BINGE ALCOHOL CONSUMPTION DOES NOT INDUCE IL-10 IMMUNE RESPONSE IN THE VENTRAL TEMENTAL AREA OF THE MOUSE BRAIN

By

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**Introduction**

Binge drinking is a pattern of behavior in which excessive amounts of alcohol are consumed in a short period of time. The National Institute on Alcohol Abuse and Alcoholism defines a binge episode as a less than 2-hour period of time when a person’s blood alcohol concentration (BAC) reaches at least 0.08 grams percent weight per volume. For a male, this corresponds to 5 drinks in a two-hour time period and 4 drinks for a female in the same time period (NIAAA, 2004). There are many negative health effects that stem from this sort of binge drinking behavior such as liver disease, high blood pressure, neurological damage, stroke, and other cardiovascular diseases. There are also many negative psychological effects associated with binge drinking including changes in mood/personality, impaired attention span, depression, anxiety, sleep pattern disturbances, and addiction (Perkins et al. 2002). Despite these negative consequences, the prevalence of adults who binge drink on a regular basis is alarmingly high. One in six US adults or over 38 million people binge drink at least 4 times a month (CDC, 2013). Studies have also shown dramatic increases in the prevalence of binge drinking behavior on college campuses (NIAAA, 2014). With such pressing pervasiveness, it is important to investigate the neurobiological mechanisms underlying the reinforcing behavior of binge alcohol consumption.

Binge drinking behavior has been shown to significantly increase the likelihood of developing alcohol addiction. Alcohol addiction is characterized by the compulsive need to obtain alcohol, the inability to control consumption, as well as the development of tolerance (Mello, 1973). Current alcohol research focuses on the factors that cause excessive drinking as well as the factors that lead to the development of dependence (Weiss and Porrino, 2002). Neuronal mechanisms that have reinforcing effects on reward seeking have been identified and studied to
try to understand why addiction develops and why addicts relapse. A number of studies have strongly implied a connection between dopamine systems and the incentivizing effects of drugs such as alcohol (Robinson and Berridge, 1993). Studies have demonstrated that addictive drugs increase dopamine neurotransmission by affecting the mesolimbic pathway (Di Chiara and Imperato, 1988). The mesolimbic pathway is a dopaminergic pathway that connects the ventral tegmental area (VTA) with the nucleus accumbens region and neurotransmission in this pathway facilitates reinforcement of rewarding stimuli and incentive salience. Furthermore, dysregulation of this reward pathway has been implicated with the development and maintenance of addiction (Robinson and Nestler, 2012). Studies have shown that low doses of ethanol activate and increase the firing of the high concentrations of dopamine containing neurons in the VTA (Brodie et al. 1990, Young et al. 1992). The sensitivity to low concentrations of ethanol shown by VTA dopaminergic neurons suggests that they are involved in the reinforcing effects of alcohol addiction (Gessa et al. 1985). While dysfunction within the mesolimbic pathway as a factor contributing to addiction is widely accepted, the underlying cause of this dysfunction is unclear.

One candidate mechanism is inflammation. Central immune signaling and the inflammatory response are critical for responding to tissue damage or disturbances of homeostasis in the brain, however, if left unresolved, excessive inflammation can become detrimental (Lobo-Silva et al. 2016). Innate immune cells of the CNS, namely microglia, are responsible for maintaining a healthy, neuroprotective amount of inflammation while preventing excessive neurodegenerative amounts of inflammation (Crott et al. 2016). Microglia maintain neuronal homeostasis and upon detection of a disturbance become activated into either cytotoxic M1 or neuroprotective M2
polarized cells (Arimoto et al. 2007). The M1 phenotype is characterized by the production of proinflammatory signals. Although crucial for fighting infections, many of the factors released by M1 microglia are damaging to neuronal cells (Fernandes et al. 2014). The M2 phenotype is characterized by the production of anti-inflammatory mediators and plays a part in tissue repair mechanisms and the cessation of inflammation. Imbalances in M1/M2 microglial activation, specifically M1 polarization, have been increasingly implicated in neurological and neurodegenerative diseases (Tang et al. 2016).

