2018-04-01

Neuroimmune-Mediated Alcohol Effects on Ventral Tegmental Area Neurons

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

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Dopamine (DA) transmission is a key player in the rewarding aspects of ethanol as well as ethanol dependence. The current dogma is that DA transmission is increased during ethanol via the inhibition of ventral tegmental area (VTA) GABA neurons and that excitation of VTA GABA neurons during withdrawal results in decreased DA transmission. Microglia, the major neuroimmune effector in the brain, may be a key mediator in this process by releasing cytokines following activation.

We evaluated the effect of ethanol on cytokine concentrations in the VTA and NAc using a cytometric bead array, and found that low dose ethanol (1.0 g/kg) decreased interleukin (IL)-10 levels, but high dose ethanol increased IL-10 levels (4.0 g/kg). We also used standard cell-attached mode electrophysiological techniques to evaluate the effects of select cytokines on VTA neuron firing rate *in vitro*. We found no change in firing rate in response to IL-6, but an increase in firing rate in VTA DA neurons response to IL-10. Consistent with the changes in firing rate, optically-evoked IPSCs were also found to be decreased in response to IL-10. *Ex vivo* voltammetry and *in vivo* microdialysis were done to determine whether IL-10 can directly result in an increase in DA release. Although *ex vivo* voltammetry showed no change in DA release, IL-10 increased DA release *in vivo*. These findings suggest that the rewarding and/or addictive effects of ethanol are mediated by cytokines, specifically the anti-inflammatory cytokine IL-10.

Keywords: GABA, dopamine, alcohol, cytokine, microglia, neuroimmune

ACKNOWLEDGEMENTS

I would like to thank my advisor, Scott Steffensen, for his mentorship and encouragement throughout the design and implementation of this project. I would also like to thank the members of my committee, Sterling Sudweeks and Sandra Hope, for their support and advice throughout my project. I would also like to thank all of our lab members for creating a productive and supportive work environment, and particularly recognize Daniel Obray, Travis Clark, Mark Woodbury, and Jordan Yorgason for their assistance with the experiments and techniques important to this study. I would also like to thank my parents for their continual support throughout my entire education and in all other aspects of my life. Finally, I would like to thank my husband, Landry, for supporting me and being the greatest motivation.

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INTRODUCTION

The Negative Consequences of Alcohol Use Disorders

Alcohol can cause serious health problems when used in excess, such as liver damage, heart problems, impaired cognitive function, increased risk of cancer, and weakened immune system. Alcoholism is the fourth leading preventable cause of death in the United States (Stahre et al., 2014). However, the social and economic consequences of alcohol use disorders can be even more harmful than the health problems, and for many people, an alcohol addiction requires outside intervention to overcome. There are three treatments currently approved by the FDA to treat alcohol use disorder (AUD): 1) Disulfiram (Antabuse), a desensitizing agent; 2) Naltrexone (Vivitrol), a mu-opioid receptor antagonist that blocks the euphoric effects of ethanol and other drugs; and 3) Acamprosate (Campral), which reduces withdrawal severity. However, 50% of subjects treated with these drugs relapse. Thus, better treatments for AUD based on an understanding of the underlying neural circuitry involved are being sought. Additional information about the neurophysiology of alcohol addiction is needed to develop better treatments to help ease the burden of alcohol use disorders.

The Mesolimbic Dopamine System

The mesolimbic dopamine (DA) system originates in a part of the midbrain called the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc) area of the striatum. This system is involved in motivation and the reinforcement of behaviors (Wise, 2008). Current dogma maintains that addiction is based on the dysregulation of the mesolimbic DA system. Ventral tegmental area DA neurons are regulated by inhibitory γ-aminobutyric acid (GABA) neurons found locally in the VTA and in other brain areas. Alcohol alters GABAergic transmission onto DA neurons, providing evidence that they play a critical role in the rewarding

properties of drugs (Gallegos et al., 1999, Theile et al., 2011). The prevailing dogma regarding ethanol effects in the brain is that synaptic transmission is depressed by acute intoxicating doses of ethanol (Berry and Pentreath, 1980, Bloom et al., 1984, Siggins et al., 1987, Deitrich et al., 1989, Shefner, 1990, Ariwodola et al., 2003), which might result from either an attenuation of excitatory glutamatergic (GLUergic) synaptic transmission (Lovinger et al., 1989, 1990, White et al., 1990, Nie et al., 1993, Roberto et al., 2004) and/or an enhancement of inhibitory GABAergic synaptic transmission (Deitrich et al., 1989, Harris and Allan, 1989, Roberto et al., 2003).

Neuroimmune Effects on the Mesolimbic Dopamine System

Neuroimmune interactions with ethanol may underlie some of the intoxicating, rewarding, and neuroadaptive effects of ethanol. The brain has a specific type of glial cell called microglia that function similarly to macrophages. Microglia are present in the healthy brain from the time of development, however, microglia are often magnified in number following monocyte migration into the adult brain when the blood-brain-barrier is disrupted in various disease states (Ginhoux et al., 2013). Microglia can also affect neurons through the release of signaling molecules called cytokines to influence neuronal health, development, and synapses. There is evidence of increased microglia expression in the VTA, NAc, and other brain areas associated with alcoholism in human post-mortem brains (He and Crews, 2008). The expression of neuroimmune related genes in VTA DA neurons was altered in mice exposed to chronic alcohol consumption (Marballi et al., 2016). Mice administered lipopolysaccharide (LPS), a bacterial signal that triggers immune system activation, show decreased firing in VTA dopamine neurons (Blednov et al., 2011) and increased DA release in the nucleus accumbens in vivo (Borowski et al., 1998). These findings suggest that neuroimmune activation alters the mesolimbic DA system and could contribute to the pathology of addiction.

Proposed Model

The premise of this project is based on the literature demonstrating that ethanol enhances DA release and that the effects of ethanol on DA transmission are mediated by neuroimmune interactions with VTA GABA neurons to modulate DA release. Figure 1 provides a theoretical model explaining how microglia may interact with the mesolimbic DA system to contribute to the rewarding actions of DA during ethanol intoxication. We propose that exposure to alcohol causes monocyte migration across the blood-brain-barrier (BBB) to activate microglia. Microglia then release select cytokines, which bind to receptors on GABA neurons to reduce inhibition onto DA neurons, which would lead to an increase in DA release that would be expected during ethanol intoxication. Although GLU NMDA receptor-mediated plasticity is clearly an harbinger of addiction, modification of GABAergic synaptic transmission is still regarded as one of the main factors underlying drug withdrawal-related phenomena (Bonci and Williams, 1996, 1997).

