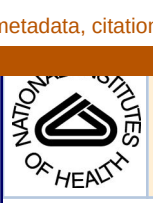

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Ethanol Administration Produces Divergent Changes in GABAergic Neuroactive Steroid Immunohistochemistry in the Rat Brain

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Abstract

Background—The 5α-reduced pregnane neuroactive steroid (3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) is a potent positive modulator of $GABA_A$ receptors capable of modulating neuronal activity. In rats, systemic ethanol administration increases cerebral cortical and hippocampal levels of 3α,5α-THP, but the effects of ethanol on 3α,5α-THP levels in other brain regions are unknown. There is a large body of evidence suggesting that 3α,5α-THP enhances ethanol sensitivity, contributes to some behavioral effects of ethanol, and modulates ethanol reinforcement and motivation to drink. In the present study, we used immunohistochemistry (IHC) to determine ethanol-induced changes in cellular 3α,5α-THP expression in brain regions associated with ethanol actions and responses.

Methods—Male Wistar rats were administered ethanol (2g/kg) or saline intraperitoneally and after 60 minutes transcardially perfused. IHC was performed on free floating sections (3–4 sections/animal/brain region) using an affinity purified anti-3α,5α-THP primary antibody and immunoreactivity was visualized with 3,3′-diaminobenzidine.

Results—Ethanol significantly increased 3α,5α-THP immunoreactivity by 24±6% in the medial prefrontal cortex, 32±12% in the hippocampal CA1 pyramidal cell layer, 52±5% in the polymorph cell layer of the dentate gyrus, 44±15% in the bed nucleus of the stria terminalis, and by 36±6% in the paraventricular nucleus of the hypothalamus. In contrast, ethanol administration significantly reduced 3α,5α-THP immunoreactivity by 25±5% in the nucleus accumbens "shore" and 21±3% in the central nucleus of the amygdala. No changes were observed in the ventral tegmental area, dorsomedial striatum, granule cell layer of the dentate gyrus, or the lateral and basolateral amygdala.

Conclusions—The results suggest acute ethanol (2g/kg) produces divergent, brain region specific, effects on cellular 3α,5α-THP levels. Regional differences in the effects of ethanol suggest there may be regional brain synthesis of 3α,5α-THP independent of the adrenal glands and novel mechanisms that reduce cellular 3α,5α-THP. Regional differences in ethanol-induced changes in 3α,5α-THP levels likely contribute to ethanol effects on neuronal function in brain.

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Keywords

Neuroactive steroid; Alcohol; Allopregnanolone; 3α,5α-THP; Neurosteroid

INTRODUCTION

Neuroactive steroids are endogenous neuromodulators capable of altering neuronal activity. Synthesis of neuroactive steroids occurs in the adrenal glands, gonads, and *de novo* in the brain. The 5α-reduced pregnane steroids,(3α,5α)-3-hydroxypregnan-20-one (3α,5α-THP or allopregnanolone) and (3α,5α)-3,21-dihydroxypregnan-20-one (3α,5α-THDOC), are positive allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors. $GABA_A$ receptors are the primary inhibitory receptor family in the brain and mediate many of the behavioral effects of ethanol. Both 3α,5α-THP and 3α,5α-THDOC enhance neuronal inhibition at a known binding site on $GABA_A$ receptor α-subunits (Hosie et al., 2006), and have corresponding behavioral effects similar to ethanol. These GABAergic neuroactive steroids are very potent positive modulators of $GABA_A$ receptors, which produce pharmacologically relevant effects at nanomolar concentrations (Morrow et al., 1987).