Drug-induced activation of central-immune signaling has been demonstrated to contribute to abusive drug behavior by enhancing the engagement of the mesolimbic reward pathway (Coller et al. 2012). Neuroimmunomodulators such as lipopolysaccharides (LPS) have been shown to lead to long-lasting activation of proinflammatory brain immune signaling as well as a prolonged increase in high ethanol preference (Blednov et al. 2011). This further suggests that neuroimmune signaling can promote ethanol ingestion and alter reward-seeking behavior. Cytokines are major mediators of the neuroimmune response and, therefore, may play a role in behavioral maladaptation within the alcohol reward/aversion system. One potential cytokine of interest present in this pathway is interleukin-10 (IL-10). IL-10 is an anti-inflammatory cytokine with wide ranging roles in immunoregulation and inflammatory responses (Moore et al. 2001). IL-10 is produced mainly by microglia and is one of the primary mechanisms by which immune cells normalize imbalances in microglial M1/M2 activation and counteract damage caused by excessive inflammation (Lobo-Silva et al. 2016). Specifically, IL-10 has also been demonstrated to limit CNS inflammation by reducing synthesis of proinflammatory cytokines, suppressing cytokine receptor expression, and inhibiting receptor activation (Strle et al. 2001; Ledeboer et al.
2002; Balasingam et al. 1996). IL-10 has also been shown to limit immune responses in the brain by inhibiting the expression of major histocompatibility complex class II (MHC-II), halting antigen presentation to T-cells (Lobo-Silva et al. 2016). Additionally, IL-10 gene polymorphisms have been associated with alcoholism. This finding further support the hypothesis that proinflammatory responses are linked to increased risk of alcohol dependence (Marcos et al. 2008). Interestingly, administration of IL-10 prior to a proinflammatory LPS injection has been shown to revert the binge behavioral effects of the LPS injection, demonstrating its potential as a therapeutic approach (Bluthe et al. 1999).

The “Drinking in the dark” (DID) method utilized in this study is a procedure that induces alcohol-preferring strains to consume enough ethanol to reach BAC’s greater than 100 mg/dl (Thiele and Navarro, 2014). This method is different than common involuntary ethanol consumption studies in that it produces pharmacologically relevant BAC’s in a time frame that can be set by the investigator. Furthermore, this pattern of heavy binge drinking more closely resembles the human behavior attempting to be modeled, giving the DID model more face validity than other alcohol disorder models. Another advantage of the DID model is that the mice are not forced to ingest the alcohol as they are in an involuntary ethanol consumption experiment. The DID method takes advantage of the increased ingestion behavior that corresponds with the animals’ dark cycle, bypassing the need to have physical interaction between the experimenter and the mouse (Thiele and Navarro, 2014). This interaction, which is necessary for involuntary administration of ethanol, introduces confounding variables into the study. This would be particularly troublesome because stress and anxiety caused by these interactions between experimenter and mouse can independently affect addictive behavior.
The goal of this study was to determine the effects of binge ethanol consumption on the levels of expression of IL-10 in the VTA of the mesolimbic pathway utilizing the DID protocol. In this study an immunohistochemical assessment is used to measure IL-10 expression in the VTA. A lack of an IL-10 anti-inflammatory immune response or a decrease in basal IL-10 levels in the VTA could be a possible factor intensifying the inflammation of the mesolimbic reward pathway that underlies the behavioral maladaptations that lead to alcoholism.