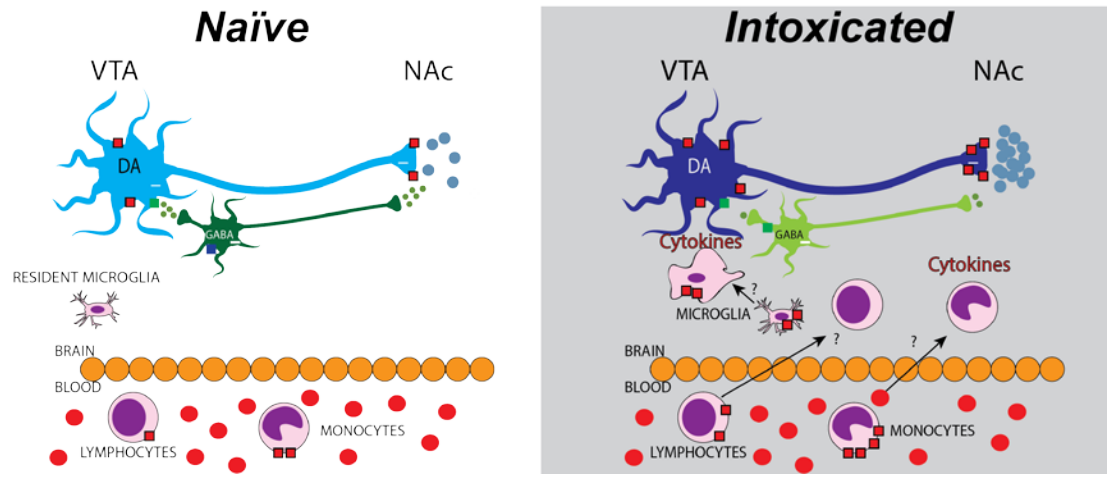

Figure 1 Theoretical Framework for Proposed Studies.

In the ethanol naive condition, DA neurons in the VTA project to the NAc via the medial forebrain bundle. The DA neurons are inhibited locally by VTA GABA neurons (Xia et al., 2011) as well as other GABA terminals projecting from other brain regions. In the ethanol intoxicated condition, the model demonstrates the potential interaction between peripheral monocytes, brain microglia and neurons. Cytokines are released from activated microglia and act on corresponding receptors in the VTA neurons to inhibit VTA GABA neurons and disinhibit DA neurons with subsequent enhancement of DA release (darker shading indicates excitation while lighter shading indicates inhibition relative to the Naïve condition). This signals ethanol intoxication and reward.

RATIONALE AND HYPOTHESES

The core hypotheses of this project is that ethanol's enhancement of DA release results from microglia-mediated cytokine disinhibition of VTA DA neurons via decreased GABAergic transmission. VTA GABA neurons are a relevant target for ethanol in the brain (Steffensen et al., 2009, Steffensen et al., 2011). The neuroadaptations of VTA GABA and DA neurons may underlie the behaviors seen in alcohol use disorders. As mentioned above, the dogma is that ethanol enhancement of DA release in the NAc underlies its rewarding effects. However, DA neurons are relatively insensitive to ethanol, and ethanol actually inhibits DA release at terminals in the NAc. To date, only a few studies have evaluated the effects of cytokines on VTA DA neurons, and none has addressed cytokine effects on VTA GABA neurons. VTA GABA neurons exhibit much more spontaneous and spike-related GABA inhibitory input than DA neurons (2-3X). Ethanol enhances GABA input to VTA GABA neurons, but not DA neurons. Although no studies have tested the effects of cytokines on VTA neurons, the anti-inflammatory cytokine, interleukin (IL)-10 has been shown to reduce GABA inhibition in the hippocampus (Suryanarayanan et al., 2016). Thus, IL-10 is a likely mediator of ethanol's effects on GABA synaptic transmission in VTA neurons. This study addresses the acute effects of ethanol, but will provide the impetus for studying the role of neuroimmune interactions in animals exposed chronically to ethanol in future studies.

Hypothesis 1: Acute Intoxicating Doses of Ethanol Enhance the Release of Cytokines in the VTA.

Hypothesis 2: Cytokines Will Modulate the Excitability of VTA Neurons *ex vivo*.

Hypothesis 3: Acute Ethanol Inhibition of NMDA Receptor-Mediated GLU Synaptic excitation and/or GABA Synaptic Inhibition of VTA GABA Neurons is Mediated via Cytokines.

METHODS

Animal Subjects

Male C57BL6 (black) mice, VGAT-ChR2-EYFP mice, and glutamate-decarboxylase-67 (GAD-67)-green fluorescent protein knock-in on a CD-1 (white albino) mice (Tamamaki et al., 2003) will be bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals will be treated in strict accordance with the Brigham Young University Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. Once weaned at PND 21, all mice will be housed in maximum groups of five and given ad libitum access to solid food and water and placed on a reverse light/dark cycle with lights ON from 10 PM to 10 AM.

Preparation of Brain Slices

All brain slice preparations will be performed in P28-60 day old C57BL/6-ChR2-VGAT and GAD-GFP knock-in mice. Brains will be extracted via anesthetization with isoflurane (5%). Upon extraction, the brain will be glued onto a cutting stage. The brain will then be sectioned in artificial cerebral spinal fluid (ACSF; in mM: 126 NaCl, 11.1 Glucose, 2.5 KCl, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 1.2 $MgCl_2$, 2.4 $CaCl_2$) perfused with 95% O_2 / 5% CO_2 . Targeting the VTA, horizontal slices (220μM thick) will be placed in an incubation chamber containing ACSF perfused with 95% O_2 / 5% CO_2 for at least 30 minutes. After 30 minutes, brain slices will then be placed in a recording tissue chamber with ACSF continuously flowing at physiological temperatures (35 °C). Cytokines were reconstituted and frozen in aliquots until ready to use: IL-10 (20ng/ml, Peprotech), IL-1B (20ng/ml, Peprotech).

