] attenuated cue-primed reinstatement of alcohol-seeking.

CEF and CEFAZ Did Not Alter Food and Sucrose Self-Administration or Weight (Experiment 3)

Because CEF and CEFAZ treatment commenced following the session on Day 5, statistical analyses were conducted only on the data during the period of antibiotic (or vehicle) treatment (Days 6–12). One animal was excluded from data analysis because his lever pressing on Day 13 was greater than two standard deviations from the mean value. A one-ANOVA was computed on active lever presses during self-administration and revealed no effect of treatment group [$F(2,17) = 2.128$, n.s.; **Figure 4A**]. We also conducted a two-way ANOVA with repeated measures on Day and this analysis revealed no significant effects [Day: $F(6,102) = 1.963$, n.s.; Day × Group: $F(12,102) = 1.375$, n.s.; Group: $F(2,17) = 2.361$, n.s.]. A one-way ANOVA was used to determine if there were group differences in the number of sucrose pellets earned on Day 13 of the experiment (**Figure 4B**) and found no effect [$F(2,17) = 1.068$, n.s.]. There was also no difference in weight over time between the three treatment groups (**Figure 4C**). A two-way RM ANOVA found an effect of Day [$F(11,102) = 9.145$, $p < 0.001$] but no Day × Group interaction [$F(12,102) = 2.507$, n.s.] or effect of Group [$F(2,17) = 1.924$, n.s.] on animal weight.

CEFAZ Did Not Attenuate the Reinstatement of Cocaine-Seeking (Experiment 4)

A two-way ANOVA computed on the amount of cocaine intake (**Figure 5A**) revealed no group differences [$F(1,12) = 0.357$, n.s.], no effect of Day [$F(11,132) = 1.562$, n.s.], and no Day × Group interaction [$F(12,132) = 0.453$, n.s.]. There were also no group differences in extinction training [$F(1,12) = 0.472$, n.s.] and no Day × Group interaction [$F(12,108) = 0.696$, n.s.] but there was an effect of Day [$F(9,108) = 19.076$, $p < 0.001$] as both groups decreased lever pressing during the course of training (**Figure 5B**). Paired-sample *t*-tests revealed significant differences between extinction and cue-primed reinstatement for both CEFAZ [$t(1,5) = 2.834$, $p < 0.05$] and vehicle-treated [$t(1,7) = 4.453$, $p < 0.01$] animals (**Figure 5C**). Paired-sample *t*-tests revealed significant differences between extinction and cocaine-primed reinstatement for both CEFAZ [$t(1,5) = 2.834$, $p < 0.05$] and vehicle-treated [$t(1,7) = 2.614$, $p < 0.05$] animals (**Figure 5D**). Thus, CEFAZ did not attenuate cue- or cocaine-primed reinstatement. Furthermore, an independent sample *t*-test showed no lever pressing differences between vehicle- and CEFAZ-treated groups during the cue [$t(1,12) = 0.272$, n.s.] and cocaine tests [$t(1,12) = 0.269$, n.s.].

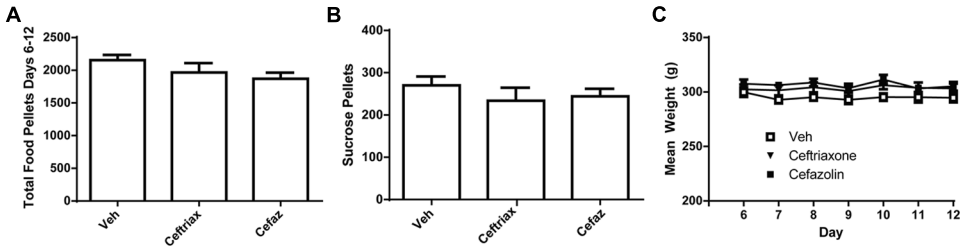

FIGURE 4 | Ceftriaxone and cefazolin did not alter the motivation to self-administer regular chow or sucrose pellets. (A) CEF and CEFAZ did not alter the self-administration of regular chow pellets. **(B)** CEF and CEFAZ did not alter the mean number of sucrose pellets self-administered. **(C)** Mean body weight did not differ between vehicle-, CEF- or CEFAZ-treated rats.

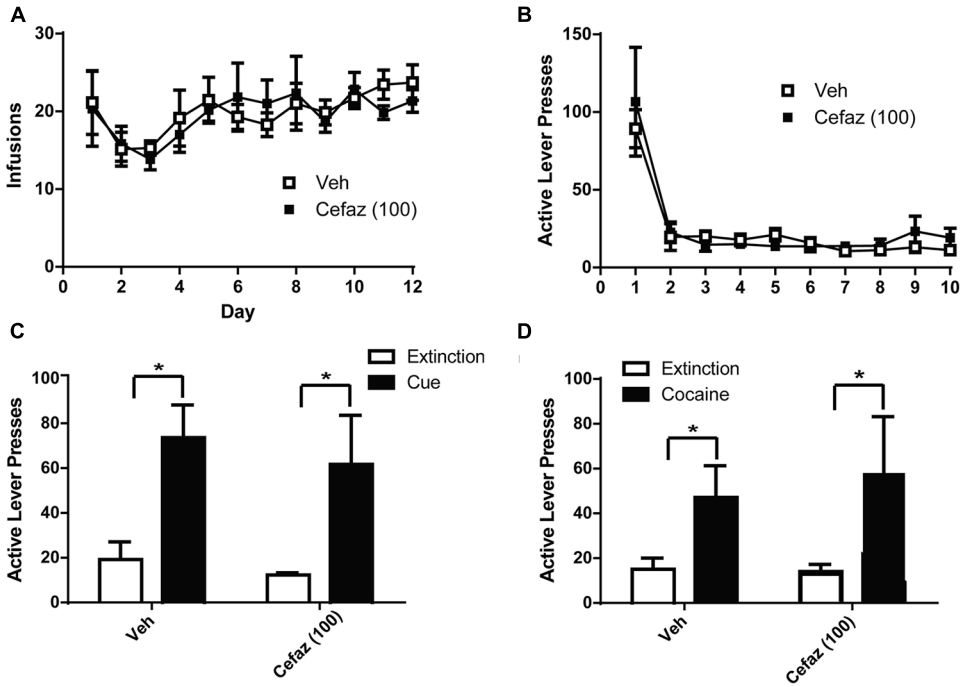

FIGURE 5 | Cefazolin did not attenuate cue- or cocaine-primed reinstatement of cocaine-seeking. (A) Animals later treated with vehicle or CEFAZ did not differ in the number of infusions attained during cocaine self-administration. **(B)** Vehicle- and CEFAZ-treated animals did not differ in active lever presses during extinction training. **(C)** CEFAZ did not attenuate cue-primed reinstatement. **(D)** CEFAZ did not attenuate cocaine-primed reinstatement. *$p < 0.05$ comparing extinction to test.

Discussion

Here, we demonstrated that training Sprague-Dawley rodents to self-administer unsweetened alcohol in the operant chamber can be accomplished using daily training sessions without lengthy (14 h; e.g., Simms et al., 2010) operant sessions on alternating days. This is the first demonstration of such an effect in the Sprague-Dawley strain. Augier et al. (2014) recently demonstrated the same effect in Wistar rats. Using the procedure outlined here (**Figure 1A**) reduced the time to complete the experiment by 4 weeks relative to a procedure utilizing intermittent (MWF) operant sessions (e.g., Simms et al., 2010). This represents a significant conservation of lab resources.

We used this operant procedure to investigate the ability of NAC, CEF, and CEFAZ to attenuate cue-primed reinstatement. Five days of NAC treatment (30 or 60 mg/kg) did not attenuate cue-primed reinstatement (**Figure 2C**). We found that chronic CEF (200 mg/kg) and CEFAZ (100 mg/kg) attenuated cue-primed reinstatement of alcohol-seeking (**Figure 3C**). Both NAC and CEF attenuate cocaine and heroin reinstatement (Baker et al., 2003; Zhou and Kalivas, 2008; Knackstedt et al., 2010; Reichel et al., 2011; Sondheimer and Knackstedt, 2011; Shen et al., 2014). Furthermore, both compounds have been documented to similarly restore glutamate homeostasis in the NAc following cocaine (see **Table 1**). Both compounds increase GLT-1 and system x_c^-/xCT expression and function (Knackstedt et al., 2010; Trantham-Davidson et al., 2012), increase basal glutamate

TABLE 1 | Summary of *N*-acetylcysteine and ceftriaxone effects on cocaine-induced adaptations in the nucleus accumbens.

Post-cocaine adaptation	Compound	
	***N*-acetylcysteine**	**Ceftriaxone**
Restore (elevate) basal glutamate	Yes Baker et al. (2003)	Yes Trantham-Davidson et al. (2012)
Prevent glutamate release during cocaine relapse	Yes Baker et al. (2003)	Yes Trantham-Davidson et al. (2012)
Increase GLT-1 expression/function	Yes (expression) Knackstedt et al. (2010) function not assessed	Yes Knackstedt et al. (2010), Trantham-Davidson et al. (2012)
Increase xCT expression/system xc- function	Yes Baker et al. (2003), Knackstedt et al. (2010)	Yes Knackstedt et al. (2010), Trantham-Davidson et al. (2012)
Stimulate mGluR2/3 function	Yes Moussawi et al. (2011)	Not yet assessed
Normalize potentiated accumbens synapses	Yes Moussawi et al. (2011)	Yes Trantham-Davidson et al. (2012)

levels (Baker et al., 2003; Trantham-Davidson et al., 2012), and normalize potentiated glutamate synapses (Moussawi et al., 2011; Trantham-Davidson et al., 2012). Because CEF and NAC modulate post-cocaine glutamate homeostasis in similar manner, and the present results reveal a dissociation between the ability of the compounds to attenuate alcohol reinstatement, CEF and CEFAZ likely attenuate alcohol reinstatement via a glutamate-independent mechanism.