Methods

Animal Handling

Six to eight-week-old C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The average weight of each mouse upon arrival was between 20 – 25 grams. Prior to undergoing any procedures, the mice were given a week to become accustomed to their new environment. Mice were individually housed in an AALAC certified vivarium. The vivarium was kept at approximately 22°C on a 12-hour reversed light-dark cycle starting with lights off at 8:00am. During all experiments, mice had ad-libitum food and water access. All procedures used herein adhered to the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drinking in Dark

A 4-day DID procedure was used to model binge alcohol consumption. During the DID cycle, the water bottles were removed from the cages of the mice and replaced with bottles containing 20% (v/v) ethanol or 3% sucrose solution. Coinciding with the name of the procedure, all bottles were put on 3 hours into the dark cycle. Mice had access to the ethanol, sucrose, or water for 2
hours before the bottles were replaced with the homecage water bottles. On the fourth day of the procedure the mice have access to the ethanol for 4 hours instead of 2. Each 4 day DID procedure is referred to as a “cycle.” The mice underwent either 1 or 3 DID cycles, with 3 days of rest in between each. After the 4-hour testing period on the final test day, blood was collected from the mice using capillary tubes after a small incision was made on the tail. Blood samples were centrifuged and the serum was used to check the blood ethanol concentration (BAC) of each mouse using an alcohol analyzer (Analox Instruments, Lunenberg, MA) to make sure the mice were accurately modeling binge consumption behavior. For each mouse, BAC’s were run in duplicates and averaged.

Immunochemistry

Upon completion of the DID procedure, the mice were anesthetically overdosed with a 0.15 mL intraperitoneal injection of ketamine (66.67 mg/mL) and xylazine (6.67 mg/mL) dissolved in 0.9% saline. They were then transcardially perfused with 1.0 M Phosphate Buffered Saline (PBS, pH = 7.4), which was immediately followed by a perfusion of 4% paraformaldehyde diluted in PBS. Extracted brains were postfix in 4% paraformaldehyde for twenty-four hours. They were then sectioned coronally into 40 µm slices using a Leica VT 1000S vibratome (Leica Microsystems, Nussloch, Germany). The brain sections were placed in a 1-in-4 series so that every fourth slice was used in the analysis. The slices were stored in ethylene glycol, a cryoprotectant, at -20°C to preserve the tissue.

For immunohistochemical analysis, slices were put through three 5 minute washes of PBS (pH = 7.4) to rinse off the ethylene glycol. The brain slices were then soaked in 0.6% hydrogen
peroxide for 5 minutes to quench naturally occurring endogenous peroxidases. This was immediately followed by another 3 rinses of PBS for 5 minutes each. Next, the slices were put in Standard Sodium Citrate (SSC) for 1 hour for antigen retrieval. This was followed by another round of three 5-minute PBS washes. The slices were then blocked from nonspecific binding using 4% goat block for 30 minutes (Sigma-Aldrich, Raleigh, NC). This was followed by another 3 five-minute PBS washes. Sections were incubated in goat IL-10 primary antibody (1:1000; R&D Systems; Minneapolis, MN) for 48 hours at 4°C. Primary antibody was washed away using 3% rabbit serum (Sigma-Aldrich, Raleigh, NC). The sections were then incubated in biotinylated rabbit-anti goat secondary antibody, which was followed by avidin-biotin-peroxidase complex (ABC elite kit, Vector Labs; Burlingame, CA). The complex was detected with chromagen 3,3’-diaminobenzidine tetrahydrochloride (Polysciences; Warrington, PA). Processed sections were mounted onto glass slides and covered with SHUR/Mount™ coverslips (Triangle Biomedical Sciences; Durham, NC).

*Image Acquisition and Analysis*

An Axio Zoom.V16 microscope (Zeiss, Oberkochen, Germany) was used to capture high definition images of each slide at 100x magnification. Images were coded to ensure experimenter blindness during analyses. The Zen Pro image processing system (Zeiss, Oberkochen, Germany) was used to analyze the imaged brain sections. The image-processing suite digitally measures the immunopositive area in the region of interest on each brain slice. First, the region of interest was determined using the contour drawing tool to trace out the VTA region. Then a threshold was set to differentiate between immune-positive pixels and the background. The program then
determines the IL-10+ area and the total area of the highlighted brain region. The immunoreactivity is presented as percent area, the IL-10+ area divided by the total regional area.