Cytokine Determination

Mice were anesthetized with isoflurane (5%) and then injected IP with saline or ethanol (1g/kg or 4g/kg). After 1 hour, brains were extracted and tissue punches containing the VTA and NAc were excised from 1 mm thick brain slices, flash frozen in liquid nitrogen, and then stored at -80ºC. Tissue was then homogenized by manual grinding and mixing in 50uL of RIPA buffer (Abcam) with a protease inhibitor cocktail (Sigma Aldrich) to help preserve cytokines. Cytokine levels were measured using the BD Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences). Samples were run on a CytoFlex (Beckman Coulter) flow cytometer and then analyzed using FlowJo.

Cell-attached, Voltage-clamp Recording of Spike Activity in Brain Slices

Cell-attached studies will use electrodes pulled from borosilicate glass capillaries (2.5-6MΩ) and then filled with an ACSF solution containing (in mM): 126 NaCl, 11.1 Glucose, 2.5 KCl, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 1.2 $MgCl_2$, 2.4 $CaCl_2$. Positive pressure will be applied to the electrode when approaching the neuron. By applying suction to the electrode, a seal (10MΩ – 1GΩ) is created between the cell membrane and the recording pipette. Spontaneous spike activity is then recorded in voltage-clamp mode with an Axon Instruments Multiclamp 700B amplifier and sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. A stable baseline recording of firing activity will be obtained for 5-10 min before adding any substances.

Whole-cell Recordings

Electrodes will be pulled from borosilicate glass capillary tubes and filled with a potassium-gluconate pipette solution [in mM: 123 K-gluconate, 0.2 EGTA, 10 HEPES, 8 NaCl, 2 Mg-ATP, 2 Na3-GTP] for oIPSC studies. Pipettes having tip resistances of 2.5 - 5MΩ, and series

resistances typically ranging from 7 to 15 MΩ will be used. Voltage clamp recordings are filtered at 2 kHz with an Axon Instruments Multiclamp 700B amplifier and digitized at 5 to 20 kHz respectively using an Axon 1440A digitizer. Axon Instruments pClamp ver10, Mini Analysis (Synaptsoft: Decatur, GA), and Igor Pro (Wavemetrics: Oswego, OR) software packages will be utilized for data collection and analysis. oIPSCs were evoked using a 470nm blue light. Using a paired-pulse stimulus, the stimulation will be adjusted to a half-max level in order to allow measurements of changes that increase or decrease oIPSC levels.

Ex Vivo Voltammetry

Slices were prepared as previously described, transferred to the recording chamber, and perfused with aCSF (34°C) at a rate of ~1.8 ml/min. Fast scan cyclic voltammetry recordings were performed and analyzed using Demon Voltammetry and Analysis software (Yorgason et al., 2011a) (RRID:SCR_014468). Carbon fiber electrodes used in voltammetry experiments were made in-house. The carbon fiber (7 μm diameter, Thornel T-650, Cytec) was aspirated into a borosilicate glass capillary tube (TW150, World Precision Instruments). Electrodes were then pulled on a P-87 Horizontal pipette puller (Sutter Instruments) and cut so that 100–150 μm of carbon fiber protruded from the tip of the glass. The electrode potential was linearly scanned as a triangular waveform from −0.4 to 1.2 V and back to −0.4 V (Ag vs AgCl) with a scan rate of 400 V/s (Yorgason et al., 2011a). Before inserting the carbon fiber electrode into the brain slice, the voltage ramp was applied every 16 ms (60 Hz) for a 5–10 min period. After this electrode conditioning period, the scanning interval was changed to 100 ms (10 Hz). If electrodes had low electrical noise (median SD < 0.07 nA averaged across six 1 s bins), then they were used for experiments. Carbon fibers were advanced completely into the tissue at a 20° angle with the tip positioned ~85 μm below the slice surface. Dopamine release was evoked through electrical

stimulation (1 pulse/min) via a glass micropipette (30 μA, monophasic^{+}, 0.5 ms). Paired pulse stimulations were performed at 0.5, 1, 2, 4, 8, 12, and 16 Hz.

Microdialysis and HPLC

Microdialysis probes (MD-2200, BASI; CMA 11, Harvard Apparatus) were inserted into the NAc through the guide cannula. Artificial cerebrospinal fluid (aCSF) containing 150 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, and 0.8 mM $MgCl_2$ in 10 mM phosphate buffer was perfused through the probe at a rate of 1.5 μl/min. Samples were collected every 20 minutes for 5 hours with IL-10 (60ng/uL, ICV) or vehicle (equivolumic, ICV) injections occurring after the first two hours had elapsed. Determination of the DA concentration in microdialysis samples was performed using a HPLC pump (Ultimate 3000, Dionex, Sunnyvale, CA, USA) connected to a coulometric detector (Coulochem III, ESA). The coulometric detector included a guard cell (5020, ESA) set at 400 mV, a screen electrode (5014B, ESA) set at -100 mV, and a detection electrodef (5014B, ESA) set at 350 mV. DA was separated using a C18 reverse phase column (HR-80, Thermo Fisher Scientific, Waltham, MA, USA). Mobile phase containing 75 mM H_2NaO_4P, 1.7 mM sodium octane sulfonate, 25 μM EDTA, 0.714 mM trimethylamine, and 10% acetonitrile was pumped through the system at a flow rate of 1.0 ml/min. DA levels following drug administration were expressed as percentage of baseline, with the baseline being determined by the average DA concentration of a minimum of 3 consecutive stable collections (collections with less than 10% variation) occurring prior to drug administration.

Statistical Analyses

All results are presented as raw mean values and percent control ± SEM. Results before and after drug exposure were compared using a two-tailed paired *t*-test. Experiments relying on variance in time or current were analyzed using an ANOVA with post hoc t-test at individual

points. Statistical significance required ≥ 95% level of confidence ($P \leq 0.05$). Analysis software includes Microsoft Excel and Igor Pro (Wavemetrics, Oswego, OR). Significance levels are indicated on graphs with asterisks *,**,*** and correspond to significance levels $P<0.05$, 0.01 and 0.001, respectively. Figures were constructed with Igor Pro software.