Cefazolin has been shown to reduce $GABA_A$ transmission (Yamazaki et al., 2002; Sugimoto et al., 2003). Positive allosteric modulation of $GABA_A$ enhances reinstatement of alcohol-seeking (Finn et al., 2008; Ramaker et al., 2014) and thus a reduction in GABA transmission by CEFAZ may be the mechanism of action by which it reduces alcohol reinstatement. CEF has not been fully evaluated for its ability to alter GABA transmission although one report exists showing that it does not alter $GABA_A$ receptor binding in the hippocampus or frontal and parietal cortices (Inui et al., 2013). CEFAZ has not been demonstrated to alter glutamate transmission: it does not alter NMDA function (Yamazaki et al., 2002; Sugimoto et al., 2003) and was not determined to potently induce GLT-1 expression as do CEF and penicillin (Rothstein et al., 2005). The data presented in **Figures 5C,D** provide evidence that CEFAZ does not alter cue- or cocaine-primed reinstatement of cocaine-seeking. Thus, the mechanism by which CEFAZ attenuates alcohol reinstatement likely does not involve modulation of glutamate neurotransmission in the NAs.

Ceftriaxone and CEFAZ are antibiotics and have the potential to produce gastrointestinal distress upon chronic administration. Oral alcohol self-administration in rodents and even alcohol-seeking during cue-primed reinstatements tests may be impacted by this side-effect. Thus we sought to determine the effects of these antibiotics on the operant self-administration of regular chow and sucrose pellets. We found no effect of either antibiotic on the self-administration of chow (**Figure 4A**) or sucrose (**Figure 4B**) pellets or on body weight during the course of CEF and CEFAZ injections (**Figure 4C**). These findings are in agreement with those of Ward et al. (2011) who found no effect of CEF on the self-administration of sweet food in mice. Interestingly, the chronic administration of NAC, which did not attenuate alcohol-seeking here, has been demonstrated to reduce operant self-administration of regular chow (Ramirez-Niño et al., 2013). Thus, we can conclude that the ability of CEF and CEFAZ to attenuate cue-primed alcohol-seeking is likely not dependent on any gastrointestinal side-effects of these compounds.

Because CEFAZ had no impact on cocaine reinstatement (**Figure 5C**), it may not be capable of restoring glutamate homeostasis after cocaine. Thus, this is not likely the mechanism by which CEF and CEFAZ attenuate cue-primed reinstatement of alcohol-seeking. In support of this, NAC, which does alter glutamate homeostasis, did not attenuate cue-primed alcohol-seeking. This conclusion is surprising in light of the evidence supporting glutamate signaling in this behavior. In alcohol-preferring (P) rats, CEF reduces the consumption of alcohol acutely and after a period of withdrawal (Sari et al., 2013; Alhaddad et al., 2014). In P rats, the ability of CEF to modulate alcohol-seeking was accompanied by increases in GLT-1 and xCT (Sari et al., 2013; Alhaddad et al., 2014). These results indicate that the modulation of glutamate homeostasis may be the mechanism by which CEF attenuates alcohol consumption, at least in P rats. However, the increased expression of these proteins was not shown to be the mechanism by which CEF exerted its behavioral effects. An alternative explanation for the failure of NAC to attenuate alcohol reinstatement in the present manuscript is that the operationalization of the alcohol-seeking response may have altered the underlying neurobiology of alcohol-seeking, rendering the manipulation of GLT-1 and xCT (and thereby glutamate levels) ineffective at preventing reinstatement.

The lack of an effect of NAC on alcohol-seeking here does not discount the involvement of glutamate in alcohol reinstatement. Antagonism of the post-synaptic mGluR5 and AMPA receptors attenuates cue-induced reinstatement (Backstrom and Hyytia, 2004; Backstrom et al., 2004; Sanchis-Segura et al., 2006; Sinclair et al., 2012). Stimulating the pre-synaptic glutamate autoreceptor mGluR2/3 also attenuates cue-induced reinstatement of alcohol-seeking (Zhao et al., 2006), presumably by decreasing pre-synaptic glutamate release. While both cocaine and alcohol reinstatement are driven by synaptic glutamate release in the NA core during the reinstatement event (McFarland et al., 2003; Gass et al., 2011) and NAC and CEF attenuate that increase during *cocaine* reinstatement (Baker et al., 2003; Trantham-Davidson et al., 2012), NAC (and potentially CEF) may not be able to attenuate glutamate release during *alcohol* reinstatement. The reason for this may be that cocaine and alcohol have opposite effects on basal glutamate levels in the NA core. Cocaine decreases (Baker et al., 2003) while alcohol increases (Melendez et al., 2005; Griffin Iii et al., 2013) basal glutamate levels. Both NAC and CEF increase basal glutamate levels after cocaine but not in drug-naïve animals

(Baker et al., 2003; Trantham-Davidson et al., 2012). After NAC treatment, this increased basal glutamate derived from system x_c- restores tone on mGluR2/3 autoreceptors (Moussawi et al., 2011), thereby restoring the ability of these receptors to exert negative feedback on glutamate release during reinstatement. It is possible that increased basal glutamate following alcohol also causes a failure in mGluR2/3 signaling that is insensitive to NAC treatment. The effects of CEF on mGluR2/3 function have not yet been investigated (see **Table 1**). Future experiments examining basal glutamate levels 2–3 weeks after cessation of alcohol self-administration in combination with NAC and CEF would provide valuable data regarding the potential value in manipulating basal glutamate to treat alcohol relapse.

Overall, these results indicate that training Sprague-Dawley rats to self-administer alcohol in the operant chamber can be accomplished without sucrose fading and by using daily training sessions without lengthy operant sessions. We found that modulating glutamate homeostasis likely will not represent a successful treatment strategy for preventing relapse to alcohol-seeking. Future studies should investigate GABA modulation as a potential mechanism of action for CEF and as a method of attenuating alcohol relapse.

Acknowledgments

This project was funded by a pilot award from the Charleston Alcohol Research Center (P50 AA010761) and NIDA R01 DA033436, both awarded to LK.

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Conflict of Interest Statement: The Reviewer Jamie Peters declares that, despite being affiliated to the same institution as the author Ana Weiland, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 19 March 2015
doi: 10.3389/fphar.2015.00049

IL-1 interacts with ethanol effects on GABAergic transmission in the mouse central amygdala

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Neuroinflammation is hypothesized to enhance alcohol consumption and contribute to the development of alcoholism. GABAergic transmission in the central amygdala (CeA) plays an important role in the transition to alcohol dependence. Therefore, we studied the effects of interleukin-1β (IL-1β), a proinflammatory cytokine mediating ethanol-induced neuroinflammation, and its interaction with ethanol on CeA GABAegic transmission in B6129SF2/J mice. We also assessed ethanol intake in B6129SF2/J mice. Intake with unlimited (24 h) ethanol access was 9.2–12.7 g/kg (3–15% ethanol), while limited (2 h) access produced an intake of 4.1 ± 0.5 g/kg (15% ethanol). In our electrophysiology experiments, we found that recombinant IL-1β (50 and 100 ng/ml) significantly decreased the amplitude of evoked inhibitory postsynaptic potentials (eIPSPs), with no significant effects on paired-pulse facilitation (PPF). IL-1β (50 ng/ml) had dual effects on spontaneous miniature inhibitory postsynaptic currents (mIPSCs): increasing mIPSC frequencies in most CeA neurons, but decreasing both mIPSC frequencies and amplitudes in a few cells. The IL-1β receptor antagonist (IL-1ra; 100 ng/ml) also had dual effects on mIPSCs and prevented the actions of IL-1β on mIPSC frequencies. These results suggest that IL-1β can alter CeA GABAergic transmission at pre- and postsynaptic sites. Ethanol (44 mM) significantly increased eIPSP amplitudes, decreased PPFs, and increased mIPSC frequencies. IL-1β did not alter ethanol's enhancement of the eIPSP amplitude, but, in IL-1β-responsive neurons, the ethanol effects on mIPSC frequencies were lost. Overall, our data suggest that the IL-1 system is involved in basal GABAergic transmission and that IL-1β interacts with the ethanol-induced facilitation of CeA GABAergic transmission.

Keywords: IL-1β, central amygdala, $GABA_A$, IPSCs, eIPSPs, interleukin, cytokine, IL-1ra

OPEN ACCESS

Edited by:
M. Foster Olive, Arizona State University, USA

Reviewed by:
Ciaran J. Faherty, Cadence Pharmaceuticals, USA
Jennifer L. Cornish, Macquarie University, Australia

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *08 November 2014*
Accepted: *28 February 2015*
Published: *19 March 2015*

Citation:
Bajo M, Varodayan FP, Madamba SG, Roberts AJ, Casal LM, Oleata CS, Siggins GR and Roberto M (2015) IL-1 interacts with ethanol effects on GABAergic transmission in the mouse central amygdala. Front. Pharmacol. 6:49. doi: 10.3389/fphar.2015.00049

Introduction

Studies of human alcoholic brains and animal models have shown a link between the neuroimmune system and the brain changes associated with acute and chronic alcohol exposure (Crews and Vetreno, 2011; Crews et al., 2011; Harris and Blednov, 2012; Szabo et al., 2012; Szabo and Lippai, 2014). In particular, the interleukin-1 (IL-1) system has emerged as an important player in alcohol drinking and the development of alcohol dependence, and as a key regulator of alcohol-induced neuroimmune responses (Crews and Vetreno, 2011;

Crews et al., 2011; Harris and Blednov, 2012; Szabo et al., 2012; Szabo and Lippai, 2014). The IL-1 system includes the cytokines IL-1α and IL-1β, the receptor IL-1R1, the IL-1R accessory protein (IL-1RAcP), and two negative regulators (a decoy receptor IL-1R2 and the IL-R1 antagonist: IL-1ra). The proinflammatory activities of the cytokines IL-1α and IL-1β are initiated by their binding to the IL-1 receptor (IL-1R1) and formation of a receptor heterodimeric complex with IL-1RAcP. After recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor to the IL-1R/IL-1RAcpP complex, signaling pathways, including NF-κB, c-Jun N-terminal kinase (JNK) and p38 MAPK, are activated (Garlanda et al., 2013; Krumm et al., 2014).