Statistics

All data were analyzed using Prism (GraphPad Software, Inc.; La Jolla, CA). One-way ANOVA tests were conducted comparing the water and ethanol groups and the water and sucrose groups for the VTA region. If a significant effect of treatment was determined, post-hoc Dunnett’s Multiple Comparison tests were used to compare sucrose or ethanol to the control water group. All data were reported as the mean ± standard error of the mean and analyses were considered significantly different if p < 0.05.

Results

Binge Alcohol Exposure Did Not Induce IL-10 Immunoreactivity in the VTA

Central immune signaling and the release of proinflammatory cytokines in the mesolimbic pathway has been shown to contribute to alcohol abuse and addiction. IL-10, a potent anti-inflammatory immunoregulator, has been shown to limit inflammation in the brain. To determine the effects of binge ethanol consumption on the neuroinflammatory immune response in the mesolimbic reward pathway, immunohistochemical assessment was used to measure IL-10 expression in the VTA following binge drinking behavior.

For the VTA, one-way ANOVA testing showed that sucrose had no effect on the IL-10 immunoreactivity ([F = 1.020, p = 0.3750]; Figure 1A), which was expected. Sucrose controls were used to demonstrate that any changes in IL-10 immunoreactivity are specific to ethanol and
do not generalize to rewarding solutions. However, ANOVA testing also indicated that EtOH levels had no effect on IL-10 expression for either the 1 or 3 DID cycle groups ([F = 1.352, p = 0.2762]; Figure 1B). These results demonstrate that IL-10 levels do not significantly change in response to binge alcohol consumption.

Figure 1. IL-10 Immunoreactivity visualized
IL-10 immunoreactivity visualized at 10x magnification in the VTA and can be seen as the dark spots on the tissue. IL-10 was visible in both the control and experimental group. These are representative images.
**Figure 2.** The effects of binge-like alcohol consumption on IL-10 immunoreactivity in the ventral tegmental area for water control groups, 1-DID cycle groups, and 3-DID cycle groups. Although the figures show trending, ANOVA testing showed no significant correlation between EtOH and IL-10 immunoreactivity.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Consumption (g/kg/day)</th>
<th>BAC (mg/dL)</th>
</tr>
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<tbody>
<tr>
<td>Ethanol 1 week</td>
<td>4.5 ± 0.4</td>
<td>54.1 ± 13.3</td>
</tr>
<tr>
<td>Ethanol 3 weeks</td>
<td>4.6 ± 0.3</td>
<td>61.3 ± 16.7</td>
</tr>
<tr>
<td>Sucrose 1 week</td>
<td>7.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Sucrose 3 weeks</td>
<td>7.7 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Ethanol consumption and blood alcohol concentrations for both the 1-week and 3-week ethanol and sucrose groups. Data was collected following completion of the DID cycles. Values shown are averages and standard deviations of each test group.

**Discussion**

Proinflammatory signaling has been linked with high alcohol preference and dependence in both mice and humans. Repeated ethanol exposure results in persistent alterations of cytokines and significant increases in the magnitude and duration of proinflammatory cytokine presence in the brain (Coller et al. 2012). Drug-induced activation of proinflammatory central immune signaling has been shown to contribute to abusive behavior specifically by enhancing the engagement of...
the mesolimbic dopamine reward pathway (Gessa et al. 1985). IL-10 is a major mediator of the neuroimmune response to binge levels of alcohol and has been shown to play a role in controlling unresolved inflammation by regulating M1/M2 microglial polarization (Lobo-Silva et al. 2016). Administration of IL-10 prior to induced proinflammatory signaling has been shown to revert binge alcohol consumption behavior, however the effect of binge alcohol consumption on basal IL-10 immunoreactivity in the mesolimbic pathway has yet to be investigated. In order to determine the effects of binge ethanol consumption on the neuroinflammatory immune response in the mesolimbic reward pathway, immunohistochemical assessment was used to measure IL-10 expression in the VTA following a DID procedure.