RESULTS

Ethanol Enhances the Release of Anti-Inflammatory Cytokines in the VTA

We evaluated cytokine release in the VTA and NAc *in vivo* following an acute injection of ethanol at two different dose levels: 1.0 g/kg and 4.0g/kg, compared to saline controls at 1 hour post injection. We evaluated IL-6, IL-10, MCP-1, IFN-Y, TNF, and IL-12p70 in brain tissue punches from the VTA and NAc using a Cytometric Bead Array Mouse

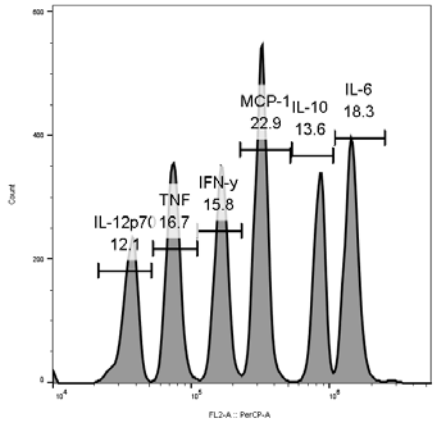

Figure 2 Flow Cytometry Bead Detection. Detection bead fluorescence intensity for the 6 different cytokine beads.

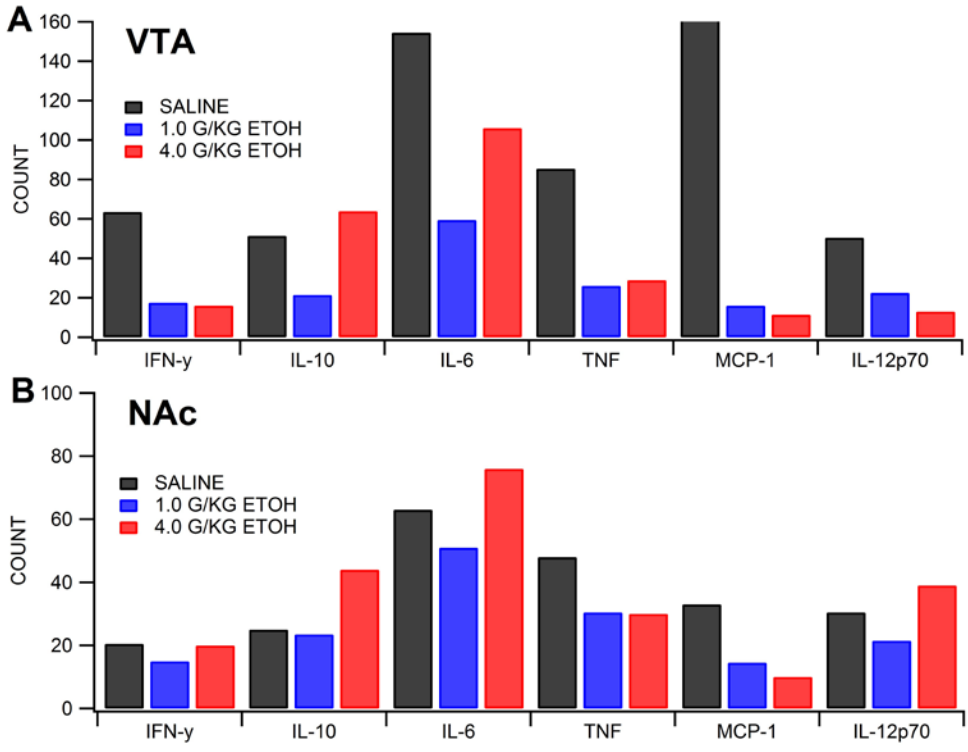

Figure 3 Effects of Ethanol on Cytokine Levels in the VTA and NAc.
(A) Ethanol decreased cytokine positive bead count for beads measuring IFN-y, IL-6, TNF, MCP-1, and IL-12p70. IL-10 levels were decreased at 1.0 g/kg, but not 4.0 g/kg. (B) Ethanol decreased the count of cytokine positive beads for TNF and MCP-1. IL-6, IFN-y, IL-10, and IL-12p70 showed mixed results with no clear inhibitory or enhancing effect.

Inflammation Kit. Cytokine levels were quite low in all samples except the positive control standards. Figure 2 shows the results from one sample with all of the beads detected. Ethanol

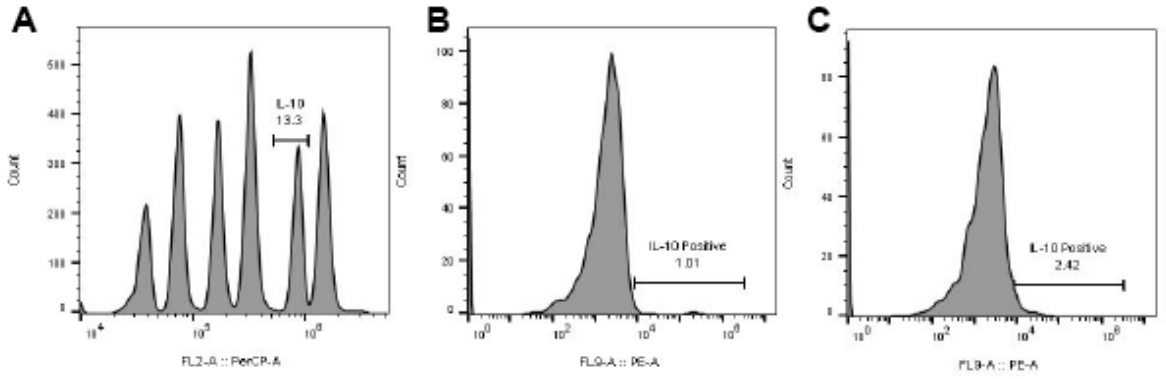

Figure 4 Fluorescence Intensity Histograms for IL-10
(A) Detection bead fluorescence intensity, indicating the peak for IL-10. (B,C) IL-10 fluorescence bead intensity for a saline VTA sample (A) and an ethanol (4.0 g/kg) VTA sample (C). Cytokine fluorescence from all samples was very low.

decreased the expression of IFN-y, IL-6, TNF, MCP-1, and IL-12p70 in the VTA (Figure 3A). IFN-y, TNF, and IL-12 are all considered to be proinflammatory cytokines, and are involved in the innate immune response. IL-6 is also considered a proinflammatory cytokine that is of particular interest in the brain due to its involvement with brain injury and disease (Erta et al., 2012). MCP-1 is a chemokine involved in the recruitment of monocytes to sites of inflammation. In the NAc results were less pronounced, as ethanol had mixed results on IFN-y, IL-6, and IL-12p70. Ethanol did decrease MCP-1 and TNF in the NAc.

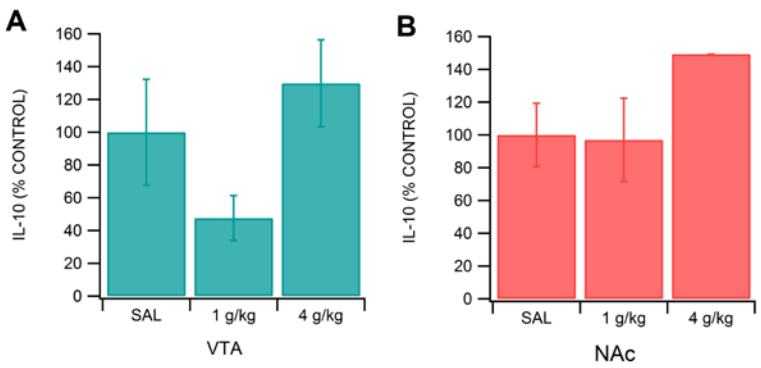

Figure 5 Effects of Acute Ethanol on IL-10 levels in the VTA and NAc.
(A) IL-10 in the VTA appears to be inhibited at 1.0g/kg but increased at 4.0 g/kg. (B) IL-10 in the NAc appears to be increased at 4.0 g/kg.

Interestingly, IL-10 levels in the VTA were increased following an ethanol exposure (Figure 4C) compared to the saline control (Figure 4B). IL-10 levels appear to be increased following high dose ethanol (4.0 g/kg, i.p.) in both the VTA (Figure 5A) and the NAc (Figure 5B), however this is not significant with the current sample size of 2. Additionally, IL-10 levels appear to be decreased following low dose ethanol (1.0 g/kg, i.p.) in the VTA (Figure 5A), but again, results were not significant given the low sample size. These results indicate a trend in IL-10 levels by ethanol dose, and suggest that IL-10 may be an important cytokine in explaining ethanol's modulation of the mesolimbic dopamine system.

IL-10, but Not IL-6, Modulates the Firing Rate of VTA Neurons

We tested the effects of select cytokines (IL-6 and IL-10) on VTA DA and GABA neuron firing rates in cell-attached mode, which is a stable recording technique that does not alter the intracellular milieu. We found that the anti-inflammatory cytokine, IL-10, increased the firing rate of VTA DA neurons by 66.45 ±

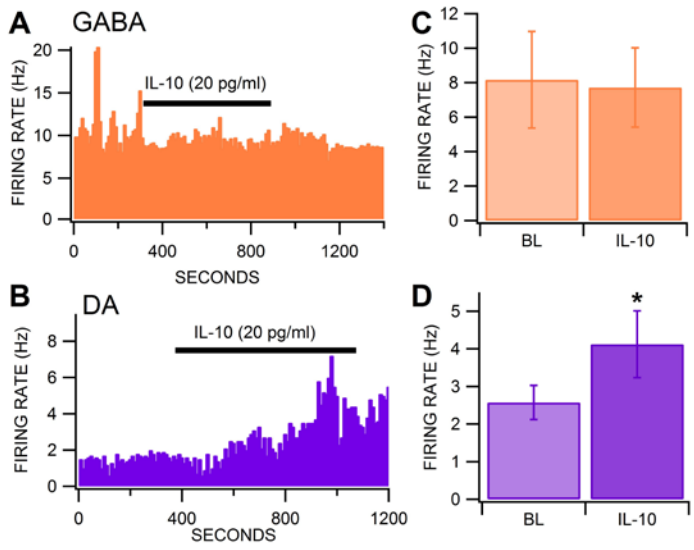

Figure 6 Effects of IL-10 on VTA Neuron Firing Rate.
(A) Representative trace of the effects of IL-10 on the firing rate of a GABA neuron. This neuron had an average firing rate of 10Hz. (B) Representative trace of the effects of IL-10 on the firing rate of a DA neuron. This neuron had an average firing rate of 1.5Hz and increased in firing frequency with IL-10. (C) IL-10 increased DA neuron firing rate but not GABA neuron firing rate.

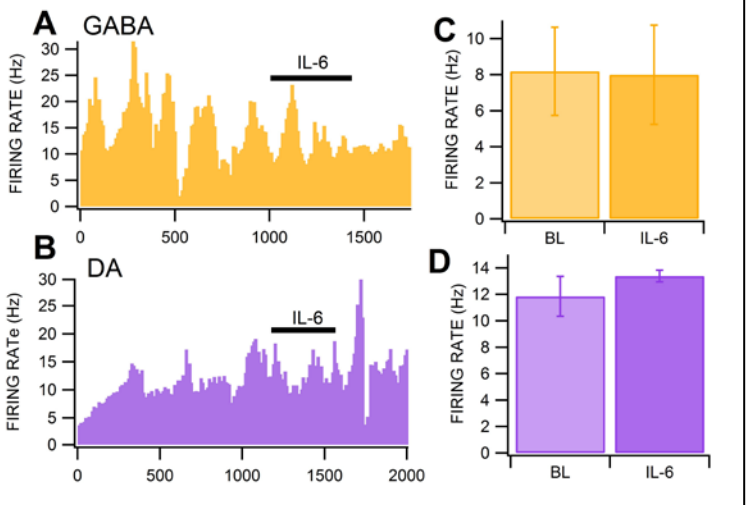

Figure 7 Effects of IL-6 on VTA Neuron Firing Rate.
(A) Representative trace of the effects of IL-6 on the firing rate of a GABA neuron. (B) Representative trace of the effects of IL-6 on the firing rate of a DA neuron. (C) IL-6 had no effect on VTA GABA or DA neuron firing rate.

23.40% (Figure 6D**,** n=13, p=0.0204, by paired t-test), but had no effect on the firing rate of VTA GABA neurons 0.63 ± 5.32% (Figure 6C, n=11, p=0.4476). However, IL-6, a pro-inflammatory cytokine, did not have any effect on firing rate of VTA GABA or DA neurons (Figure 7C, GABA: 1.17 ± 11.24%, n=5, p=0.9061; Figure 7D, DA: 113.98 ± 9.48% n=4, p=0.3349). These results suggest that IL-10 is modulating the firing rate of VTA DA neurons by an alternative mechanism than by changing the firing rate of VTA GABA neurons. Because the IL-6 recordings shows no significant change in firing rate, it suggests that the effect of increasing firing rate is specific to IL-10 or perhaps it is an effect of anti-inflammatory cytokines.

Effects of IL-10 on Optogenetically-Evoked IPSCs on VTA DA Neurons

Based on the findings that IL-10 increased DA neuron firing rate, we tested the effects of IL-10 on optogenetically evoked IPSCs (oIPSC) on DA neurons. Previous studies have shown that IL-10 inhibits GABAergic synaptic inhibition in the hippocampus (Suryanarayanan et al., 2016), suggesting that IL-10 may be acting through a similar mechanism to increase VTA DA neuron firing rate. We used a channelrhodopsin-2 (ChR2) vesicular GABA transporter (VGAT) mice to activate GABA neurons and then recorded from DA neurons to study the effects of IL-10 on GABA transmission on DA neurons. We found that IL-10 decreased the amplitude of the

oIPSC on DA neurons by 21.69 ± 6.31 % (Figure 8, n=10, p=0.0397). This suggests that IL-10 may contribute to the disinhibition of DA neurons by attenuating GABAergic synaptic transmission on VTA DA neurons.

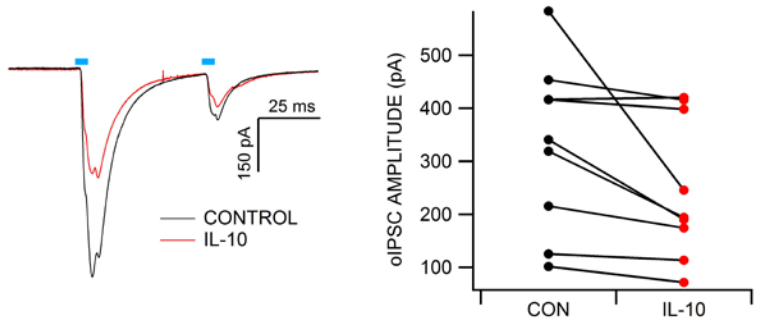

Figure 8 Effects of IL-10 on Optogenetic IPSCs on VTA DA Neurons.
(A) Representative trace of the effects of IL-10 on the optogenetically-evoked IPSC in a VTA DA neuron. This neuron had an IPSC that was decreased from 340pA to 190pA during IL-10 perfusion. (B) IL-10 decreased the amplitude of oIPSCs in 9 out of 10 neurons.

Effects of IL-10 on DA Release

Based on previous evidence of increased DA neuron firing and decreased GABAergic synaptic inhibition, the next question was whether DA release in the nucleus accumbens occurs in response to IL-10. Therefore, we tested the effects of IL-10 on *ex vivo* evoked DA release. A paired-pulse protocol of evoked DA release was used to determine if the interstimulus interval combined with IL-10 had an effect on DA release (Figure 9A). From this data, a subtraction was performed (Figure 9B) to determine whether IL-10 had an effect on evoked DA release. In slices

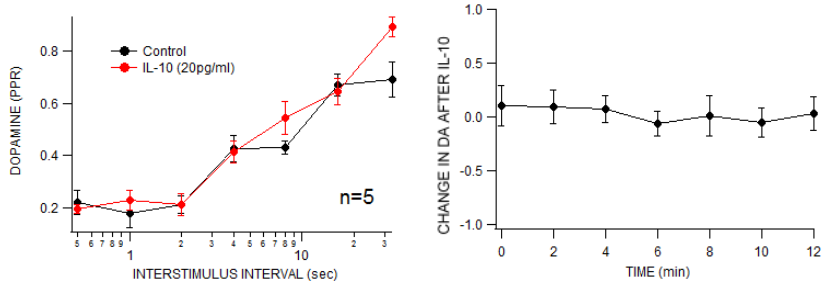

Figure 9 Effects of IL-10 on Evoked DA Release in the NAc Ex Vivo Using Fast Scan Cyclic Voltammetry.
(A) A paired-pulse ratio protocol was tested and then repeated with the superfusion of IL-10. There was no significant difference between the control and IL-10 at any of the stimulation intervals. (B) There was no significant change in DA after IL-10 application as a subtraction from IL-10 from baseline.

containing NAc terminals, without connections to the cell bodies in the VTA, IL-10 had no effect on evoked DA release. Additionally, there was no interstimulus interval where a significant difference was seen between control and IL-10. This suggests that IL-10 has no effect on DA neuron terminals in the NAc. Dopamine release was also measured *in vivo* by microdialysis, following an intracerebroventricular injection of IL-10 (60ng/uL/mouse). IL-10 caused a 60.31 ± 2.14% increase in DA release in the NAc at 40 minutes following IL-10 administration (Figure 10, n=5, Significant at 20, 40 and 60 minutes (20: $t_{(4)}$= 3.0730, p=0.0372, 40: $t_{(4)}$= 4.5435, p=0.0200, 60: $t_{(4)}$= 3.1051, p=0.0360) all other points not significantly different from baseline (p>0.05)). This suggests that the change in firing observed *ex vivo* due to IL-10 contributes to increased DA release in the NAc observed *in vivo.*

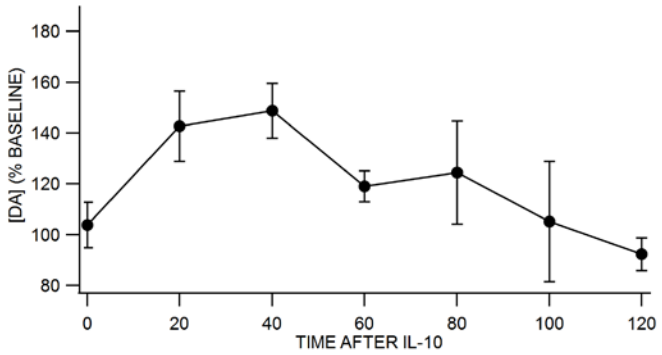

Figure 10 Effects of IL-10 on In Vivo DA Release.
(A) DA release in the NAc was measured by microdialysis. IL-10 (60ng/uL/mouse ICV) increased DA release in the NAc.

DISCUSSION

The purpose of this study was to investigate the effects of cytokines on VTA neurons to identify the potential role of cytokines in alcohol addiction. We began with the intention of investigating several different cytokines, however, we quickly focused on IL-10. Although cytokines were detected at very low levels, IL-10 appears to be elevated in the VTA following ethanol exposure (4.0 g/kg). The pro-inflammatory cytokines (IFN-γ, IL-6, TNF, MCP-1, IL-12p70), that were measured appears to be decreased following ethanol exposure (1.0 g/kg and 4.0 g/kg). IL-10 modulates the firing of VTA DA neurons by decreasing GABAergic synaptic inhibition onto VTA DA neurons. However, IL-6 had no effect on GABA or DA neuron firing rate. Preliminary data also suggests that IL-10 increases DA release in the NAc *in vivo*, although increased release is not seen in *ex vivo* slice preparations. Thus, IL-10 may mediate some of the reinforcing effects of alcohol.

Three hypotheses were tested in this study related to the interaction between cytokines and alcohol neural circuitry. The first hypothesis was that acute intoxicating doses of ethanol would enhance the release of cytokines in the VTA. We found that IL-10 levels were elevated in the VTA following a 4.0 g/kg ethanol exposure, which supports the hypothesis. However, most of the cytokines measured decreased in the VTA, which modifies the hypothesis to clarify that anti-inflammatory, but not pro-inflammatory cytokines increase following a 4.0 g/kg ethanol exposure. The second hypothesis was that cytokines would modulate the excitability of VTA neurons *ex vivo*, which appears to be true for IL-10 but not IL-6. IL-10 modulated the excitability of VTA DA neurons. Further studies could investigate whether any other cytokines (particularly anti-inflammatory cytokines modulate VTA neuron excitability). The third hypothesis was that acute ethanol inhibition of GLU synaptic excitation or GABA synaptic inhibition of VTA GABA

neurons is mediated by cytokines. This hypothesis depends heavily on finding a change in excitation as measured in hypothesis 3, but since the change observed was on VTA DA rather than GABA excitability, we chose to test the effects of IL-10 on VTA DA neuron GABA synaptic transmission and found that IL-10 inhibits GABAergic synaptic transmission. However, we did not test whether GLUergic synaptic transmission is modified by IL-10, but we hope to look at GLUergic synaptic transmission in future studies.

Ethanol Effects on Cytokines

Cytokines have clearly been shown to be involved in the brain's ethanol response. Ten daily doses of ethanol (5g/kg, i.g.), potentiated LPS-induced production of pro-inflammatory cytokines (TNFα, IL-1β and MCP-1) in the brain (Qin et al., 2008). The same study reported that IL-10 levels were decreased in the brain one week following 10 ethanol doses. Chronic ethanol exposure also decreased IL-10 levels in the basolateral amygdala, but not in the central amygdala (Marshall et al., 2017). However, *in vitro* ethanol exposure increased the IL-10 production by human monocytes (Mandrekar et al., 1996). More recently, IL-10 was found to be elevated in the hippocampus one hour following acute ethanol gavage (5 g/kg) (Suryanarayanan et al., 2016). These studies suggest that alcohol affects the amount of IL-10 in the brain and that specific regions, time points, and ethanol doses may result in increases or decreases in IL-10 production.

Our results did not show any significant difference in cytokine levels in the VTA or NAc. However, we do see a trend of increasing IL-10 at 4.0 g/kg in both the VTA and NAc that could be significant with a larger sample size. This is somewhat consistent with findings of other labs following acute ethanol exposure in other brain areas (Mandrekar et al., 1996, Suryanarayanan et al., 2016). Conversely, a low dose of ethanol actually decreased IL-10 levels in the VTA. This is similar to the results obtained from chronic ethanol exposure in other labs, which suggests that

perhaps that there is a similar mechanism between chronic ethanol exposure and low dose ethanol exposure that both decrease IL-10 levels. Another study reported that IL-10 levels were elevated after 12 days of alcohol withdrawal (Schunck et al., 2015), which may indicate that extended withdrawal causes a rebound in IL-10 after the initial decline. In the future, we hope to repeat this study to increase the sample size based on the promising trend in IL-10 levels.

In addition, we observed general decreases in all of the pro-inflammatory cytokines measured (IFN-γ, IL-6, TNF, MCP-1, IL-12p70). A decrease in pro-inflammatory cytokines is consistent with an increase in the anti-inflammatory cytokine, IL-10. One of the effects of IL-10 is to decrease the production of pro-inflammatory cytokines, which may explain some of the decrease. However, IL-10 also decreases one hour following a 1.0g/kg ethanol exposure, suggesting that perhaps ethanol inhibits cytokine production more generally at low doses. Although we observed a decrease in many of the pro-inflammatory cytokines in the VTA, they may affect synaptic transmission and be involved in the response to ethanol. Pro-inflammatory cytokines could be more important in a chronic ethanol exposure paradigm, or during withdrawal from ethanol.

We had a hard time trying to create a protocol for measuring cytokines in specific brain areas using flow cytometry. The first time we performed the cytokine assay we did not detect cytokines in anything other than the positive control. We suspected that our lack of detection was due to inadequate tissue homogenization. The second time we tried the cytokine assay, we added more steps to prepare and homogenize the tissue, including flash freezing the tissue in liquid nitrogen immediately following extraction and adding protease inhibitors in a cell lysis buffer to the tissue. This yielded low levels of detectable cytokine, which could be due to low cytokine

levels in the brain, although in the future we hope to increase detection levels by further refinement of the protocol.

Cytokine Effects on Synaptic Transmission

Many cytokines have been shown to modulate synaptic transmission in various regions of the brain. IL-1 receptor (IL-1R) activation can potentially reduce GABA neuron excitability through a number of mechanisms. First, IL-1R activity has been shown to reduce phosphorylation of the AMPA receptor, resulting in AMPA receptor internalization (Lai et al., 2006). Second, IL-1R activation can also reduce voltage-gated Ca2+ channel activity (Plata-Salaman and Ffrench-Mullen, 1992, Campbell and Lynch, 1998). Additionally, IL-1β affects the sensitivity of CB1 receptors in the striatum that control both GABA and glutamate transmission (De Chiara et al., 2013). Similarly, IL-1R has also been shown to reduce GABA inhibition onto Purkinje neurons in the cerebellum (Pringle et al., 1996). The IL-1R also seems to be involved in the development of LTP, suggesting that it can modulate glutamatergic synapses (del Rey et al., 2013). Currently, we have not tested the effects of IL-1R activation on VTA neuron firing rate, but it is a good avenue for future studies.

Additional evidence suggests that IL-6, a pro-inflammatory cytokine, may affect synaptic transmission. IL-6 has been shown to enhance excitatory postsynaptic potentials in the hippocampus (Nelson et al., 2012). Additionally, overexpression of IL-6 alters the effects of acute ethanol on field excitatory postsynaptic potentials and LTP (Hernandez et al., 2016). We tested the effects of IL-6 on firing rate. No difference was observed on the firing rate of DA or GABA neurons, although these results are not entirely conclusive due to the low sampling of neurons and the abnormally high firing rate of the putative DA neurons. However, because no difference was seen, we did not perform any further experiments with IL-6.

Alternatively, less is known about the role of the opposing anti-inflammatory cytokine IL-10 in the brain, particularly in regards to how it may be involved in the response to ethanol. A polymorphism in the IL-10 gene is associated with alcoholism, suggesting that IL-10 may play a role in the neural plasticity associated with alcoholism (Marcos et al., 2008). Recent evidence has shown that IL-10 inhibits mini IPSCs in the hippocampus (Suryanarayanan et al., 2016). IL-1β inhibits LTP induction, and this effect is prevented by IL-10 (Kelly et al., 2001). We observed that IL-10 increased the excitability of VTA DA, but not GABA neurons. The increase in firing rate is consistent with the IL-10 decreasing oIPSC amplitude in VTA DA neurons. This suggests that decreased inhibition explains some, if not all, of the increased excitability of DA neurons. However, we do not know exactly how IL-10 is decreasing GABAergic inhibition.

IL-10 receptor (IL-10R) activation is mediated by the Jak/STAT pathway, and leads to decreased cytokine production and decreased inflammation (Murray, 2006). Currently the expression of IL-10 receptors in the brain is not well characterized. IL-10Rs have been shown to be expressed in the brain on neurons in the hippocampus, cortex, and spinal cord as well as microglia (Lim et al., 2013). It seems likely that IL-10Rs are expressed in the VTA as well, but whether they are expressed on GABA or DA neurons or both, remains to be elucidated. IL-10 effects on GABA synaptic transmission could be due to IL-10 effects on either presynaptic GABA terminals or DA neurons. Recent evidence has shown that IL-10 inhibits GABAergic synaptic transmission. For example, IL-10 inhibits both the frequency and amplitude of mini IPSCs in the hippocampus, at least partially due to the postsynaptic effects of PI3K on GABA(A)R expression (Suryanarayanan et al., 2016). IL-10 has also been shown to modulate GLUergic synaptic plasticity. IL-1β inhibits LTP induction, and this effect is prevented by IL-10 (Kelly et al., 2001).

IL-10 Increases DA Release

Increased VTA DA neuron firing logically results in increased DA release in the NAc. Many drugs of abuse act in the VTA to cause increases in DA release, such as opioids, nicotine, and alcohol (Deitrich et al., 1989, Balfour, 2009, Fields and Margolis, 2015). Alcohol is known to have effects on many different neurotransmitter receptors in many different brain regions (Crews et al., 1996, Olsen et al., 2014). Much of the rewarding properties of alcohol are not well understood, although the VTA is an important target. Our findings suggest that IL-10 (and perhaps other cytokines) may be mediating some of the rewarding and reinforcing properties of alcohol. DA release is thought to correlate to the reinforcement of behavior and conditioned preferences (Salamone and Correa, 2002, Wise, 2008). In this study, we have shown that IL-10 increases DA release, which is suggestive of reward. However, behavioral studies are necessary to determine whether IL-10 alone is rewarding. Other studies have shown behavioral effects of anti-inflammatory cytokines on ethanol drinking behaviors. For example, IL-10 in the basolateral amygdala decreased ethanol consumption, but not sucrose consumption or performance in an open-field test (Marshall et al., 2017). Interestingly, another study found that antagonizing the IL-1 receptor in the basolateral amygdala also decreased ethanol consumption (Marshall et al., 2016). These results suggest that anti-inflammatory cytokines play a role in ethanol related behaviors. Future studies will address the behavioral effects of IL-10 on the rewarding properties of ethanol.

Neuroimmune and Alcohol Interactions

The effects of ethanol on the brain are closely linked to neuroimmune interactions both systemically and through the effects of activated microglia in the brain. Minocycline, a microglia inhibitor, led to decreased alcohol drinking in mice (Agrawal et al., 2011), suggesting that microglia mediate the effects of alcohol in the brain. Additionally, chronic or intermittent exposure

to ethanol can lead to the sensitization, or partial activation, of microglia through the upregulation of toll like receptors, priming them for further activation (Alfonso-Loeches and Guerri, 2011). Alcohol can also activate microglia directly through Toll-like receptor 4 (Fernandez-Lizarbe et al., 2009, Bajo et al., 2016). Different types of microglial activation occur depending on the extent and duration of alcohol exposure. Some studies show an anti-inflammatory response after acute binge alcohol exposure lacking full microglial activation and BBB disruption, but a pro-inflammatory response after chronic alcohol exposure (Marshall et al., 2013, Zhao et al., 2013, Chastain and Sarkar, 2014), suggesting that different types of alcohol exposure lead to different microglial phenotypes and therefore different cytokine production. The activation of microglia, either as a result of monocyte invasion or from peripheral signaling molecules, or by the direct effects of ethanol on brain microglia seems to play an important role in explaining ethanol's rewarding effects on the brain.

Our results affirm the importance of the neuroimmune system in mediating alcohol's effects, and particularly implicate IL-10 as a key mediator in these effects. Future studies should investigate the effects of other cytokines, particularly anti-inflammatory cytokines in the mesolimbic dopamine system in order to fully understand these effects. Additionally, more pharmacological research is needed to identify whether cytokines affect glutamatergic synaptic transmission in the VTA. Further research about the mechanism of IL-10's modulation of DA neuron activity will clarify the role of IL-10 in the neural circuitry of alcohol use disorders, and could potentially lead to novel pharmacological and behavioral treatments for alcoholism.

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