Interleukin-1 and its receptor (IL-1R1) are expressed throughout the brain (Hagan et al., 1993; Ericsson et al., 1995; Quan et al., 1996, 1998; Taishi et al., 1997; Cartmell et al., 1999; French et al., 1999; Gayle et al., 1999; Parker et al., 2000; Hosoi et al., 2002; Johnson et al., 2004; Heida and Pittman, 2005) in both neurons (Allan et al., 2005) and glial cells (Blanco et al., 2005; Blanco and Guerri, 2007). Several studies have reported changes in the expression of genes encoding components of the IL-1R1 signaling pathways in the brains of mice with a genetic predisposition to alcohol consumption (Mulligan et al., 2006; Blednov et al., 2012). Additionally, polymorphisms in the genes encoding the IL-1R antagonist (IL-1ra; *Il1rn*) and IL-1β (*Il1b*), but not IL-1α (*Il1a*) and IL1-R1 (IL-1R1 type 1; *Il1r*), have been associated with a susceptibility to alcoholism or ALD (alcohol liver disease) in Spanish men (Pastor et al., 2005). Behavioral studies indicate a reduction in alcohol drinking and/or preference in *Il1rn* knockout mice (Blednov et al., 2012) and suggest an important role of the IL-1 system in alcohol's effects. IL-1β levels are increased in alcoholics, as well as animal models of chronic alcohol exposure (Valles et al., 2004; Qin et al., 2008; Lippai et al., 2013a,b), and intracerebroventricular administrations of IL-1β potentiate alcohol withdrawal-induced anxiety (Breese et al., 2008). Conversely, administration of IL-1ra prevented and protected against alcohol-induced neuroinflammation (Lippai et al., 2013b), and reduced alcohol-induced sedation and motor impairment recovery time in mice (Wu et al., 2011).

As the central nucleus of the amygdala (CeA) plays a critical role in mediating alcohol-related and anxiety-like behaviors (Gilpin et al., 2014), it is likely that the IL-1 signaling system modulates ethanol's effects on CeA function. In fact, we reported recently that the IL-1ra regulates baseline GABAergic transmission in the CeA and is critical for the effects of ethanol at these synapses (Bajo et al., 2014a). Additionally, immune challenges, such as systemic IL-1β or LPS administration, are known to activate the CeA (Dayas et al., 2001; Frost et al., 2001; Konsman et al., 2008). Moreover, IL-1R1 is expressed in the amygdala under basal conditions (Frost et al., 2001), while both IL-1β and IL-1ra are induced in the CeA by excitotoxic stimuli or systemic immune challenge (Eriksson et al., 2000; Konsman et al., 2008). This is particularly significant as the activation of IL-1R1 modulates synaptic transmission and plasticity (Zeise et al., 1992; Bellinger et al., 1995; Dunn et al., 1999; O'Connor and Coogan, 1999; Rothwell and Luheshi, 2000; Lin et al., 2006), glutamate and GABA release (Miller et al., 1991; Murray et al., 1997; Feleder et al., 1998; Sama et al., 2008; Mishra et al., 2012), and membrane expression of GABA receptors (Serantes et al., 2006; Wang et al., 2012).

As neuroinflammation plays an important role in alcohol use disorders and other psychiatric disorders (e.g., depression, PTSD; Jones and Thomsen, 2013), there are concerted efforts to develop new therapeutic strategies using compounds with anti-inflammatory properties to treat these disorders. Therefore, understanding the molecular and cellular mechanisms that mediate normal and pathological neuroimmune responses in the key brain regions involved in the pathogenesis of psychiatric disorders is critical for the evaluation of potential candidate drugs and their clinical use. Here, we examined the effects of IL-1β on GABAergic transmission in the CeA, as well as its actions on ethanol-induced facilitation of GABAergic transmission. We recorded from B6129SF2/J mice because they have been used previously as a control for *Il1r* KO mouse studies assessing the role of IL-1R1 in various biological phenomena[1] (for list of publications). Because alcohol-related behaviors in these mice have not been studied, we also characterized the B6129SF2/J strain for alcohol drinking and preference.

Materials and Methods

Animal Treatment

Male B6129SF2/J ($n = 80$; 29.5 ± 0.3 g) mice were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle (lights on at 6:00 pm) with food and water available *ad libitum*. We conducted all care procedures in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Institutional Animal Care and Use Committee policies of The Scripps Research Institute.

Slice Preparation

The mice (10–16 weeks old at the time of electrophysiological recordings) were anesthetized with 3% isoflurane, decapitated, and the brains quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF: composition in mM: NaCl, 130; KCl, 3.5; NaH_2PO_4, 1.25; $MgSO_4.7H_2O$, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10) and ice-cold oxygenated high-sucrose cutting solution (composition (in mM): sucrose, 206; KCl, 2.5; $CaCl_2$, 0.5; $MgCl_2$, 7; NaH_2PO_4, 1.2; $NaHCO_3$, 26; glucose, 5; HEPES, 5; pH7.3–7.4) gassed with 95% O_2 and 5% CO_2. We cut coronal slices containing the CeA using a Leica 1000S vibratome cutter (Campden, Lafayette, IN, USA).

Intracellular Recordings

We incubated the slices (400 μm) in an interface configuration for 30 min, and then completely submerged and continuously superfused (flow rate of 2–4 ml/min) them with warm (31°C), O_2/CO_2-gassed ACSF. We added drugs to the ACSF from

[1] http://jaxmice.jax.org/strain/003018.html

stock solutions to obtain known concentrations in the superfusate. We recorded from CeA neurons with sharp micropipettes containing 3 M KCl (65–80 MΩ resistance) using current-clamp mode. Data were acquired with an Axoclamp-2B preamplifier (now Molecular Devices, Sunnyvale, CA, USA) and stored for offline analysis via pClamp 10.2 software (Molecular Devices). We evoked pharmacologically isolated $GABA_A$ergic IPSPs by stimulating locally within the medial subdivision of the CeA with a bipolar stimulating electrode, while continuously superfusing the glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM) and DL-2-amino-5-phosphonopentanoic acid (DL-AP5, 30 μM), and the $GABA_B$ blocker CGP 55845A (1 μM).

We held the CeA neurons near their resting membrane potentials (RMPs ranging from –65 to –85 mV (mean: –78.6 + 0.7 mV, $n = 73$), and applied hyperpolarizing and depolarizing current steps (200 pA increments, 750 ms duration) to generate voltage-current curves. To determine half-maximal IPSP amplitudes, we examined input/output (I/O) curves by measuring evoked IPSP amplitudes at five stimulus strengths ranging from the threshold to maximum stimulation. Subsequent analyses were done with averages of two IPSPs evoked with the half-maximal stimuli. We measured the IPSP amplitudes before (baseline), during (up to 20 min) and after (washout for 10–25 min) drug application, and determined the percent change in IPSP amplitude at each stimulus intensity using the equation: (Vdrug/Vcontrol) * 100.

We examined paired-pulse facilitation (PPF) using 100 ms interstimulus intervals and the stimulus strength was adjusted so that the amplitude of the first IPSP was 50% of the maximal determined from the I/O relationship. We calculated the PPF using the equation: (2nd IPSP amplitude/1st IPSP amplitude) * 100. We took PPF measurements before drug superfusion (baseline), during (10–20 min) and after drug washout (10–25 min).

Whole-cell Patch-Clamp Recording

After cutting, the slices (300 μm) were incubated in O_2/CO_2-gassed ACSF for 30 min at 32°C, followed by incubation for 30 min at room temperature. We performed whole-cell patch-clamp recording in voltage clamp mode, as described previously (Bajo et al., 2011). Briefly, we used infrared/DIC visualization of CeA neurons (Dodt and Zieglgansberger, 1990), followed by digitization and image enhancement via an upright, fixed-stage Olympus microscope. We used micropipettes with an input resistances of 3–6 MΩ (access resistance <20 MΩ, compensated 60–80%) filled with an internal solution (composition in mM: KCl, 145; EGTA, 5; $MgCl_2$, 5; HEPES, 10; Na-ATP, 2; Na-GTP, 0.2; the latter two added fresh on the day of recording), pH 7.3–7.4. We isolated spontaneous miniature $GABA_A$-mediated IPSCs (mIPSCs) pharmacologically by applying blockers of glutamatergic (20 μM DNQX, 30 μM DL-AP5) and $GABA_B$ receptors (1 μM CGP 55845A), and adding 0.5 μM tetrodotoxin (TTX) to the bath. We used the Multiclamp 700B and pClamp 10.2 software (Molecular Devices) for data acquisition. Recombinant mouse IL-1β, recombinant IL-1ra and ethanol were added to the ACSF from stock solutions in known concentrations. We took all measures before drug (baseline) and during drug superfusion (12–15 min).

Ethanol Drinking Procedure

This procedure was adapted from that of Blednov et al. (2005). Mice were allowed to acclimate for 1 week to individual housing. Two drinking tubes were continuously available, Monday–Friday, to each mouse and fluid consumption was measured daily. One bottle of water was available across weekends. Food was available *ad libitum* and mice were weighed each week. After 4 days of water consumption (on Monday, off Friday; water in both tubes), mice were offered 3% ethanol (v/v) versus water on the following Monday–Friday. We changed tube positions every day to control for position preferences. Over the following 4 weeks mice received 6, 9, 12, and 15% ethanol in this same manner. Following this, mice received 15% ethanol for 2 h per day (starting 3 h after lights off) for 5 days in order to examine limited access two bottle-choice (2BC) drinking. The quantity of ethanol consumed (g/kg body weight/24 h or 2 h) was calculated for each mouse and averaged across each 4–5 day measurement period.

Data Analysis and Statistics

To analyze data acquired from intracellular and whole-cell recordings, we used Clampfit 10.2 (Molecular Devices) and MiniAnalysis 5.1 software (Synaptosoft, Leonia, NJ, USA), respectively. We used GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) software for all statistical analysis. We accepted statistical significance at the $p < 0.05$ level using one-way ANOVA and *t*-tests. The data are presented as percentile changes in mean ± SEM.

Drugs

We purchased CGP 55845A, DNQX, and DL-AP5 from Tocris Biosciences (Ellisville, MI, USA), recombinant mouse IL-1β from Biolegend (San Diego, CA, USA), recombinant human IL-1ra from Peprotech (Rocky Hill, NJ, USA), and TTX from Calbiochem (San Diego, CA, USA). We obtained ethanol from Remet (La Mirada, CA, USA).

Results

Ethanol Drinking and Preference of B6129SF2/J Mice

Although the B6129SF2/J mice have been used previously as controls for *Il1r* KO mouse studies assessing the role of IL-1R1 in various biological phenomena, their ethanol drinking and preference behavior is unknown. Therefore, we used 2BC tests with unlimited (24 h) and limited (2 h) ethanol access to determine their voluntary drinking and preference. The average daily ethanol intake with 24 h access, measured for a period of 5 days, ranged from 9.24 to 12.65 g/kg for the ethanol concentrations tested (3, 6, 9, 12, and 15%; **Figure 1A**). There were no significant differences in ethanol intake between the tested concentrations, nor was there a correlation between the intake and ethanol concentration ($R^2 = 0.7$). During 2 h limited access, the average ethanol (concentration 15%) intake was 4.1 ± 0.5 g/kg

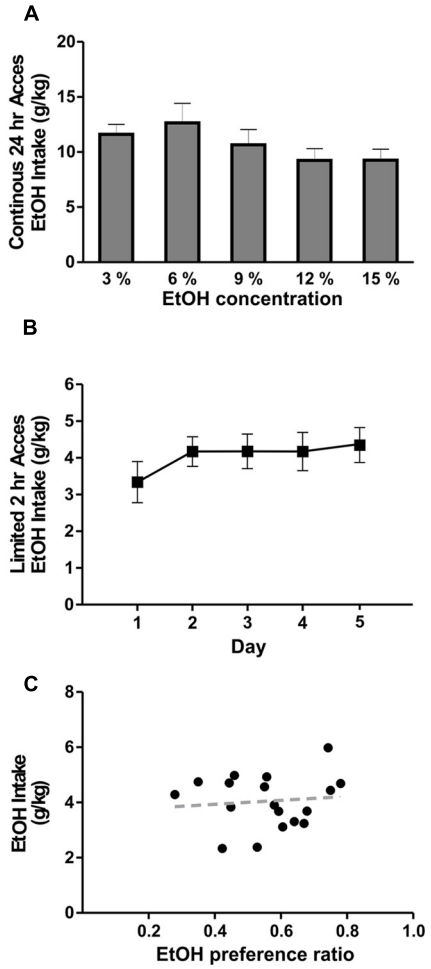

FIGURE 1 | Ethanol drinking behavior of B6129SF2/J mice. The ethanol intake of B6129SF2/J mice was tested using unlimited (24 h) and limited (2 h) 2-bottle choice (2BC) paradigms. **(A)** The intake of 3, 6, 9, 12, and 15% ethanol was measured by 2BC with unlimited access to ethanol. On average, the mice consumed 11.6 ± 0.9 g/kg/day of 3% ethanol solution, 12.7 ± 1.8 g/kg/day of 6% ethanol solution, 10.7 ± 1.1 g/kg/day of 9% ethanol solution, 9.2 ± 1.1 g/kg/day at 12% ethanol solution, and 9.3 ± 1.0 g/kg/day of 15% ethanol solution. There was significant main difference in ethanol intake between the ethanol concentrations [$F_{(4,19)} = 2.5$, $n = 20$], but Tukey *post hoc* analysis did not reveal significant differences between specific ethanol concentrations. **(B)** For limited access measurements of ethanol consumption, we used 15% ethanol solution and intake was measured for 2 h daily (starting 3 h after lights off) for a period of 5 days. Consumption was 3.3 ± 0.6 g/kg of ethanol on day 1; 4.2 ± 0.4 g/kg of ethanol on day 2; 0.4.2 ± 0.5 g/kg on days 3 and 4; 4.4 ± 0.5 on day 5. Repeated measure one-way ANOVA showed no significant difference in ethanol intake between testing days [$F_{(4,17)} = 0.7$, $n = 18$]. **(C)** Ethanol intake of individual mice is plotted as a function of preference. The average ethanol preference ratio (volume of ethanol consumed/total volume of fluid consumed) was 0.56 ± 0.05. There was no significant correlation ($R^2 = 0.01$) between ethanol preference and intake in B6129SF2/J mice.

($n = 18$; **Figure 1B**) and the average ethanol preference ratio was 0.56 ± 0.05 (volume of ethanol consumed/total volume of fluid consumed). We did not observe a significant correlation between ethanol preference and ethanol intake ($R^2 = 0.01$) in the individual B6129SF2/J mice (**Figure 1C**). These data indicate that B6129SF2/J mice drink a substantial amount of ethanol and have a modest preference for ethanol.

IL-1β Decreased eIPSP Amplitudes in the CeA

We tested the effects of recombinant mouse IL-1β (5, 50, and 100 ng/ml) on $GABA_A$ receptor-mediated eIPSPs in the CeA. None of the tested concentrations significantly altered the current–voltage relationships, resting membrane potentials, or resistance (data not shown). In the majority of CeA neurons, high IL-1β concentrations (50 and 100 ng/ml) significantly decreased eIPSP amplitudes, by 27.8 ± 6.0% ($n = 11$; *t*-test: $p < 0.01$) and by 21.6 ± 6.7% ($n = 5$; *t*-test: $p < 0.05$), respectively (**Figure 2A**). However, 5 ng/ml IL-1β had no significant effect on the mean amplitudes of the eIPSPs (to 89.8 ± 5.0% of baseline, $n = 7$). The significant decreases in eIPSPs were not associated with changes in the PPF ratio, although there was a trend toward an increase in the PPF ratio by IL-1β at 100 ng/ml (50 ng/ml: 103.7 ± 7.2% of baseline, $n = 8$; 100 ng/ml: 130.1 ± 19.4% of baseline, $n = 4$; **Figure 2B**). Thus, these results indicate that IL-1β reduces GABAergic transmission, likely via postsynaptic mechanisms.

IL-1β had Dual Effects on mIPSC Frequencies and Decreased mIPSC Amplitudes in CeA Neurons

We performed whole-cell recordings of mIPSCs in CeA neurons while superfusing 50 ng/ml of IL-1β. Here, we present the combined results of all experiments where IL-1β was applied to the naïve slice for 12–15 min (**Figure 3**). We found that IL-1β had dual effects (Δ > 15% from baseline) on mIPSC frequencies and amplitudes, and so we examined its effects on mIPSC frequencies and amplitudes separately. We found that IL-1β significantly increased the mean mIPSC frequency by 50.7 ± 10.1% in 13 of 21 CeA neurons (**Figure 3A**). In 6 of 21 cells, we observed a significant decrease in the mean mIPSC frequency by 44.1 ± 9.5%, which was associated with a significant decrease in the mean mIPSC amplitude (76.7 ± 6.2% of baseline) and an increase in mIPSC rise time (114.4 ± 4.7% of baseline; **Figure 3B**). In the remaining 2 neurons, IL-1β had no effect on mIPSC frequency (data not shown). Using the changes in mIPSC amplitude as the parameter for the division of the data, we found a significant decrease in the mIPSC amplitude in 8 of 21 cells (72.2 ± 6.2% of baseline) and no effects in 10 of 21 cells. In the remaining three CeA neurons, IL-1β increased both the mIPSC amplitude by 46.0 ± 14.7% and frequency by 62.8 ± 26.7% (data not shown). Since changes in mIPSC frequencies suggest an altered probability of vesicular transmitter release, and changes in mIPSC amplitudes may reflect modulation of postsynaptic $GABA_A$ receptors (De Koninck and Mody, 1994; Otis et al., 1994), our data indicate that IL-1β alters spontaneous action potential-independent GABA transmission through both presynaptic and postsynaptic mechanisms of action. Importantly, the parallel changes in mIPSC frequencies and amplitudes of individual CeA neurons suggest that acute IL-1β acts in a cell-specific manner.

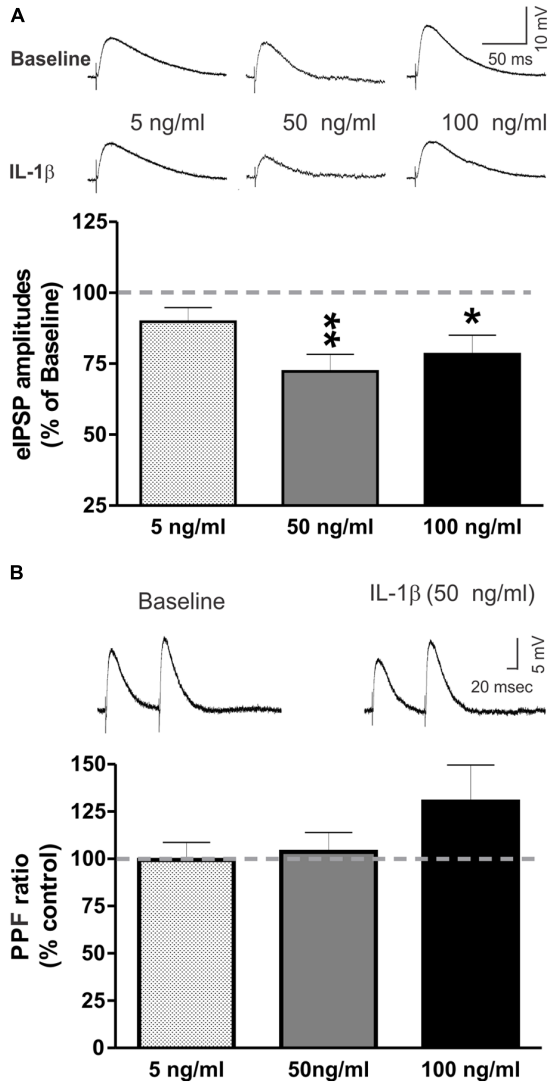

FIGURE 2 | IL-1β decreases evoked $GABA_A$-receptor mediated IPSPs in the CeA. (A) Recombinant mouse IL-1β decreased the mean amplitude of evoked IPSPs (eIPSPs) by 27.8 ± 6.0% ($n = 11$; *t*-test: $p < 0.01$) at 50 ng/ml and by 21.6 ± 6.7% ($n = 5$; *t*-test: $p < 0.05$) at 100 ng/ml. A lower concentration of IL-1β (5 ng/ml) had no significant effects on the eIPSPs (89.8 ± 5.0% of baseline, $n = 7$). (Top) Representative eIPSPs taken during baseline and IL-1β superfusion. (Bottom) Summary of the maximal effects of IL-1β elicited by the tested doses of IL-1β as compared to baseline. The statistical significance (*$p < 0.05$ and **$p < 0.01$) was calculated by *t*-test. **(B)** IL-1β did not alter significantly paired-pulse facilitation (PPF; using a 100 ms interstimulus interval) at any of the tested concentrations (5 ng/ml: 99.4 ± 9.3% of baseline, $n = 6$; 50 ng/ml: 103.7 ± 7.2% of baseline, $n = 8$; 100 ng/ml: 130.1 ± 19.4% of baseline, $n = 4$). (Top) Representative recordings of PPF from a CeA neuron superfused with 50 ng/ml IL-1β. (Bottom) The PPF results are summarized on the bar graph with the PPF ratio during IL-1β superfusion compared to the baseline levels.

IL-1ra Modulates CeA mIPSCs and Blocks the Effects of IL-1β on mIPSCs

To examine the role of IL-1R1 in the effects of IL-1β in the CeA, we used an IL-1R1 antagonist (recombinant IL-1ra) to block IL-1β's actions on GABAergic transmission. Here, we present the results of experiments where IL-1ra (100 ng/ml) was applied to the naïve slice for 12–15 min, and the subset of these experiments where IL-1β was subsequently co-applied for 12–15 min (**Figure 4**). We observed transient IL-1ra effects with maximal cellular responses within 9–15 min of drug application. Similar to the IL-1β effects, the IL-1ra-induced changes in mIPSC frequency and/or amplitude varied among individual CeA neurons. In the majority (67%) of CeA cells, IL-1ra decreased significantly the mean mIPSC frequency by 31.3 ± 2.1% (**Figure 4A**). In the remaining cells, IL-1ra significantly increased the mIPSC frequency by 34.1 ± 7.7% (**Figure 4A**). These changes in the mIPSC frequencies were not associated with significant changes in mIPSC amplitudes or kinetics. On the other hand, when we used the change in mIPSC amplitude ($\Delta > 15\%$) as the criterion for cell grouping, we found that IL-1ra increased significantly the mean mIPSC amplitude by 27.9 ± 4.5% in 39% of the cells and decreased the mean mIPSC amplitude by 21.7 ± 5.9% in 28% of CeA neurons (**Figure 4B**). These changes in mIPSC amplitudes were not associated with significant changes in mIPSC frequencies or kinetics. In the rest of the cells (33%), IL-1ra did not alter significantly the mean mIPSC frequency, amplitude or kinetics. These results indicate that IL-1R1 plays a role in basal GABAergic transmission in the CeA.

We also examined whether IL-1ra prevents the IL-1β-induced changes in mIPSCs. In order to do this, we grouped the neurons into two groups based on their IL-1ra-induced changes in mIPSC frequencies, and compared their mean mIPSC characteristics to the average mIPSC characteristics observed after 12–15 min of IL-1ra and IL-1β co-application. Co-application with IL-1β did not induce significant differences in the mIPSCs compared to IL-1ra alone (**Figure 4C**). These results suggest that IL-1β modulates mIPSCs via IL-1R1.

Ethanol Increased eIPSPs and mIPSCs in the CeA Via a Predominantly Presynaptic Mechanism

Ethanol (44 mM) had no significant effects on the intrinsic membrane properties (resting membrane potential, the current–voltage relationship, resistance) of CeA neurons (data not shown; see also Roberto et al., 2003). Superfusion of 44 mM ethanol increased the mean eIPSP amplitude by 22.5 ± 5.9% in CeA neurons and significantly decreased the PPF ratio (82.2 ± 4.0% of baseline), suggesting that ethanol acts via presynaptic mechanisms (**Figure 5A**). This finding is supported by ethanol's facilitation of the mean mIPSC frequency by 40.7 ± 17.5% (**Figure 5B**). Although ethanol had no effect on the mean mIPSC amplitude, it significantly increased the mIPSC rise (by 14.7 ± 4.2%) and decay (by 25.4 ± 7.0%) times.

Co-Application of Ethanol Reversed the IL-1β-Induced Decrease in the Mean eIPSP Amplitude

We then examined the interaction between IL-1β and ethanol on GABAergic transmission in the CeA, by superfusing IL-1β (50 ng/ml) for 15–20 min, followed by co-application of IL-1β and ethanol (44 mM) for an additional 15–20 min. Using

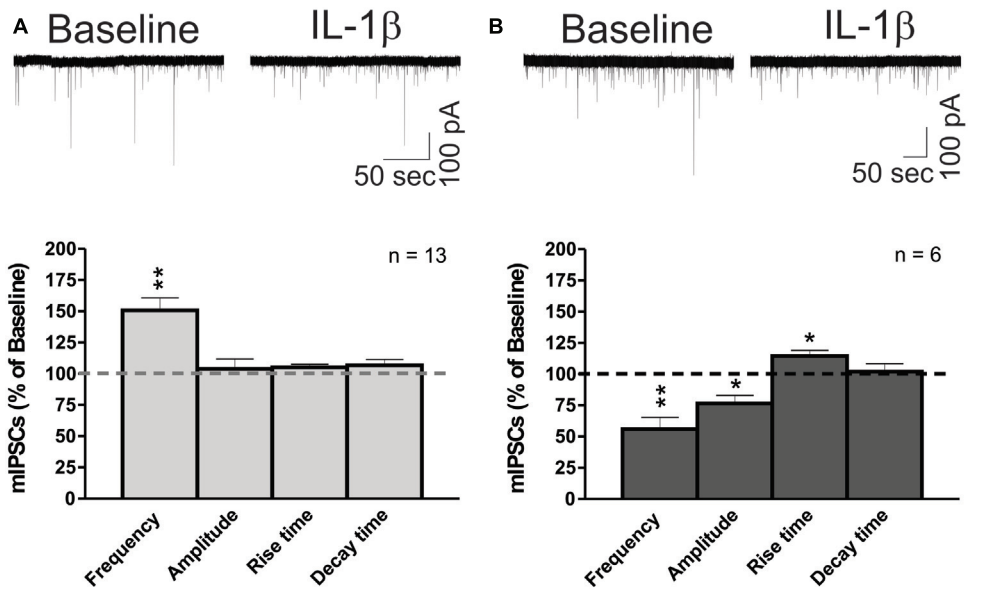

FIGURE 3 | IL-1β has dual effects on miniature IPSCs in CeA neurons. **(A)** IL-1β increases mIPSC frequencies in the CeA. (Top) A representative whole-cell voltage clamp recording showing an increase in mIPSC frequency induced by 50 ng/ml IL-1β superfusion. (Bottom) The bar graphs present normalized mIPSC parameters from 62% of CeA neurons (13 of 21 cells) responding to IL-1β with an increase in mIPSC frequency (150.7 ± 10.0% of baseline). mIPSC amplitude (104.0 ± 7.8% of baseline) and kinetics were not significantly changed in these CeA neurons, although some cells showed individual changes in mIPSC amplitude. We calculated statistical significance (**$p < 0.01$) by *t*-test. **(B)** IL-1β decreases mIPSC frequencies and amplitudes in CeA neurons. (Top) Representative recording of a CeA neuron responding to IL-1β with a decrease in mIPSC frequency. (Bottom) Acute application of IL-1β significantly decreased the mIPSC frequency (55.9 ± 9.5% of baseline) and amplitude (76.7 ± 6.2% of baseline) in 29% of CeA neurons (6 of 21 cells). In addition, IL-1β increased the mIPSC rise time (114.3 ± 4.7% of baseline) in these neurons. Statistical significance (*$p < 0.05$) and (**$p < 0.01$) were calculated by *t*-test.

intracellular recording, we found no significant changes in the membrane properties induced by IL-1β or co-application of IL-1β and ethanol (data not shown). IL-1β alone significantly decreased the mean eIPSP amplitude (84.8 ± 4.7% of baseline), whereas co-application with ethanol reversed the IL-1β-induced decrease in the mean eIPSP amplitude back to 115.4 ± 5.3% of the original baseline (**Figures 6A,B**). Ethanol co-application significantly increased the mean eIPSP amplitude in comparison to the IL-1β effect, but not with respect to the baseline level (**Figure 6C**). In addition, we did not observe significant changes in the PPF ratio following superfusion with IL-1β alone or co-application of IL-1β and ethanol (**Figure 6D**). These results indicate that IL-1β and ethanol modulate CeA eIPSPs via different mechanisms, though the occlusion of ethanol's PPF effects suggest that IL-1β may interfere with the downstream mechanisms mediating ethanol-facilitated GABA release.

IL-1β Occluded Ethanol Effects on CeA mIPSCs

Finally, we investigated the potential interaction between IL-1β and ethanol on action potential-independent vesicular GABA release. In the majority of CeA neurons (6 of 10 cells), IL-1β alone, as well as its co-application with ethanol, significantly increased the mean mIPSC frequency (to 145.9 ± 14.6% and 142.4 ± 8.9% compared to baseline, respectively; **Figure 7A**). There were no significant changes in the mean mIPSC amplitudes or kinetics with IL-1β or co-application of IL-1β and ethanol in these cells (**Figure 7A**). However, in 3 of 10 cells, IL-1β alone decreased the mIPSC frequency by 53.5 ± 11.3%, and subsequent co-application of ethanol did not alter this IL-1β-induced decrease in mIPSC frequency (remained at 59.2 ± 7.9% of baseline; **Figure 7B**). Although there was a trend toward a decrease in mIPSC amplitudes by IL-1β and co-application of IL-1β and ethanol (82.15 ± 9.7% and 88.14 ± 11.8 of baseline, respectively) in these three neurons, it did not reach statistical significance. Finally, there was one CeA cell that showed no IL-1β effect on mIPSC frequency, but co-application of ethanol increased the mIPSC frequency to 127.6% of baseline. In this neuron, the mIPSC amplitude was decreased by IL-1β alone (by 30.8%) and also by ethanol co-application (by 32.1%).

Discussion

In the present study, we investigated cytokine IL-1β modulation of GABAergic transmission and its interaction with ethanol-induced facilitation of GABA signaling in the CeA of B6129SF2/J mice. Behaviorally, B6129SF2/J mice have a moderate preference for alcohol and consume a substantial amount of alcohol. At the cellular level, IL-1β modulation of CeA GABAergic transmission is characterized by a reduction of evoked IPSPs, mediated predominantly by postsynaptic mechanisms, and by predominantly presynaptic dual effects on spontaneous miniature IPSCs in a cell-specific manner. The IL-1β effects on mIPSCs appear to be mediated by IL-1R1. Moreover, IL-1R1 regulates basal mIPSCs in

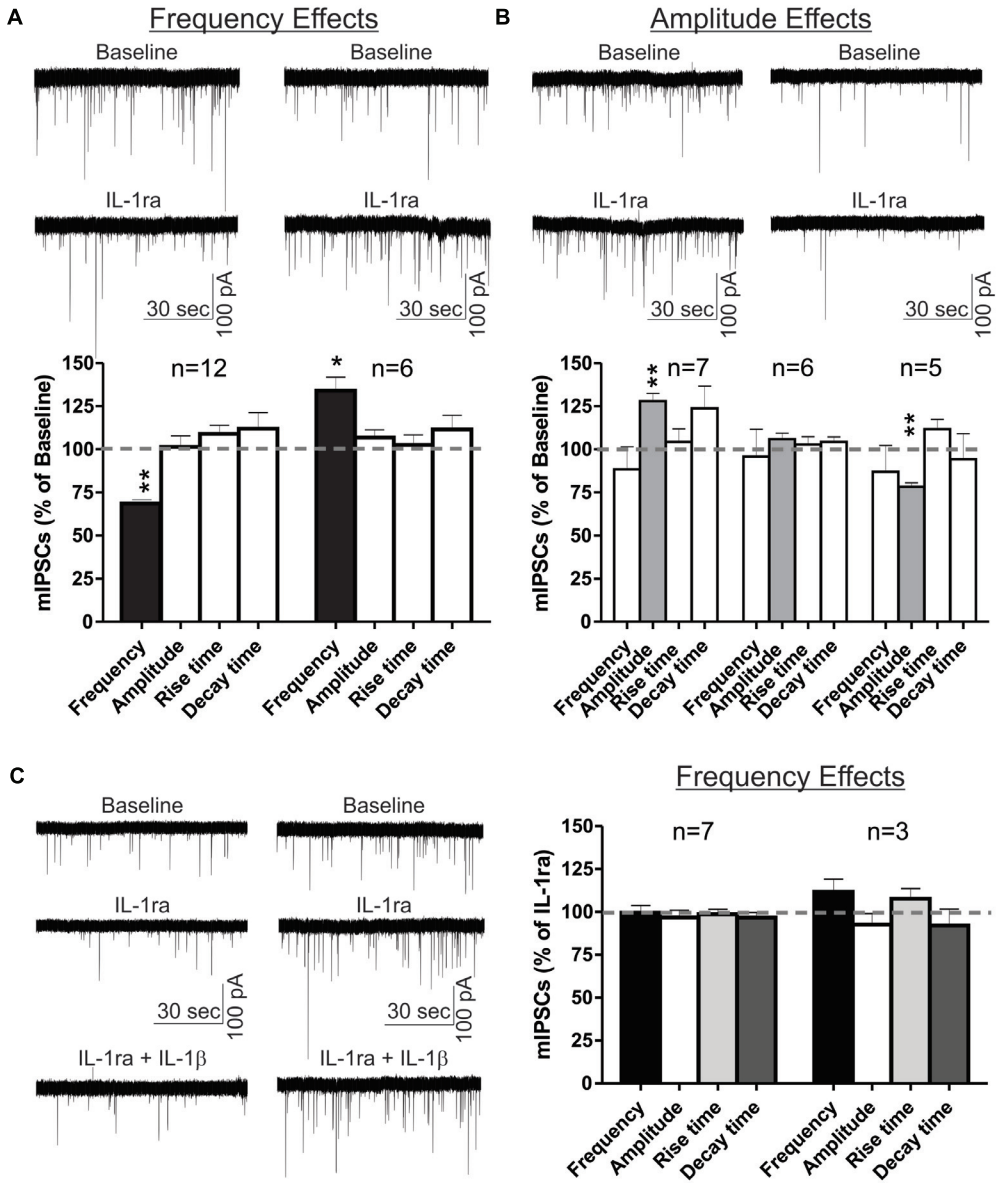

FIGURE 4 | IL-1ra has dual effects on basal mIPSCs and prevents IL-1β-induced modulation of mIPSCs in the CeA. IL-1ra-induced changes in mIPSC frequencies and/or amplitudes vary among CeA neurons, indicating cell-specific differences in IL-1ra modulation of GABAergic transmission. **(A)** Dual IL-1ra-induced changes in mIPSC frequencies. (Top) Representative recordings of two CeA neurons showing a decrease (left column) or increase (right column) in mIPSC frequencies following IL-1ra application (100 ng/ml). (Bottom) Summary bar graph showing IL-1ra decreased significantly (*t*-test, $p < 0.01$) the mean mIPSC frequency by 31.3 ± 2.1% in 12 of 18 (67%) neurons. In the remaining CeA cells (6 of 18), IL-1ra increased the mIPSC frequency by 34.1 ± 7.7% (*t*-test, $p < 0.05$). The changes in mIPSC frequencies were not associated with significant changes in mIPSC amplitudes or kinetics. **(B)** The IL-1ra induced changes in the mIPSC amplitudes were also variable. (Top) Representative recordings of two cells responding to IL-1ra with increased (left column) or decreased (right column) mIPSC amplitudes. (Bottom) Summary bar graph showing IL-1ra increased (*t*-test, $p < 0.01$) mIPSC amplitudes by 27.9 ± 4.5% in 7 of 18 cells (39%) and decreased (*t*-test, $p < 0.01$) by 21.7 ± 5.9% in 5 of 18 cells (28%). There were no significant changes in the mean mIPSC frequencies and kinetics across all cell groups. The statistical significance ($^*p < 0.05$) and ($^{**}p < 0.01$) was calculated by *t*-test. **(C)** To examine the effects of IL-1ra on the IL-1β-induced modulation of mIPSCs, we compared the mIPSC parameters recorded within 9–15 min of 100 ng/ml IL-1ra and 50 ng/ml IL-1β co-application to the last 6 min (9–15 min) of IL-1ra application alone. We divided the CeA neurons into two groups according to their cellular responses (mIPSCs frequency) to IL-1ra alone: the cells that responded to IL-1ra with decreased mIPSC frequency [by 31.8 ± 3%; $F_{(2,23)} = 7.5$, $p < 0.05$; $n = 7$] and the cells that responded to IL-1ra with increased mIPSC frequency [by 27.9 ± 7%; $F_{(2,8)} = 1.7$, $p < 0.05$; $n = 3$]. (Left) Representative recordings from two CeA neurons responding to IL-1ra with decreased (left column) or increased (right column) mIPSCs frequencies. (Right) IL-1ra prevented the IL-1β-induced modulation of mIPSCs, as there were no significance differences in mIPSCs after co-application of IL-1ra and IL-1β compared to IL-1ra alone. The statistical significance was set at ($^*p < 0.05$) and was calculated by repeated measurement one-way ANOVA followed by a Tukey *post hoc* test.

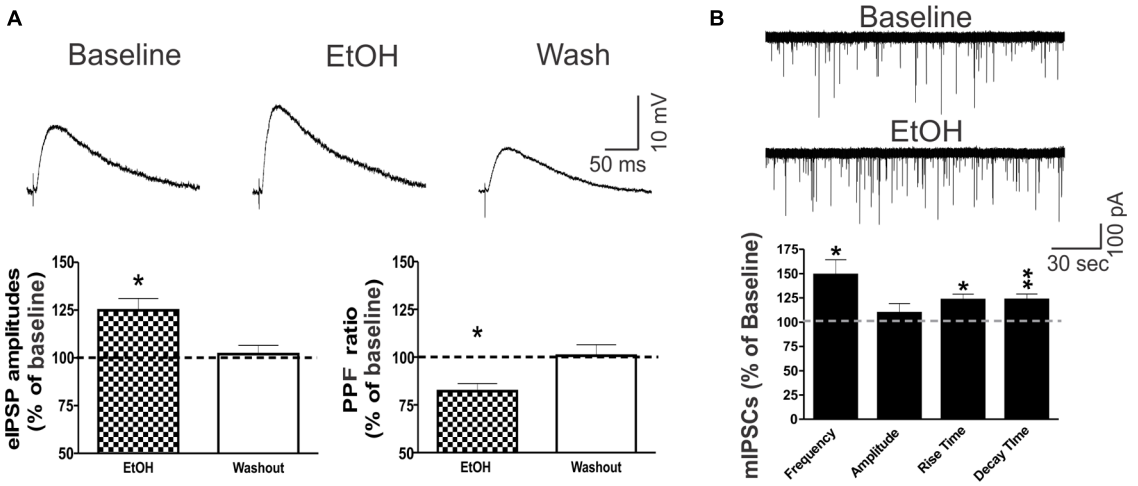

FIGURE 5 | Ethanol potentiates CeA GABAergic transmission. **(A)** Ethanol potentiated eIPSPs via a presynaptic mechanism in CeA neurons. (Top) Representative recordings of eIPSPs from a CeA neuron showing an ethanol-induced increase in eIPSP amplitude that is reversed upon drug washout. (Bottom) On average, 44 mM ethanol significantly increased the mean eIPSP amplitude by 22.5 ± 5.9% (left column: $n = 8$; *t*-test: $p < 0.05$) and decreased the PPF ratio to 82.2 ± 4.0% of baseline in six of eight neurons (right column: $n = 6$; *t*-test: $p < 0.05$), indicating that ethanol-induced eIPSP potentiation is mediated by increased GABA release. **(B)** Ethanol increases spontaneous miniature GABA transmission in the CeA by both pre- and postsynaptic mechanisms. (Top) Representative mIPSC recordings from a CeA neuron showing an ethanol-induced increase in frequency. (Bottom) Superfusion of 44 mM ethanol induced a significant increase in the mean mIPSC frequency (140.7 ± 17.5% of baseline), but had no effect on the mean amplitude (102.6 ± 9.5% of baseline; $n = 4$, *t*-test: $p < 0.05$), supporting the finding that ethanol's mechanism of action is predominantly presynaptic. However, ethanol significantly altered mIPSC kinetics, with a 14.7 ± 4.2% increase in the rise time and a 25.4 ± 7.0% increase in the decay time, indicating additional postsynaptic changes (*t*-test: $^{*}p < 0.05$ and $^{**}p < 0.01$).

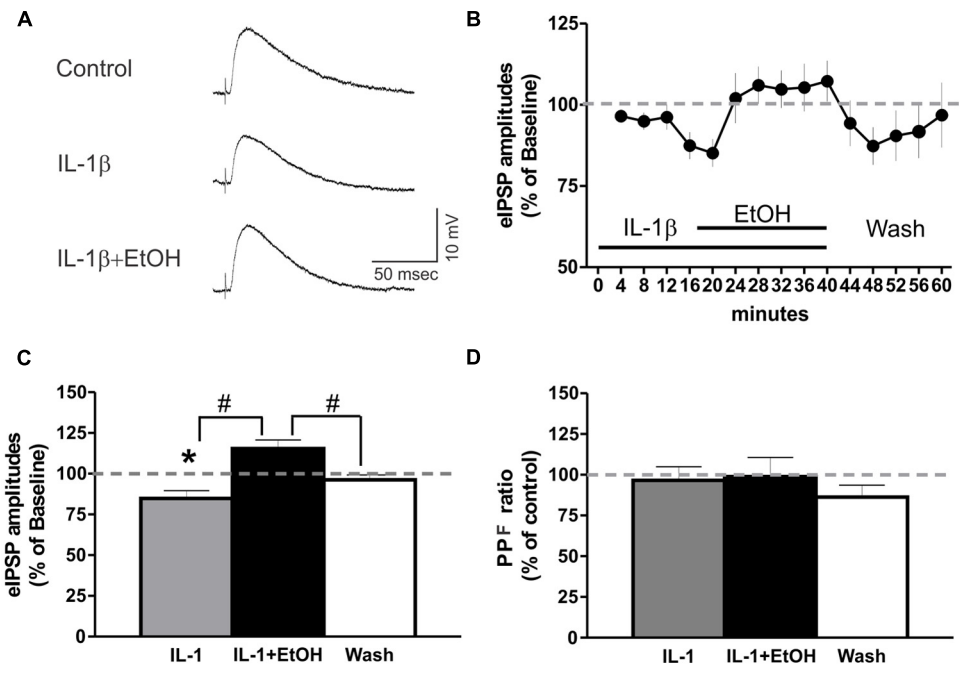

FIGURE 6 | IL-1β and ethanol have opposing effects on eIPSP amplitudes. **(A)** Representative eIPSPs from a CeA neuron showing a 50 ng/ml IL-1β-induced decrease in eIPSP amplitude, and its subsequent reversal to baseline levels by the addition of ethanol (44 mM). **(B)** Time course presenting the averaged eIPSP amplitudes over 3 min bin periods. **(C)** Co-application of ethanol reversed the IL-1β-induced decrease in mean eIPSP amplitude [84.8 ± 4.7% of baseline, $n = 9$; $F_{(2,26)} = 12.1$, $p < 0.01$] to slightly above baseline levels (115.4 ± 5.3% of baseline). Statistical significance [$p < 0.05$; *(comparisons to baseline) and $^{\#}$(comparison of the effects of ethanol plus IL-1β co-application to IL-1β alone or washout)] was calculated by repeated measurement one-way ANOVA followed by a Tukey *post hoc* test. **(D)** There were no significant effects on the PPF ratio (100 ms interstimulus interval) of IL-1β alone, or when it was co-applied with ethanol [$F_{(2,23)} = 0.24$].

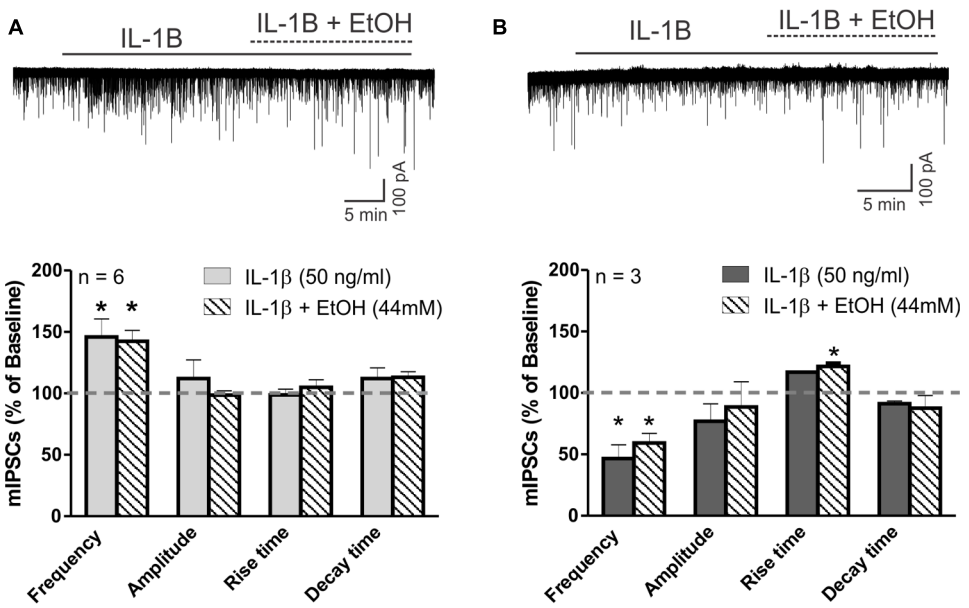

FIGURE 7 | IL-1β occludes ethanol's facilitation of mIPSCs. (A) The effects of ethanol on mIPSCs are blocked by IL-1β in cells that previously showed increased mIPSC frequency in the presence of IL-1β alone. (Top) Voltage clamp recordings of mIPSCs from a CeA neuron showing an IL-1β-induced increase in mIPSC frequency that is unaltered by the addition of ethanol. (Bottom) Summary of the normalized mIPSC maximal effects of IL-1β (50 ng/ml) alone, and IL-1β and ethanol (44 mM) co-application. IL-1β alone significantly increases mIPSC frequency by 45.9 ± 14.6% in 6 of 10 CeA neurons. The co-application of ethanol did not further change the mIPSC frequency (142.4 ± 8.9% of baseline; [$F_{(2,5)} = 6.9$, $p < 0.05$; Tukey *post hoc* test]. There were no differences in mIPSC amplitudes and kinetics across all treatments. Statistical significance (*$p < 0.05$) was calculated by one-way ANOVA followed by a Tukey *post hoc* test. **(B)** Ethanol's effects on mIPSC frequency are blocked by IL-1β in cells that previously showed decreased mIPSC frequency in the presence of IL-1β alone. (Top) Representative recordings from a CeA neuron showing a reduction in mIPSC frequency elicited by IL-1β and the co-application of IL-1β and ethanol. (Bottom) In 3 of 10 CeA neurons, mIPSC frequency was significantly decreased by IL-1β (46.5 ± 11.3% of baseline) alone, as well as with the co-application of IL-1β and ethanol [59.2 ± 7.9% of baseline; $F_{(2,2)} = 35.5$, $p < 0.05$; Tukey *post hoc* test]. There was no significant difference between the effects of IL-1β alone and co-application of IL-1β and ethanol (Tukey *post hoc* test, $p < 0.05$), but co-application of IL-1β and ethanol significantly increased the mean rise time of mIPSCs [116.4 ± 5.4% of baseline; $F_{(2,2)} = 7.9$, $p < 0.05$; Tukey *post hoc* test]. The statistical significance (*$p < 0.05$) was calculated by one-way ANOVA followed by a Tukey *post hoc* test.

the CeA. The interaction of IL-1β and ethanol is likely to occur presynaptically, and is characterized by the occlusion of ethanol's facilitation of vesicular GABA release by IL-1β.

B6129SF2/J mice have been used previously as controls for *Il1r* KO mice (#003018, Jackson Laboratories) in studies characterizing the role of IL-1R1 in various physiological and pathological processes[2] (for a list of publications). Alcohol drinking behavior in B6129SF2/J mice has not been determined, despite the fact that different mice strains exhibit a range of alcohol drinking behaviors in terms of alcohol consumption and preference (Rhodes et al., 2007; Yoneyama et al., 2008). Since the genetic background of B6129SF2/J mice is based on C57BL/6J and 129S1/SvImJ mice, we expected to find similarities in the alcohol drinking phenotype of B6129SF2/J mice to those two strains, particularly the C57BL/6J mice. Our behavioral data showed that ethanol intake and ethanol preference of B6129SF2/J mice are similar to the values reported for C57BL/6J mice (Yoneyama et al., 2008).

Cytokines, including IL-1, play an important role in the regulation of both excitatory and inhibitory neurotransmission in the central nervous system (Camacho-Arroyo et al., 2009). IL-1R1 is expressed on glial cells and neurons, and thus, the overall effect of IL-1 on synaptic transmission is a combination of the direct effects of IL-1 binding to neuronal IL-1R1 and the indirect effects mediated by other signaling molecules generated and released by both neurons and glia in response to IL-1/IL-1R1 binding (e.g., cytokines, chemokines, ATP, etc.). The IL-1β effects on GABAergic transmission appear to be brain region specific, as IL-1 increases GABAergic transmission in some regions (e.g. hypothalamus, hippocampus; Miller et al., 1991; Plata-Salaman et al., 1998; Tabarean et al., 2006) and decreases it in others (e.g., basolateral amygdala, cerebellum; Yu and Shinnick-Gallagher, 1994; Pringle et al., 1996).

In our study we determined a concentration response curve for IL-1β, and found that only higher concentrations of IL-1β (>5 ng/ml) were effective in the modulation of CeA GABAergic transmission. The fact that the effective doses in our study are higher than in other brain regions (often in pg/ml range) may be caused by regional differences in the IL-1 system, especially in the expression of IL-1R1 (Wong and Licinio, 1994; Yabuuchi et al., 1994; Ericsson et al., 1995). To examine the role of IL-1R1 in the IL-1β effects, we used a recombinant IL-1R1 antagonist

[2]http://jaxmice.jax.org/strain/003018.html

(IL-1ra). IL-1ra blocks the effects of IL-1β on mIPSCs, indicating that IL-1R1 mediates the IL-1β-induced modulation of CeA GABAergic transmission. We also observed a transient modulation of mIPSCs by IL-1ra alone, indicating that IL-1R1 regulates basal mIPSCs. In agreement with this finding, we reported recently an important role of IL-1ra and the IL-1 system in basal CeA GABAergic transmission. In that study, we observed an increase in the frequency of the spontaneous action potential-dependent IPSCs in IL-1ra deficient mice, but mISPC frequencies (action potential-independent IPSCs) were not affected (Bajo et al., 2014a). In addition to the compensatory mechanism associated with knockout technology and the different strains of mice used in the two studies (B6129SF2/J vs. C57Bl6J), the transiency of the IL-1ra effects on mIPSC in the current study may explain the lack of differences between baseline mIPSC frequencies of IL-1ra deficient mice and wild-type controls. Overall, both studies indicate that IL-1ra and the IL-1 system are involved in the regulation of basal GABAergic transmission in the mouse CeA. In the hippocampus, IL-1R1 also plays a critical role in baseline neuronal activity, (Hellstrom et al., 2005), while IL-1ra alone had no effects in neurons from the paraventricular nucleus of the hypothalamus (Ferri and Ferguson, 2003) or the spinal cord (Liu et al., 2013). Collectively, these findings further support that the regional specificity of the IL-1 system-dependent regulation of neuronal activities may underlie the brain region differences in the neuropathology associated with neuroinflammation.

Additionally, in our study, the IL-1β and IL-1ra effects on GABAergic transmission occurred in a majority of CeA neurons, and the effects were characterized by a duality of responses in individual CeA neurons. Other groups have reported a similar duality in their results, with electrophysiological studies revealing that IL-1β only affects synaptic transmission in a portion of neurons in the amygdala, cerebellum, hippocampus, and hypothalamus (Miller et al., 1991; Yu and Shinnick-Gallagher, 1994; Pringle et al., 1996; Plata-Salaman et al., 1998; Tabarean et al., 2006). It is therefore plausible to speculate that the cell-specific IL-1β and IL-1ra effects that we observed are determined by neuronal type, especially given the considerable heterogeneity of CeA neurons in terms of their biochemical and electrophysiological properties (Chieng et al., 2006; Herman et al., 2013) and their likely IL-1R1 expression. We classified each CeA neuron according to the electrophysiological criteria used in our previous studies on cell-type specific tonic GABA conductance in the CeA (Herman et al., 2013; Herman and Roberto, 2014), but did not observe any correlation between the IL-1β or IL-1ra effects and cell-type (low-threshold bursting, late spiking and regular spiking CeA neurons). Instead, it is likely that the cell-specificity of the IL-1β and IL-1ra effects are determined by the CeA neuronal expression of IL-1R1 (pre- versus postsynaptic expression), signaling pathways and/or other biochemical properties. Although our findings strongly indicate that the IL-1β effects on GABAergic transmission are mediated by IL-1R1, we cannot rule out completely that some of the IL-1β effects in the CeA may be caused by indirect actions of IL-1β via other signaling molecules (Camacho-Arroyo et al., 2009).

In this study, we have corroborated in B6129SF2/J mice our previous findings on ethanol's facilitation of GABAergic transmission in the mouse CeA (mostly C57BL6/J; Bajo et al., 2008, 2014b; Kang-Park et al., 2009; Cruz et al., 2011; Herman et al., 2013). In CeA slices from B6129SF2/J mice, ethanol potentiated both evoked and spontaneous CeA GABAergic transmission predominantly via presynaptic mechanisms, but also had limited postsynaptic effects. To investigate the potential interaction between ethanol and IL-1β, we pretreated slices with IL-1β, and then co-applied ethanol and IL-1β. Ethanol, in the presence of IL-1β, was still able to potentiate evoked GABAergic transmission, despite the IL-1β-induced reduction in evoked IPSP amplitudes. However, the effects of ethanol co-application with IL-1β were not significantly different when compared to the original baseline levels. These data suggest that the mechanisms of action of IL-1β and ethanol on evoked GABA transmission are different, in line with our findings that IL-1β acts via predominantly postsynaptic mechanisms, whereas ethanol acts presynaptically. In the case of spontaneous GABAergic transmission in the CeA, IL-1β has dual effects, either increasing or decreasing vesicular GABA release. Notably, ethanol co-application with IL-1β did not facilitate further vesicular GABA release in the neurons that had previously responded to IL-1β with an increase in mIPSC frequency. Ethanol also failed to increase GABA release in the neurons that responded to IL-1β with decreased mIPSC frequency, suggesting an occlusion of ethanol's effects by IL-1β pretreatment. The differences in eIPSP and mIPSC findings on the interaction of IL-1β and ethanol are likely to originate from differences in the forms of GABA release involved in each kind of synaptic transmission (Mathew et al., 2008; Fredj and Burrone, 2009). Specifically, mIPSCs are recorded in the presence of TTX to block Na^+ channels and consequently, the generation of action potentials. In contrast, evoked IPSPs require stimulation of a synaptic network, and thus, action potential-dependent release (Farrant and Nusser, 2005).

Conclusion

Our data collectively demonstrate that B6129SF2/J mice show an ethanol phenotype similar to that of C57BL6J mice, both behaviorally and electrophysiologically, in the CeA. IL-1β modulation of CeA GABAergic transmission is complex and characterized by dual and cell-specific modulations of presynaptic GABA release and postsynaptic $GABA_A$ receptor activity. With regard to the IL-1β effects on ethanol-induced facilitation of CeA GABAergic transmission, our data indicate that IL-1β interacts with ethanol presynaptically to occlude ethanol's enhancement of GABA signaling. Understanding these complex interactions of acute ethanol with IL-1 on GABAergic transmission are critical for shedding light on the potential role of the IL-1 neuroimmune system in the development of alcohol dependence and addiction.

Acknowledgments

We thank Dr. Marian L. Logrip for help with statistical analyses. This work was supported by NIH/NIAAA INIA West Consortium U01-AA013498 to MR and GRS, U01-AA013498-14S1 to MR, AA013517 to MR and MB, and AA020893 to AR.

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