After the DID cycles there was no significant increase in IL-10 immunoreactivity in the VTA. These results suggest that the lack of a neuroimmune anti-inflammatory response in the VTA could be a factor intensifying the inflammation of the mesolimbic reward pathway that underlies the behavioral maladaptations that lead to alcoholism.

However, it is too early to make any definitive conclusions because interactions between central immune signaling and alcohol response are extremely complicated with many interplaying factors. To achieve a global understanding of the homeostatic environment of the brain, it is necessary to understand how localized regions come together to work as system. The scope of this study was limited to the VTA of the mesolimbic reward pathway. The effects of binge ethanol consumption on central immune signaling and alcohol abuse can further be explored by investigating other regions of this pathway. Immunohistochemical assessment could be conducted on the nucleus accumbens region which has also been shown to be implicated in
alcohol addiction (Hyman et al. 2006). Additionally, there are many other proinflammatory and anti-inflammatory cytokines that are activated by alcohol exposure. Future studies could investigate the role of other immunoregulators such as IL-1, IL-2, IL-6, IL-8, IL-12, and IL-13 which have all been shown to elevated in alcoholics (Gonzalez-Quintela, 2000). Understanding the roles each of these cytokines play and how they interact with each other can further elucidate the link between increased central immune signaling and alcohol dependence.

Future works could more accurately model the long-term nature of human binge-drinking behavior by conducting the DID procedure on the mice for a longer period of time. Studies could have groups that undergo more than 3 DID cycles to see how prolonged binge behavior effects IL-10 immunoreactivity in the VTA. Previous studies have found that seven days after ethanol exposure, there is a significant increase in IL-10 expression levels (Marshall et al, 2013). There was no immediate change in IL-10 expression after ethanol consumption, similar to what was found in the VTA for this study. Future studies should look at other time points to see how IL-10 immunoreactivity fluctuates over longer period of binge behavior or during a period of abstinence in the brain regions of interest.

IL-10 is one of the most widely studied suppressive molecules, however much of this research is focused on IL-10 in the peripheral nervous system. Knowledge on IL-10 expression and regulation in the CNS is much more limited. Recent studies have shown that CNS homeostasis is maintained by active surveillance by the innate immune system (Lampron et al. 2013). The most widely studied component of the CNS innate immune system is the microglia, the macrophage of the brain. Upon detection of disturbances in the homeostatic environment, microglia polarize into
either the proinflammatory M1 phenotype or the anti-inflammatory M2 phenotype. While the M1 phenotype is important for fighting pathogens, unregulated, non-resolving microglial inflammation is not neuroprotective and may lead to neurodegeneration. Imbalanced polarization towards the M1 phenotypes has been implicated in neurodegenerative diseases including, neuropathic pain, Alzheimer's and Parkinson’s (Kwilasz et al. 2015). However, studies pioneering IL-10 as a new therapeutic approach aimed at correcting microglial imbalances have been met with conflicting results. Many of these problems are thought to stem from a lack of bioavailability in specific locations (Lobo-Silva et al. 2016). For example, intracranial administration of IL-10 improved outcomes for Experimental Autoimmune Encephalomyelitis, an animal model of brain inflammation, however, systemic delivery of IL-10 did not. Thus, a deep understanding of the temporal and spatial expression of IL-10 is necessary to further explore its potential as a therapy. Considering IL-10’s systemic importance and the promising results of studies investigating its efficacy as a therapeutic approach for controlling neuroinflammation, this lack of knowledge impedes the development of more sophisticated and effective immune modulatory strategies.

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REFERENCES:


