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Neuroactive Steroid (3 α ,5 α)3-hydroxypregnan-20-one (3 α ,5 α -THP) and Pro-inflammatory Cytokine MCP-1 Levels in Hippocampus CA1 are Correlated with Voluntary Ethanol Consumption in Cynomolgus Monkey

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Abstract

Background—Neuroactive steroids such as (3 α ,5 α)3-hydroxypregnan-20-one (3 α ,5 α -THP, allopregnanolone) are potent neuromodulators that enhance GABAergic neurotransmission and produce inhibitory neurobehavioral and anti-inflammatory effects. Chronic ethanol consumption reduces 3 α ,5 α -THP levels in human plasma, but has brain-region and species-specific effects on CNS levels of 3 α ,5 α -THP. We explored the relationship between 3 α ,5 α -THP levels in the hippocampus and voluntary ethanol consumption in the cynomolgus monkey following daily self-administration of ethanol for 12 months and further examined the relationship to HPA axis function prior to ethanol exposure. We simultaneously explored hippocampus levels of monocyte

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MCB, ALM, KAG were responsible for the study concept and design. KAG was responsible for all animal procedures. MCB, CSR, PP, and JBD contributed to the acquisition of animal data. MCB, CSR, PP, KAG, and ALM conducted the data analysis and interpretation of findings. MCB drafted the manuscript. CSR, PP, JBD, KAG, and ALM provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved the final version for publication.

chemoattractant protein 1 (MCP-1), a pro-inflammatory cytokine that plays an important role in the neuroimmune response to ethanol, following chronic self-administration.

Methods—Monkeys were subjected to scheduled induction of water and ethanol consumption (0–1.5 g/kg) over four months, followed by free access to ethanol or water for 22 hours/day over twelve months. Immunohistochemistry was performed using an anti-3 α ,5 α -THP or anti-MCP-1 antibody. Prolonged voluntary drinking resulted in individual differences in ethanol consumption that ranged from 1.2 – 4.2 g/kg/day over 12 months.

Results—Prolonged ethanol consumption increased cellular 3 α ,5 α -THP immunoreactivity by 12 $\pm$ 2% ($p < 0.05$) and reduced MCP-1 immunoreactivity by 23 $\pm$ 9% ($p < 0.05$) in the hippocampus CA1. In both cases, the effect of ethanol was most pronounced in heavy drinkers that consumed 3 g/kg for 20% of days. 3 α ,5 α -THP immunoreactivity was positively correlated with average daily ethanol intake (Spearman $r = 0.75$, $p < 0.05$) as well as dexamethasone inhibition of HPA axis function (Spearman $r = 0.9$, $p < 0.05$). In contrast, MCP-1 immunoreactivity was negatively correlated with average daily ethanol intake (Spearman $r = -0.78$, $p < 0.05$) as well as dexamethasone suppression of HPA axis function (Spearman $r = -0.76$, $p < 0.05$). Finally, 3 α ,5 α -THP and MCP-1 immunoreactivity were inversely correlated with each other (Spearman $r = -0.68$, $P < 0.05$).

Conclusions—These data indicate that voluntary, long-term ethanol consumption results in higher levels of 3 α ,5 α -THP, while decreasing levels of MCP-1 in the CA1 hippocampus, and that both changes may be linked to HPA axis function and the magnitude of voluntary ethanol consumption.

Keywords

neuroactive steroids; neuroimmune; cytokine; chronic ethanol; monkey

Introduction

Ethanol (EtOH) consumption leads to many and varied responses throughout the brain. A primary effect of ethanol administration on neural tissue is modulation of various neurotransmitter systems in the brain, namely that of γ -aminobutyric acid (GABA). GABA type A receptors belong to a family of receptors that serve as ligand-gated chloride ion channels. The heteropentameric GABA_A receptor acts to mediate much of the synaptic inhibition within the central nervous system. One class of molecules that has activity at these receptors are neuroactive steroids such as 3 α -reduced metabolites of progesterone, the most potent of which is (3 α ,5 α)-3-hydroxypregnan-20-one (3 α ,5 α -THP or allopregnanolone). 3 α ,5 α -THP acts as a positive allosteric modulator of GABA_A receptors by potentiating sites within α subunits to enhance GABAergic activity, which produces pharmacological effects similar to those of ethanol administration. In studies of human and animal models, systemic administration of GABAergic neuroactive steroids leads to a number of varied pharmacological responses. Consistent with their actions at the GABA_A receptor, those responses include analgesic, anesthetic, antidepressant, anxiolytic, anticonvulsant, and sedative effects (Kavaliers, 1988, Belelli et al., 1989, Carl et al., 1990, Bitran et al., 1991, Khisti et al., 2000). Systemic ethanol has been shown to increase both brain levels and

serum levels of neuroactive steroids (Serra et al., 2003, VanDoren et al., 2000) and many of the pharmacological responses associated with both ethanol and neuroactive steroids are inhibited by enzymatic inhibition of steroidogenesis or adrenalectomy.

Chronic ethanol consumption leads to species-specific effects on neuroactive steroids. Human alcoholics have reduced 3 α ,5 α -THP levels (Romeo et al., 1996) in their plasma, while Sprague Dawley rats do not (Janis et al., 1998). Male rats that have become dependent on ethanol, however, exhibit tolerance to the induction of circulating 3 α ,5 α -THP levels by ethanol. These rats also had reduced 3 α ,5 α -THP in both cerebral cortex and in hippocampus (Cagetti et al., 2004). Multiple brain regions have been shown to have reduced 3 α ,5 α -THP levels in C57BL/6J mice dependent on alcohol, including the prefrontal cortex, ventral tegmental area (VTA), and lateral amygdala, while the hippocampus CA3 exhibits an increase in levels of this neuroactive steroid (Maldonado-Devincci et al., 2014). While acute ethanol administration does not alter 3 α ,5 α -THP levels in cerebral cortex, hippocampus, or plasma of cynomolgus monkeys (Porcu et al., 2010), chronic consumption leads to reductions in lateral amygdala, basolateral amygdala, and plasma in these monkeys (Beattie et al., 2017). In a brain region-specific manner, neuroactive steroid levels in response to ethanol may be contributing to ethanol tolerance and increased consumption.

Ethanol consumption results in increased pro-inflammatory signaling in the brain that is linked with alcohol use disorders and changes in drinking behavior (Blednov et al., 2005, Blednov et al., 2012, Agrawal et al., 2011). In humans, chronic, high-dose ethanol consumption can lead to direct suppression of multiple immune responses. It has also been reported that alcoholism can lead to increased incidences of infectious diseases (Romeo et al., 2007). Innate immune system activation by ethanol is thought to occur via activation of the toll-like receptor (TLR) family of receptors. The signaling pathway initiated by TLR4 regulates many pro-inflammatory cytokines in the central nervous system (CNS). TLR4 knockout mice do not show increased expression of multiple pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) seen in their wild-type counterparts (Alfonso-Loeches et al., 2010). One cytokine, monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) has been implicated in mediating both inflammation and alcohol drinking behavior in mice (Blednov et al., 2005), and has been found to be increased in the amygdala, VTA, substantia nigra (SN), and hippocampus of postmortem human alcoholic brains (He and Crews, 2008). Alcohol-preferring P rats have innately elevated levels of TLR4 and MCP-1, and inhibiting gene expression of either factor results in reduced binge alcohol consumption (June et al., 2015). It is currently unknown how dysfunction of neuroimmune signaling pathways may contribute to alcohol dependence. Similarly, it is unknown what relationship exists between neuroactive steroids such as 3 α ,5 α -THP and neuroimmune factors like MCP-1.

In this study, we investigated the effects of long-term ethanol exposure on hippocampus levels of 3 α ,5 α -THP and MCP-1. Hippocampus was chosen as a neuroanatomical site of action in part because the neuroactive steroid levels in response to ethanol in previous studies reveal alterations inconsistent with those found in other brain regions. Due to similarities in brain regions between humans and monkeys, these types of studies on non-human primates are essential toward understanding the complex biomedical disease processes affecting populations throughout the world. Cynomolgus macaques voluntarily

self-administer ethanol at levels great enough to achieve intoxication with daily intake ranges similar to those seen in human social drinkers through those with alcohol use disorder (AUD) (Grant et al., 2008), indicating that they are an important translational model for studying neural adaptive responses related to excessive drinking. Approximately half of the monkeys (4 of the 9 alcohol-consuming animals) showed a ‘heavy-drinking’ phenotype of at least 3.0g/kg/day (or >12-drink equivalent) over the 12 months of free access. The animals consumed ethanol and maintained blood ethanol concentrations similar to human alcoholics (Majchrowicz and Mendelson, 1970), allowing us to assess a distribution that better reflects a population of humans, where only some will become “heavy-drinkers” given free access. We have previously reported on reductions in both circulating and amygdalar levels of 3 α ,5 α -THP after chronic ethanol consumption (Beattie et al., 2017), and here we see changes in hippocampus levels indicating a region-specific relationship between neuroactive steroids and ethanol consumption.

Materials and Methods

Animals

Monkeys (*Macaca fascicularis*, 50 to 62 months of age, weight 3.84 to 5.74 kg, n = 9) used in these studies are the same monkeys previously used in numerous studies to assess neuroendocrine mechanisms associated with ethanol self-administration (Beattie et al., 2017, Porcu et al., 2006a, Jimenez et al., 2017). The monkeys were housed individually in cages that were adjacent to three other conspecifics in quarters maintained within a narrow range of temperature (68 to 72F), humidity (65%), and a 12-hour light cycle (lights on at 7:00 AM). Monkeys were acclimated to the laboratory personnel, and then trained to present their leg for blood collection via saphenous or femoral venipuncture for subsequent assays of plasma steroids. All primate handling procedures were performed in accordance with the NIH guidelines, the Commission on Life Sciences, National Research Council (1996) Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington), and approved by Wake Forest University animal care and use committee. As detailed training procedures were previously published (*vide supra*), they are not detailed here.

Induction of Ethanol Self-Administration

As previously described, a group of 10 monkeys were trained to operate a drinking panel in daily 60-minutes sessions and then induced to drink water and later ethanol (4% w/v in water). A separate group of 10 control monkeys were similarly trained, but housed without access to ethanol and did not undergo scheduled induction of any fluids. Monkeys undergoing scheduled induction of ethanol were trained to press the panel for food delivery for their meals, but pellet delivery was contingent upon consumption of a predetermined volume of either water or 4% (w/v) ethanol. Initially, the monkeys were induced to drink water (150 to 227 ml, based on body weight) prior to food delivery for 30 days. Next, induction of ethanol self-administration was initiated with ethanol (4% w/v) as the only fluid available. The monkeys were induced to drink 0.5 g/kg ethanol per day for the first month, 1 g/kg/day for the next month, and finally 1.5 g/kg/day for 30 days.

Chronic Ethanol Self-Administration

Following 120 days of scheduled induction, ethanol and water were freely available and food was available in meals during each daily 22-hour session (11:00 am – 9:00 am the following day). Details of these procedures were previously published (Vivian, 2001).

Pharmacological Profiling

A particular advantage of using cohorts studied previously involves the opportunity to examine the relationship between hypothalamic-pituitary-adrenal (HPA) axis function prior to ethanol exposure and subsequent effects of ethanol in various brain regions (Beattie et al., 2017). Each monkey in this cohort participated in endocrine profiling as previously described (Porcu et al., 2006a). These studies were designed to reflect clinical assessments commonly available in human research protocols. Eight pharmacological challenges were conducted over the course of 4 weeks: naloxone (2 doses), corticotrophin releasing factor (CRF), adrenocorticotrophic hormone (ACTH), dexamethasone, ethanol (2 doses, 1 and 1.5 g/kg), and saline. Here, we report data from the saline and ethanol challenges as well as the dexamethasone suppression test in relation to the post-mortem 3 α ,5 α -THP levels in hippocampus.

Tissue Acquisition

As previously described, monkeys underwent a state-of-the-art necropsy protocol (Davenport et al., 2014) within hours of their final access to ethanol in which the brain was removed, cut into 3 blocks and frozen in isopentane at –35°C. The temporal pole containing the hippocampus was cut from the brain block and shipped by the Monkey Alcohol Tissue Research Resource. The tissue block was subsequently post-fixed in 4% paraformaldehyde for 24 hr at 4°C, and then stored in 30% sucrose until they were sectioned at 40 μ m on a freezing microtome.

Immunohistochemistry

Immunohistochemistry was performed on free-floating sections (6 to 8 sections/animal/brain region) using an affinity purified 3 α ,5 α -THP sheep antibody as previously described (Beattie et al., 2017) or a commercial MCP-1 antibody. Briefly, sections were rinsed in PBS, incubated in 1% hydrogen peroxide to block endogenous peroxidase activity, blocked in 10% normal rabbit serum (Vector Laboratories, Burlingame, CA) in PBS to reduce background staining and then incubated with antibody for 48 hr at 4°C. The 3 α ,5 α -THP antiserum (purchased from Dr. R.H. Purdy) was used at a 1:2,500 dilution while the MCP-1 antibody (Abcam, Cambridge, MA) was used at 1:200 dilution. The Vectastain Elite ABC kit (Vector Laboratories) was utilized for staining for each antibody, and immunoreactivity was visualized with 3,3'-diaminobenzidine (Sigma-Aldrich) using the manufacturer's recommended protocol.

IHC Analyses

Brain-region immunoreactivity was visualized with an Olympus CX41 light microscope (Olympus America, Center Valley, PA), images were captured with a digital camera (Regita model; QImaging, Burnaby, BC), and analyzed using Bioquant (Nashville, TN) image

analysis to obtain linear integrated optical density for immunoreactivity assessment. The microscope, camera, and software were background corrected to eliminate nonspecific labeling and normalized to preset light levels to ensure fidelity of data acquisition. Positive pixel counts of immunoreactivity were quantified from a circumscribed field, delineated as a brain region, divided by the area of the region in square millimeters, and expressed as pixels/mm². Data from 6 to 8 alternate sequential sections per animal per brain region from a single hemisphere were used to average 1 value per monkey. The experimenter was blind to the condition of each animal when analyses were conducted.

Statistics

Raw pixel densities (pixels/mm²) were analyzed between ethanol naïve controls and ethanol-consuming monkeys using Student's t-test for comparisons in each experiment. Data were further analyzed with alcohol consumption levels as a factor (3: control, heavy, non-heavy) using a one-way analysis of variance (ANOVA) (Statistica; StatSoft Inc., Tulsa, OK). Post-hoc analyses were conducted with the Newman-Keuls test. Nonparametric correlations were performed between 3 α ,5 α -THP or MCP-1 raw pixel densities (pixels/mm²) and individual drinking levels (average daily drinking g/kg) for each monkey using Spearman correlations.

Results

Chronic ethanol consumption increases 3 α ,5 α -THP in CA1 hippocampus

3 α ,5 α -THP immunoreactivity was examined in CA1 hippocampus following 12 months of voluntary ethanol consumption and compared to hippocampal 3 α ,5 α -THP immunoreactivity in control animals that were similarly housed, but without access to ethanol. Overall, EtOH-consuming monkeys showed higher 3 α ,5 α -THP immunoreactivity in CA1 hippocampus compared to control animals (Fig 1a). An increase of cellular 3 α ,5 α -THP immunoreactivity (12 $\pm$ 2%; $t(18)=3.8$, $p<0.05$) was observed in the hippocampus CA1 of monkeys that chronically consumed ethanol. The effect of ethanol was largely driven by those animals that drank heavily (≥ 3 g/kg/day for $\geq 20\%$ of days) [$F(2,17)=13.3$, $p<0.05$]. Indeed, monkeys classified as heavy drinkers displayed increased (16 $\pm$ 2%; $p<0.05$) 3 α ,5 α -THP immunoreactivity vs. controls (Fig 1b), while those classified as non-heavy drinkers (< 3 g/kg for $\geq 20\%$ of days) showed no effect of ethanol consumption. 3 α ,5 α -THP immunoreactivity in the hippocampus of heavy drinkers was also increased (10 $\pm$ 2%; $p<0.05$) when compared to non-heavy drinkers (Fig 1b).

Average daily drinking is correlated with 3 α ,5 α -THP levels in CA1 hippocampus

To assess the relationship between ethanol intake and 3 α ,5 α -THP immunoreactivity we examined Spearman correlation coefficients. 3 α ,5 α -THP immunoreactivity in the hippocampus (Fig 2a) was positively correlated ($r=0.76$, $p<0.05$) with voluntary ethanol consumption (average daily ethanol intake [g/kg]) after 12 months of daily drinking (Fig 2b). These data suggest that 3 α ,5 α -THP immunoreactivity in the CA1 region of hippocampus is dependent upon ethanol dose.

Chronic ethanol consumption reduces MCP-1 levels in CA1 hippocampus

Next, we assessed MCP-1 immunoreactivity in the hippocampus of the same monkeys. MCP-1 immunoreactivity was reduced ($-23 \pm 9\%$; $t(17) = 2.1$, $P < 0.05$) in monkeys that chronically consumed ethanol vs. control subjects (Fig 3a). The greatest reductions were found in heavy drinkers [$F(2,18) = 5.3$, $P < 0.05$] that displayed significantly reduced (-42 ± 14 percent; $P < 0.05$) MCP-1 immunoreactivity (Fig 3b) compared to controls or non-heavy drinkers ($35 \pm 10\%$; $p < 0.05$; Fig 3b). Non-heavy drinkers did not show a significant reduction in MCP-1 immunoreactivity compared to controls.

Average daily drinking is correlated with MCP-1 levels in hippocampus

To assess the relationship between ethanol intake and MCP-1 immunoreactivity we examined Spearman correlation coefficients. MCP-1 immunoreactivity in the hippocampus was inversely correlated ($r = -0.78$, $P < 0.05$) with voluntary ethanol consumption [average daily ethanol intake (g/kg)] after 12 months of voluntary drinking (Fig 4c). Changes in MCP-1 immunoreactivity in the hippocampus appear to be proportional to the average amount of ethanol consumed. These data suggest that MCP-1 immunoreactivity in the CA1 region of hippocampus is also dependent upon ethanol dose.

3 α ,5 α -THP levels are inversely correlated with MCP-1 levels in CA1 hippocampus

Since ethanol intake appeared to influence both 3 α ,5 α -THP and MCP-1 immunoreactivity in the same animals and these factors have previously been shown to influence ethanol consumption in rodents, we examined the correlation between these factors following 12 months of ethanol self-administration. MCP-1 levels were inversely correlated with 3 α ,5 α -THP levels ($r = -0.68$, $P < 0.05$) in the CA1 region of the hippocampus (Fig 5). This result indicates a potential relationship between 3 α ,5 α -THP and MCP-1 levels that may influence heavy ethanol consumption.

Neither 3 α ,5 α -THP or MCP-1 levels in CA1 hippocampus are correlated with serum levels of 3 α ,5 α -THP

We have previously reported that chronic ethanol consumption reduces circulating 3 α ,5 α -THP levels in these same animals, but that no correlation was found between circulating 3 α ,5 α -THP levels and average daily intake of ethanol (Beattie et al. 2017). Here we compared 3 α ,5 α -THP levels in the CA1 hippocampus and serum levels of 3 α ,5 α -THP levels and found no correlation (data not shown). Similarly, we compared hippocampal levels of MCP-1 with circulating 3 α ,5 α -THP and found no correlation (data not shown). Thus, circulating levels of 3 α ,5 α -THP are not indicative of hippocampal levels of 3 α ,5 α -THP or MCP-1 or the average daily ethanol intake of cynomolgus monkeys.

Postmortem 3 α ,5 α -THP and MCP-1 levels correlate with HPA axis function prior to ethanol exposure

A previous study in these same monkeys showed that dexamethasone-induced changes in circulating pregnenolone levels prior to ethanol exposure were correlated with subsequent alcohol intake (Porcu et al., 2006). We subsequently found that post-mortem 3 α ,5 α -THP levels in the lateral amygdala of these monkeys was inversely correlated with

dexamethasone-induced changes in circulating pregnenolone measured prior to ethanol exposure (Beattie et al., 2017). To extend this relationship we examined postmortem levels of 3 α ,5 α -THP in the hippocampus and found a strong correlation with dexamethasone-induced changes in circulating pregnenolone levels (Fig 6a; $r=0.93$, $p<0.05$).

Dexamethasone-induced changes in plasma pregnenolone levels prior to ethanol exposure were predictive of postmortem 3 α ,5 α -THP levels in the hippocampus. Similarly, MCP-1 levels in CA1 hippocampus were inversely correlated with dexamethasone-induced changes in circulating pregnenolone levels (Fig 6b; $r=-0.76$, $p<0.05$) indicating another factor that was predicted by dexamethasone sensitivity prior to ethanol exposure.

Since pregnenolone is the precursor of 3 α ,5 α -THP and pregnenolone administration increases 3 α ,5 α -THP levels in rats and humans (Porcu et al., 2009, Marx et al., 2009), we examined whether pregnenolone levels following saline injection or acute ethanol gavage (1.5g/kg) prior to ethanol exposure were related to postmortem levels of hippocampal 3 α ,5 α -THP following long-term ethanol consumption. No correlation was observed between postmortem 3 α ,5 α -THP and circulating pregnenolone after saline or acute ethanol administration to the ethanol-naïve monkey (data not shown).

Discussion

This study demonstrates increased 3 α ,5 α -THP and decreased MCP-1 immunoreactivity in the CA1 region of hippocampus following prolonged voluntary ethanol consumption in male cynomolgus monkeys. 3 α ,5 α -THP immunoreactivity is inversely proportional to the average daily intake of ethanol following chronic exposure, while MCP-1 immunoreactivity was directly proportional to average daily drinking. Additionally, 3 α ,5 α -THP and MCP-1 immunoreactivity were inversely correlated in this brain region. The hippocampus is responsible for many aspects of learning and memory and also plays a part in modulating the mesolimbic dopamine system, thought to be important for the development of alcohol use disorders, via projections to other limbic brain regions, including the cortex and amygdala. Ethanol is known to affect multiple aspects of hippocampal function including neurotransmission, synaptic plasticity, cell signaling, and adult neurogenesis (Morris et al., 2010).

The finding that chronic ethanol consumption increases 3 α ,5 α -THP immunoreactivity in monkey hippocampus is consistent with studies in C57BL/6J mice where chronic intermittent ethanol exposure produced increases in CA3 hippocampal 3 α ,5 α -THP immunoreactivity (Maldonado-Devincci et al., 2014), though findings in Sprague-Dawley rats indicate reduced 3 α ,5 α -THP in whole hippocampus following chronic ethanol consumption (Cagetti et al., 2004). The physiological significance of these changes is unknown, but it's been shown in isolated rat hippocampus, that ethanol acutely increases the concentration of 3 α ,5 α -THP as well as the amplitude of GABA_A receptor-mediated IPSCs recorded from CA1 pyramidal neurons (Sanna et al., 2004). While neuroactive steroids are generally thought to bind and activate GABA_A receptors leading to local inhibition, some neuronal populations within the hippocampus may be acting in an excitatory manner in the presence of 3 α ,5 α -THP. GABA_A receptors expressed on presynaptic glutamatergic nerve terminals in rat CA3 hippocampus modulate neurotransmitter release in response to 3 α ,5 α -

THP (Iwata et al., 2013). Thus, an increase in 3 α ,5 α -THP at this site could result in increased excitability. 3 α ,5 α -THP also increases dendritic spine density in cultured hippocampal neurons (Shimizu, 2015). As dendritic spines are the postsynaptic receptive regions enriched in excitatory synapses, certain neuron populations within the hippocampus may act in an excitatory manner following neuroactive steroid interactions. 3 α ,5 α -THP can exert either inhibitory or excitatory effects, depending on the site of action.

The effects of chronic ethanol intake in CA1 hippocampus are clearly distinct from the decreases in 3 α ,5 α -THP immunoreactivity that were reported in amygdala of the same monkeys (Beattie et al., 2017) as well as C57BL/6J mice (Maldonado-Devincci et al., 2014). GABA_A receptors in the amygdala of cynomolgus macaques display altered GABA potency following chronic ethanol self-administration as a result of selective reductions in subunit mRNA expression (Floyd et al., 2004). Ethanol-induced changes in receptor function are driven by these alterations in subunit expression and are largely brain-region specific. In the putamen of cynomolgus monkeys, prolonged intermittent ethanol consumption decreases GABAergic inhibitory synaptic transmission while increasing glutamatergic excitatory transmission (Cuzon Carlson et al., 2011). The activity of 3 α ,5 α -THP on GABA_A receptor modulation, whether pre- or postsynaptic, regulates neuronal excitability differently across brain regions. Taken with our previous study, this work reveals differential regulation of 3 α , 5 α -THP levels after long-term, voluntary ethanol consumption between the amygdala and the hippocampus (Beattie et al., 2017). A similar, differential relationship has been observed in studies conducted in C57BL/6J mice showing reductions in 3 α ,5 α -THP levels in the lateral amygdala after chronic ethanol consumption as well as increases in hippocampus CA3 (Maldonado-Devincci et al., 2014). In rats, acute ethanol treatment (2g/kg) leads to reductions in 3 α ,5 α -THP levels the central nucleus of the amygdala, while the CA1 pyramidal cell layer of the hippocampus showed increases in local 3 α ,5 α -THP levels (Cook et al., 2014). These observations are consistent with our findings that long-term voluntary ethanol consumption leads to divergent effects in distinct brain regions.

Ethanol consumption, withdrawal and stress are known to activate the innate immune system, resulting in increased neuroimmune signaling molecules. Thus, it was surprising to find a decrease in MCP-1 in monkey CA1 hippocampus in the present study. Previous studies have shown increased MCP-1 in other brain regions in rodent models of ethanol consumption and withdrawal (Knapp et al., 2016). In orbitalfrontal cortex of human postmortem alcoholics, high-mobility group box 1 (HMGB1), a cytokine-like molecule that activates the innate immune system via the TLR pathways, as well as TLR2, TLR3, and TLR4 mRNA levels are increased and those levels correlate with lifetime drinking (Crews et al., 2013). The differential effect on MCP-1 in our study may be due to the precise anatomical localization to CA1 hippocampus, which has not been previously examined or it may relate to the observed increase in 3 α ,5 α -THP immunoreactivity, since there was an inverse correlation between MCP-1 and 3 α ,5 α -THP. Further studies are needed to explore these questions.

The physiological significance of altered MCP-1 in CA hippocampus is unclear. MCP-1 can cause activation and migration of microglia potentially resulting in increased secretions of proinflammatory cytokines. It's been shown that MCP-1 knockout mice display reductions

in proinflammatory cytokine production such as IL-1 β (interleukin-1 beta) and TNF α (tumor necrosis factor alpha) (Rankine et al., 2006). MCP-1 also regulates the release of several neurotransmitters, including glutamate, GABA, and dopamine, and binding to its receptor (CCR2) can lead to cross-desensitization of GABA_A receptors (Rostene et al., 2007). CCR2 activation by MCP-1 results in inhibition of GABA_A receptor activity that is likely occurring through a direct receptor-receptor interaction (Gosselin et al., 2005). Thus, the effects of alcohol-induced changes in MCP-1 could involve such mechanisms. Further studies are required to better understand the differential regulation of MCP-1 across the CNS after chronic alcohol exposure.

The relationship between neuroactive steroid changes and neuroimmune activation following ethanol administration is not yet understood. In this study, an inverse relationship was present between 3 α ,5 α -THP levels and MCP-1 levels in CA1 hippocampus in the cynomolgus monkey following ethanol consumption. Both 3 α ,5 α -THP and cytokines have been implicated in HPA axis dysfunction associated with various disease states, such as depression and drug addiction (Hueston and Deak, 2014, Girdler and Klatzkin, 2007, Kaminski et al., 2005, Morrow et al., 2006). Following 12 months of ethanol self-administration, cynomolgus monkeys show an inverse correlation between plasma MCP-1 and cortisol as well as changes in other immune-related plasma proteins in relation to HPA axis function (Helms et al., 2012). In rat hippocampus and frontal cortex, chronic stress-induced glucocorticoids regulate expression of TNF α , IL-1 β , and NF- κ B (Munhoz et al., 2006). Known to be present in microglia, MCP-1 has been shown to localize to the cell bodies of some neuron populations. Here we found that MCP-1 exhibited neuronal staining patterns similar to those found with 3 α ,5 α -THP immunostaining, indicating a potential relationship between cytokines and neuroactive steroids following ethanol administration. This relationship, as well as the ability of neuroimmune factors to regulate HPA axis function could provide an important link between stress response and ethanol consumption.

Earlier studies showed that dexamethasone regulation of circulating pregnenolone in these monkeys was predictive of subsequent ethanol intake (Porcu et al., 2006b). Indeed, the monkeys that exhibited dexamethasone suppression of plasma pregnenolone were classified as non-heavy drinkers in the subsequent 12 month voluntary drinking period. In contrast, the monkeys that exhibited dexamethasone enhancement of plasma pregnenolone were later classified as heavy drinkers. In the current study, we extended this analysis to the post-mortem hippocampal 3 α ,5 α -THP levels and found that dexamethasone-induced changes in plasma pregnenolone were directly correlated. We also found an inverse correlation between hippocampus CA1 MCP-1 levels and dexamethasone-induced changes in plasma pregnenolone. It is unclear, however, whether elevated hippocampal 3 α ,5 α -THP, or reduced MCP-1 content preceded or was a consequence of chronic heavy alcohol consumption. What is clear is the presence of a relationship between HPA axis function, neuroimmune regulation, and drinking behavior. Future studies will be required to determine the mechanistic relationships involved in these interactions. From a clinical perspective, these relationships may help identify risk factors or biomarkers for heavy drinking behaviors and allow possible interventions, whether proactive or reactive.

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Figure 1.

(a) Effects of chronic ethanol exposure on 3α,5α-THP immunoreactivity in the hippocampus in control (clear bars) or ethanol (EtOH) drinking monkeys (black bars). (b) Effect of drinking levels on 3α,5α-THP immunoreactivity in the hippocampus in heavy drinkers (HD) and non-heavy drinkers (NHD, represented by grey bar). Data depicted are mean positive pixels/mm² ± SEM. *P<0.05 compared to control; #P<0.05 compared to NHD.

Figure 2.

(a) Representative photomicrographs (10x) of cellular 3α,5α-THP immunoreactivity in coronal slices of hippocampus from non-heavy and heavy drinking cynomolgus monkeys following 12 months of voluntary access to ethanol. (b) Correlation between average daily ethanol consumption and 3α,5α-THP immunoreactivity ($r = 0.76$, $P < 0.05$). (c) Atlas image of cynomolgus monkey hippocampus. The red box indicates the location of the representative photomicrograph within the hippocampus CA1.

Figure 3.

(a) Effects of chronic ethanol exposure on MCP-1 immunoreactivity in the hippocampus CA1 in control (clear bars) or ethanol (EtOH) drinking monkeys (black bars). (b) Effect of drinking levels on MCP-1 immunoreactivity in the hippocampus CA1 in heavy drinkers (HD) and non-heavy drinkers (NHD). Data depicted are mean positive pixels/mm² ± SEM. *P<0.05 compared to control; #P<0.05 compared to NHD.

Figure 4.

(a) Representative photomicrographs (10x) of cellular MCP-1 immunoreactivity in coronal slices of hippocampus from non-heavy and heavy drinking cynomolgus monkeys following 12 months of voluntary access to ethanol. (b) Negative correlation between average daily ethanol consumption and MCP-1 immunoreactivity ($r = -0.78$, $P < 0.05$). (c) Atlas image of cynomolgus monkey hippocampus CA1. The red box indicates the location of the representative photomicrograph within the hippocampus.

Figure 5.
Negative correlation between 3 α ,5 α -THP immunoreactivity and MCP-1 immunoreactivity in the hippocampus CA1 of cynomolgus monkeys following voluntary 12 month alcohol consumption ($r = -0.68$, $P < 0.05$).

Figure 6.

(a) Dexamethasone-induced PREG in plasma prior to ethanol exposure is predictive of post-mortem 3α,5α-THP immunoreactivity in hippocampus CA1 ($r = 0.93$, $P < 0.05$). (b) Dexamethasone-induced PREG is predictive of post-mortem MCP-1 levels in hippocampus CA1 ($r = -0.76$, $P < 0.05$). EtOH, ethanol; PREG, pregnenolone.