A large body of evidence suggests increased levels of 3α,5α-THP and 3α,5α-THDOC following ethanol administration contribute to both electrophysiological and behavioral effects of ethanol in rodents. In rats, ethanol activates the hypothalamic-pituitary-adrenal (HPA) axis (Boyd et al., 2010) leading to physiologically significant increases of 3α,5α-THP and 3α,5α-THDOC in the blood plasma, cerebral cortex, and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000; Porcu et al., 2009). Adrenalectomy or inhibition of 5α-reduced steroid synthesis with the 5α-reductase (5α-R) inhibitor finasteride reduces some of the behavioral effects of ethanol, including the hypnotic (Khisti et al., 2003), anxiolytic-like (Hirani et al., 2005), anticonvulsant (VanDoren et al., 2000), and anti-depressant-like (Hirani et al., 2002) effects in rats. Furthermore, finasteride reduces some of the subjective effects of alcohol in healthy men (Pierucci-Lagha et al., 2005). Finasteride also blocks ethanol inhibition of neuron firing in the medial septum (VanDoren et al., 2000), hippocampus (Tokunaga et al., 2003), hippocampal slice (Sanna et al., 2004), and long-term potentiation (LTP) in the hippocampal slice preparation (Tokuda et al., 2011). Taken together, these findings suggest ethanol-induced elevations of 3α,5α-THP and 3α,5α-THDOC contribute to many of the physiological and behavioral effects of ethanol. However, outside of the cerebral cortex and hippocampus, it is not known if ethanol increases levels of 3α,5α-THP in other brain regions that contribute to ethanol's myriad of pharmacological effects.

GABAergic neuroactive steroids are involved in stress responses; therefore, steroid concentrations may be altered in stress related neurocircuitry following ethanol administration. GABAergic neuroactive steroids are increased in the plasma and cerebral cortex following environmental stress (Purdy et al., 1991; Barbaccia, 1996), including ethanol administration. GABAergic neuroactive steroids contribute to negative feedback on the HPA axis at the level of the hypothalamus in rats (Owens et al., 1992; Patchev et al., 1994; Patchev et al., 1996), but not in C57BL/6J mice (Sarkar et al., 2011). Furthermore,

recent evidence in C57BL/6J mice suggests GABAergic neuroactive steroids are involved in initiation of the stress response (Sarkar et al., 2011). In the current study, we investigated ethanol-induced changes of 3α,5α-THP in the paraventricular nucleus (PVN) of the hypothalamus, the bed nucleus of the stria terminalis (BNST), and amygdala due to the involvement of these regions in stress, emotion, and ethanol responses (Armario, 2010; Cui et al., 2012; Koob, 2013).

Ethanol and 3α,5α-THP both impair learning and memory performance in a similar manner (Matthews et al., 2002), which may be due to modulation of activity of specific cellular populations in the hippocampus (Matthews et al., 2002; Tokunaga et al., 2003; Tokuda et al., 2011). *In vitro*, ethanol increases 3α,5α-THP immunoreactivity in hippocampal pyramidal cells (Tokuda et al., 2011), and finasteride prevents ethanol's inhibitory effect on these cells in anesthetized rats (Tokunaga et al., 2003). Therefore, we examined the cellular layer specificity of ethanol effects on 3α,5α-THP levels in the hippocampal formation.

Systemic and intracerebroventricular administration of exogenous 3α,5α-THP or systemic administration of the longer acting 3α,5α-THP analogue, ganaxolone, alter ethanol consumption in rodents. Both 3α,5α-THP and ganaxolone have been shown to produce biphasic effects on ethanol consumption, with low doses increasing consumption and high doses decreasing consumption (Janak et al., 1998; Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007; Besheer et al., 2010). The mechanisms by which 3α,5α-THP affects ethanol consumption are not clear, but these effects could be due to modulation of dopamine release in mesocorticolimbic circuitry (Motzo et al., 1996; Rouge-Pont et al., 2002). Therefore, we examined the effects of ethanol on 3α,5α-THP immunoreactivity in the ventral tegmental area (VTA), nucleus accumbens (NAc), striatum, and medial prefrontal cortex (mPFC).

Previous studies measuring ethanol-induced changes of 3α,5α-THP in the brain have used radioimmunoassay (RIA) or gas chromatography-mass spectroscopy (GC-MS), which lack the sensitivity to determine levels in discrete cell layers or brain regions with low levels of 3α,5α-THP. Although ethanol increases levels of 3α,5α-THP in the cerebral cortex and hippocampus, it is unknown whether ethanol alters levels of 3α,5α-THP in other brain regions. Immunohistochemistry (IHC) using an anti-3α,5α-THP primary antibody was used to examine ethanol-induced changes in cellular 3α,5α-THP levels. This technique has been used to determine the cellular distribution of 3α,5α-THP in the rat brain (Saalmann et al., 2007) and displays the sensitivity to detect changes in immunoreactivity that are associated with corresponding changes in electrophysiological measurements (Saalmann et al., 2006; Tokuda et al., 2011). We examined ethanol-induced changes in cellular 3α,5α-THP expression across multiple brain regions that are implicated in alcohol use disorders. This approach provides brain region specificity of neuroactive steroid measurements that has not previously been reported following ethanol administration, and may reveal novel mechanisms of ethanol action.

METHODS

Subjects

Adult male Wistar rats (~250 g/7–8 per group) were purchased from Harlan Laboratories (Indianapolis, IN, USA). The animals were housed in Plexiglass cages (2 to 4 per cage) with food and water available *ad libitum*. The colony room was maintained on a normal 12 hr light-dark cycle (light onset at 0700 hr) and at a constant temperature of 22 ± 2°C and relative humitity of 65%. The animals were allowed 1 week to acclimate to the colony room. Following acclimation, the animals were habituated to handling and intraperitoneal (i.p.) saline injections for 5 days. Experiments were conducted between 0800 and 1300 hr to minimize potential circadian fluctuation in neuroactive steroid levels.

Ethanol (2g/kg, 20%v/v in saline) or saline were administered by i.p. injection 60 minutes before transcardial perfusion. Animal care and handling procedures followed National Institutes of Health Guidelines under University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee approved protocols.

Antibody specificity tests

The specificity of the affinity purified 3α,5α-THP antibody was measured by RIA. Standards of 3α,5α-THP, 3α,5α-THDOC, (3α,5β)-3-hydroxypregnan-20-one (3α,5β-THP), pregnenolone, progesterone and (3α)-3-hydroxy-4-pregnen-20-one (3α-HP) were diluted in 95% ethanol at an initial concentration of 0.1 mg/ml. The compounds were serially diluted to a range of 40.0 – 0.0049 ng/ml, no more than 24 hours before the assay. RIAs were repeated three times for each compound, following previously described methods (Janis et al., 1998). Briefly, 5 μl of each concentration of the tested compounds were mixed with 10,000 CPM of [^{3}H]-3α,5α-THP and a 1:500 dilution of the affinity purified 3α,5α-THP antiserum. Unbound [^{3}H]- 3α,5α-THP was removed by centrifugation after adding dextran-coated charcoal. The supernatant was mixed with Ecoscint H (National Diagnostics) and [^{3}H]-3α,5α-THP was measured in a scintillation counter. The resulting curves were analyzed using a one-site competition model (Prism, GraphPad Software, La Jolla, CA, USA) for EC_{50} values. We observed cross reactivity with 3α, hydroxy-4-pregnen-20-one (3α-HP; 41±0.14%), (3α,5β)-3-hydroxypregnan-20-one (3α,5β-THP; 22±0.43%), progesterone (14±1.95%), 3α,5α-THDOC (11±0.29%), and pregnenolone (9±1.61%) as expected from previous reports using a different antibody preparation (Janis et al., 1998; VanDoren et al., 2000; Khisti et al., 2003; Boyd et al., 2010).

Immunohistochemistry

Fifty minutes after 2g/kg ethanol or saline injection the animals were anesthetized with pentobarbital (100mg/kg, i.p.; Professional Compounding Centers of America, Houston, TX, USA) and transcardially perfused approximately 1hr following ethanol or saline injection with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Tissue was post-fixed in 4% paraformaldehyde for 24hr at 4°C, sectioned coronally on a vibrating microtome at 40μm, and stored at −30°C until further processing.

Immunohistochemical assays were performed using a procedure modified from (Saalmann et al., 2007). No detergents or organic solvents were used to prevent the possibility of neuroactive steroid leeching. Free floating brain sections (3–4 sections/animal/brain region) were rinsed in PBS, followed by incubation in 1% hydrogen peroxide to block endogenous peroxidase activity, and then blocked using 10% rabbit serum in PBS. Next, the tissue was incubated in sheep affinity purified anti-3α,5α-THP antiserum (targeted against 3α,5α-THP carboxymethyl ether coupled to bovine serum albumin; purchased from Dr. R.H. Purdy) at a 1:2500 dilution for 48 hr at 4°C. Following rinsing in PBS, tissue was incubated in a rabbit anti-sheep biotinylated secondary antibody (1:200; Vector laboratories, Burlingame, CA, USA) for 1 hr. After rinsing in PBS, avidin biotin amplification was performed with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB; Polysciences, Inc., Warrington, PA, USA and Sigma-Aldrich, St. Louis, MO, USA) using the manufacturers' recommended procedures.

Immunohistochemical analysis

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA, USA) and images were captured with a digital camera (Regita model, Q Imaging, Burnaby, BC). Image analysis software (Bioquant Life Sciences version 8.00.20; Bioquant Life Sciences, Nashville, TN, USA) that utilizes linear integrated optical density was used for comparing relative changes in immunoreactivity between groups. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Immunoreactive positive pixel count measurements were calculated from a circumscribed field (e.g., brain region), divided by the area of the region in square millimeters, and expressed as positive pixels/mm^2. Data were acquired from 3–4 sections/animal/brain region, and data were averaged within a brain region for an individual animal to obtain one value per subject. Inter-rater reliability was determined by calculating the intraclass-correlation coefficient for two raters blind to the experimental conditions. The polymorph dentate gyrus (DG), NAc, and mPFC were chosen randomly from 6 brain regions for analysis and values of $r = 0.85$ ($p < 0.001$), $r = 0.78$ ($p < 0.01$), and $r = 0.77$ ($p < 0.005$) were obtained, respectively. Intraclass-correlation coefficient was calculated using MATLAB (MathWorks, Natick, MA, USA). Immunoreactivity was measured separately for each brain region and statistically analyzed using Student's t-test (Prism, GraphPad Software, La Jolla, CA, USA) to compare the ethanol versus saline group within each brain region.

Brain region analyses were performed using histological coordinates as follows: mPFC (+3.00 to +2.20 AP), amygdala [central nucleus (CeA); lateral amygdala; basolateral amygdala; −2.56 to −3.14AP], NAc (+ 1.70 to + 1.00AP), dorsomedial striatum [DMS (+1.70 to + 1.00 AP)], VTA (−5.20 to −6.04 AP), hippocampus [pyramidal cell layer of CA1(−2.56 to −3.14 AP), polymorph (−2.56 to −3.14AP) and granule (−2.56 to −3.14AP) cell layers of the DG, BNST (−0.26 to −0.40 AP), hypothalamus [PVN,(−1.60 to −2.12 AP)]. All analyses were based on coordinates relative to bregma in the Rat Brain Atlas (Paxinos and Watson, 1998).

RESULTS

Ethanol-induced increases of cellular 3α,5α-THP

Previous studies using RIAs have shown ethanol-induced increases in 3α,5α-THP levels in the rat cortex and hippocampus (Barbaccia et al., 1999; VanDoren et al., 2000). Therefore, we first examined these regions to determine if the elevations displayed sub-region or cellular layer specificity. Ethanol administration (2g/kg, i.p.) increased 3α,5α-THP immunoreactivity in the mPFC [24±6%; $t(13)=2.996$, $p < 0.01$]. The effects of ethanol appear uniform across the cortical cell layers (Fig. 1A). In hippocampus, ethanol increased 3α,5α-THP immunoreactivity in the pyramidal cells of the CA1 region [32±12%; $t(14)=2.401$, $p < 0.05$] (Fig. 1B), and the polymorph cell layer of the DG [52±5%; $t(14)=5.288$, $p < 0.001$] (Fig. 2A), but had no effect on cellular 3α,5α-THP in the granule cell layer of the DG (Fig. 2B), indicating cellular layer specificity in the response to ethanol.

Ethanol also increased 3α,5α-THP immunoreactivity in brain regions involved in stress responses. We examined ethanol effects on cellular 3α,5α-THP levels in the PVN due to its role in modulating HPA axis activity. We also investigated ethanol's effects on cellular 3α, 5α-THP in the BNST due to its role as an interface between stress and reward circuitry and involvement in ethanol responses (Cui et al., 2012). Ethanol administration (2g/kg, i.p.) increased 3α,5α-THP immunoreactivity in both the PVN [36±6%; $t(14)=3.406$, $p < 0.01$] (Fig. 3A) and BNST [44±15%; $t(13)=2.346$, $p < 0.05$] (Fig. 3B).

Ethanol-induced decreases of cellular 3α,5α-THP in regions associated with ethanol reinforcement and consumption

Multiple studies suggest that 3α,5α-THP alters ethanol consumption (Morrow et al., 2001; Ford et al., 2005; Ford et al., 2007) and reinforcement (Janak et al., 1998; Janak and Michael Gill, 2003), so we examined the effects of ethanol administration (2g/kg, i.p.) in regions that are known to contribute to these phenomena. Ethanol administration did not alter 3α,5α-THP immunoreactivity in the VTA. Interestingly, ethanol administration decreased 3α,5α-THP immunoreactivity in the NAc [−22±5%; $t(12)=3.214$, $p < 0.01$] (Fig. 4A) but had no effect on 3α,5α-THP in the DMS (Fig. 4B) or VTA (Fig. 4C). The ethanol-induced reduction of 3α,5α-THP in the NAc was only detected in the NAc "shore" (core-shell border), as no changes in 3α,5α-THP were found in the NAc core or the NAc shell (data not shown), indicating sub-region specificity of ethanol effects on 3α,5α-THP levels in the ventral striatum. Ethanol administration reduced 3α,5α-THP immunoreactivity in the CeA [−21±3%; $t(14)=2.508$, $p < 0.05$] (Fig. 5A), but there was no change in 3α,5α-THP in the lateral (Fig. 5B) or basolateral amygdala (Fig. 5C). Therefore, ethanol produces sub-region specific effects on 3α,5α-THP in amygdala regions as well.

DISCUSSION

The goal of the present study was to use IHC to examine ethanol-induced changes in cellular 3α,5α-THP expression in multiple brain regions implicated in alcohol use disorders. Our findings support previous evidence that ethanol increases 3α,5α-THP concentrations in the rat cerebral cortex and hippocampus using RIA or GC-MS. We extend previous findings by

showing that ethanol increases cellular 3α,5α-THP in the mPFC, CA1 pyramidal cell layer, and polymorph cell layer of the DG but not in granule cells of the DG. Therefore, ethanol-induced increases of cellular 3α,5α-THP in the hippocampus are isolated to specific cellular populations. We also show that ethanol increases cellular 3α,5α-THP levels in the BNST and the PVN of the hypothalamus. Interestingly, acute ethanol administration reduced cellular 3α,5α-THP levels in the NAc "shore" (core-shell border) and the CeA. To our knowledge, this is the first example of acute ethanol reducing 3α,5α-THP levels in the rat brain or periphery. We also determined that ethanol does not alter 3α,5α-THP levels in the VTA, DMS, or the lateral or basolateral amygdala. Therefore, ethanol produces divergent brain region and cellular layer specific changes in 3α,5α-THP concentrations.

The immunohistochemical technique used in the present study provides brain region and cell layer specificity of ethanol-induced changes in 3α,5α-THP. A similar IHC assay was used to demonstrate that 3α,5α-THP is located primarily in GABAergic and glutamatergic projecting principle neurons, but not in interneurons or glia (Saalmann et al., 2007). The present results indicate similar relative intensity of basal staining across brain, with strong labeling of cortical and hippocampal cell layers as well as the striatum. There are some important differences between measurements of 3α,5α-THP with IHC compared to RIA measurements. IHC measures cellular levels of 3α,5α-THP but RIA measures absolute levels of neuroactive steroids. Therefore, if there are significant pools of extraneuronal 3α, 5α-THP present in brain tissue, one might predict IHC measurements would differ from results obtained by RIA. Our current IHC results in the mPFC, CA1 pyramidal, and DG polymorph cell layer show that ethanol increased relative 3α,5α-THP immunoreactivity by 24±6%, 32±12%, and 52±5%, respectively. However, RIA studies measuring ethanol-induced increases of 3α,5α-THP in whole cerebral cortex and hippocampus have found larger increases of 3α,5α-THP. For example, increases of 5–17 fold (Barbaccia et al., 1999),~3 fold (VanDoren et al., 2000; Khisti et al., 2004), and ~2 fold (Boyd et al., 2010) have been observed in the cerebral cortex, while studies examining whole hippocampus have found 4–11(Barbaccia et al., 1999) and ~6 fold (Khisti et al., 2004) increases in 3α,5α-THP concentrations. The discrepancies in magnitude of ethanol-induced increases of 3α,5α-THP could be due to relatively small increases of 3α,5α-THP in the mPFC when compared to the whole cortex, and similarly, small relative increases of 3α,5α-THP in the CA1 pyramidal cells and the polymorph cell layer of the DG compared to whole hippocampus. It is important to note we are using an affinity purified 3α,5α-THP antiserum that appears to be more specific in detecting 3α,5α-THP. The only other study examining ethanol's effects on 3α,5α-THP using the affinity purified 3α,5α-THP antiserum used IHC with immunofluorescent detection and found over a 200% increase in fluorescent intensity in CA1 pyramidal cells (Tokuda et al., 2011) compared to a 32±12% increase in immunoreactivity in the present study using DAB detection. Although ethanol increased 3α, 5α-THP using both fluorescent and DAB visualization, the magnitude of 3α,5α-THP changes may differ due to differences in immunohistochemical methodology.

The 3α,5α-THP antisera used in the present study was found to cross react with 3α-HP (41±0.14%) and 3α,5β-THP (22±0.43%). This is not a major limitation since both of these steroids have similar GABAergic activities as 3α,5α-THP. Furthermore, there is no evidence that 3α-HP (Griffin and Mellon, 2001) or 3α,5β-THP are present in the brain in appreciable

concentrations. In addition, ethanol does not elevate 3α,5β-THP levels in rat plasma (Porcu et al., 2010). Therefore, 3α,5α-THP is the most likely endogenous antigen immunolabelled using this approach. A potential limitation of the study is that only one dose of ethanol was examined. In the cerebral cortex, a threshold dose of ethanol (1.3–1.5 g/kg) is required to produce an increase of 3α,5α-THP and increasingly higher doses produce less of an increase of 3α,5α-THP (VanDoren et al., 2000; Boyd et al., 2010). However, since only one dose was used it is not known if a similar threshold dose is needed to produce increases or decreases of 3α,5α-THP in the cortical and subcortical brain regions examined in the present study. Similarly, it is not clear if ethanol produces a biphasic effect on 3α,5α-THP levels in these brain regions.

The diverse effects of ethanol administration on cellular 3α,5α-THP levels suggest that ethanol may alter local synthesis and/or metabolism of 3α,5α-THP in the rat brain. These results suggest ethanol-induced changes of cellular 3α,5α-THP in the brain are not due exclusively to adrenal gland derived elevations of neuroactive steroids or steroid precursors, which would be expected to increase 3α,5α-THP throughout the brain. However, we observed no change in 3α,5α-THP levels after acute ethanol administration in the granule cell layer of the DG and the DMS, which both display dense labeling of 3α,5α-THP(Saalmann et al., 2007) as well as the biosynthetic enzymes 5α-R and 3α-hydroxysteroid dehydrogenase (3α-HSD) needed for 3α,5α-THP synthesis (Agis-Balboa et al., 2006). Furthermore, there was a reduction of cellular 3α,5α-THP in the NAc and the CeA. Based on these findings our data suggest there are unknown mechanisms contributing to ethanol's effects on 3α,5α-THP concentrations. In the cerebral cortex, ethanol-induced elevations of 3α,5α-THP are dependent on the pituitary-adrenal axis (Khisti et al., 2003; O'Dell et al., 2004; Boyd et al., 2010). *In vitro*, however, there is evidence for ethanol-induced brain synthesis of 3α,5α-THP in hippocampal slices (Sanna et al., 2004; Tokuda et al., 2011). Thus, it is not known if ethanol alters brain synthesis of 3α,5α-THP *in vivo* independent of circulating steroids. Therefore, it will be important to determine the role of circulating steroids in the current observations, which may provide insight into this important question.

The most striking finding in the present study is that ethanol produces brain region and cellular layer specific changes in 3α,5α-THP concentrations. These data suggest ethanol alters local synthesis of 3α,5α-THP in the rat brain. A possible mechanism that may explain these findings is ethanol may change local expression and/or activity of steroidogenic enzymes. Steroidogenesis is initiated by cholesterol transport to cytochrome P450 side chain cleavage (P450scc), resulting in conversion of cholesterol to pregnenolone. Previous work from our laboratory has shown that *de novo* adrenal synthesis of the cholesterol transporter, steroidogenic acute regulatory protein (StAR), is necessary for ethanol-induced increases of 3α,5α-THP in the cerebral cortex (Boyd et al., 2010). Acute ethanol administration increases StAR and P450scc mRNA in the rat frontal cortex and hypothalamus, and StAR mRNA is also increased in the hippocampus (Kim et al., 2003). The synthesis of 3α,5α-THP from progesterone is accomplished by the sequential actions of 5α-R and 3α-HSD. Acute ethanol administration increases 5α-R type 1 (5α-RI) and 3α-HSD mRNA in the frontal cortex and 3α-HSD mRNA in the hypothalamus, but no change in transcript expression of either

enzyme were detected in the hippocampus (Kim et al., 2003). Unfortunately, there are no data examining the effects of acute ethanol on expression of these enzymes in the NAc or CeA where we observed reductions in 3α,5α-THP. However, chronic intermittent ethanol administration reduces 3α,5α-THP levels in the hippocampus, which is associated with concurrent decreases in 5α-RI and 3α-HSD mRNA expression (Cagetti et al., 2004). Taken together, ethanol-induced changes in steroidogenic enzyme expression may underlie the divergent changes in 3α,5α-THP levels observed in the rat brain. Additionally, ethanol may directly or indirectly change the activity of steroidogenic enzymes, which could alter steroid concentrations. Ultimately, ethanol may alter the expression and/or activity of enzymes involved in steroid synthesis and metabolism, resulting in local changes in neurosteroid levels that are dependent on which enzymes are expressed in a particular cell.

Another possible explanation for the reduction in cellular 3α,5α-THP levels observed in the NAc and CeA is extracellular redistribution of 3α,5α-THP. Neuroactive steroids have been proposed to act on membrane bound receptors by a paracrine or autocrine mechanism (Herd et al., 2007), or by lateral diffusion through the cell membrane to access transmembrane neuroactive steroid binding sites on $GABA_A$ receptors (Akk et al., 2007). Although controversial, we cannot rule out the possibility that ethanol leads to a “release” or redistribution of 3α,5α-THP into the extracellular space, perhaps in a brain region specific manner.

Significance of ethanol-induced changes in cellular expression of 3α,5α-THP

Evidence suggests ethanol-induced increases of 3α,5α-THP contribute to the behavioral effects of ethanol in rodents and some of the subjective effects of alcohol in humans. Contribution of 3α,5α-THP to the pharmacological effects of ethanol is likely due to potentiation of $GABA_A$ receptors across multiple brain regions. The localization of 3α,5α-THP and the biosynthetic enzymes 5α-R and 3α-HSD in principle output neurons suggests a major role of 3α,5α-THP may be to modulate neural circuitry (Agis-Balboa et al., 2006; Saalmann et al., 2007). The neurophysiological effects of 3α,5α-THP have been best characterized in hippocampal pyramidal cells. *In vivo*, ethanol (1.5g/kg) reduces spontaneous activity of hippocampal pyramidal cells in anesthetized rats, which is prevented by finasteride pre-administration (Tokunaga et al., 2003). *In vitro*, ethanol (50, 100 mM) increases 3α,5α-THP in isolated hippocampal tissue and amplitude of $GABA_A$ receptor mediated spontaneous and evoked IPSCs in CA1 pyramidal cells (Sanna et al., 2004), the latter of which is prevented by pretreatment with finasteride. Ethanol (60 mM) also inhibits LTP in CA1 pyramidal cells, with concomitant increases in 3α,5α-THP immunostaining (Tokuda et al., 2011). Furthermore, pretreatment with finasteride blocks ethanol’s inhibition on LTP, suggesting that ethanol’s ability to inhibit LTP is mediated by 3α,5α-THP. In the present study, ethanol increased 3α,5α-THP in the polymorphic cell layer but not the granule cell layer of the DG. Although we did not distinguish which polymorphic cells exhibit increased 3α,5α-THP, the most prominent cell type in this layer is the mossy cell. Mossy cells in the polymorphic layer project to the molecular and granule cell layers of the DG(Amaral et al., 2007), the latter of which constitutes the only projection from the DG to the hippocampus. Considering the major role the hippocampus and DG play in learning and

memory, 3α,5α-THP induction by ethanol may contribute to ethanol's effects on memory and cognition.

Ethanol-induced changes in brain concentrations of 3α,5α-THP may also modulate neuronal activity in other brain regions examined in the present study. *In vivo* evidence suggests ethanol-induced increases of 3α,5α-THP modulate neuronal activity outside of the hippocampus as well. For example, finasteride prevents ethanol (1.5g/kg) inhibition of spontaneous firing of medial septal/diagonal band of Broca neurons (VanDoren et al., 2000). In addition to hippocampal pyramidal cells, we observed increases of cellular 3α,5α-THP in the polymorph DG, mPFC, BNST, and PVN of the hypothalamus. The physiological consequences of 3α,5α-THP increases may alter neuronal activity and synaptic plasticity in these regions and related circuitry. Clearly, studies are needed to examine how increases of 3α,5α-THP in these brain regions may contribute to the physiological and behavioral effects of ethanol.

Ethanol-induced reductions of cellular 3α,5α-THP were observed in the NAc and CeA. These observations are particularly interesting because these two brain regions are strongly associated with ethanol reinforcement and consumption (McBride, 2002; Gonzales et al., 2004). A reduction in cellular 3α,5α-THP would presumably reduce the amount of $GABA_A$ receptor mediated neuronal inhibition, which may increase firing of mesolimbic dopamine neurons and CeA circuitry involved in ethanol reinforcement. In the NAc, reductions of 3α,5α-THP were isolated to the "shore" (core-shell border), as no changes in 3α,5α-THP were detected exclusively in the shell or core. This is an interesting finding since ethanol associated cues and operant self-administration of ethanol both increase dopamine release in the "shore" following operant training (Howard et al., 2009), but in the core or shell only ethanol associated cues increase dopamine release following operant training. It is important to note, however, that acute systemic ethanol administration increases dopamine release in the NAc core and shell (Imperato and Di Chiara, 1986; Yim et al., 2000) where ethanol did not alter 3α,5α-THP levels. Administration of 3α,5α-THP produces biphasic effects on ethanol consumption (Janak et al., 1998; Ford et al., 2005; Ford et al., 2007; Finn et al., 2010) and dopamine release in the NAc (Motzo et al., 1996; Rouge-Pont et al., 2002). Furthermore, 3α,5α-THP modulates ethanol's effects on mPFC dopamine content (Dazzi et al., 2002). Therefore, investigating possible interactions of 3α,5α-THP and dopaminergic activity in the mesocorticolimbic system may be valuable in the effort to reduce ethanol consumption via neuroactive steroid modulation.

The current data show that ethanol produces divergent brain region and cell layer specific changes in cellular 3α,5α-THP concentrations. These divergent effects suggest that ethanol-induces local regulation of 3α,5α-THP levels. These changes in 3α,5α-THP levels likely contribute to the neurophysiological and behavioral effects of ethanol in rats. Examining the mechanisms by which ethanol alters levels of 3α,5α-THP across the brain may lead to new therapeutic strategies for treating alcohol use disorders.

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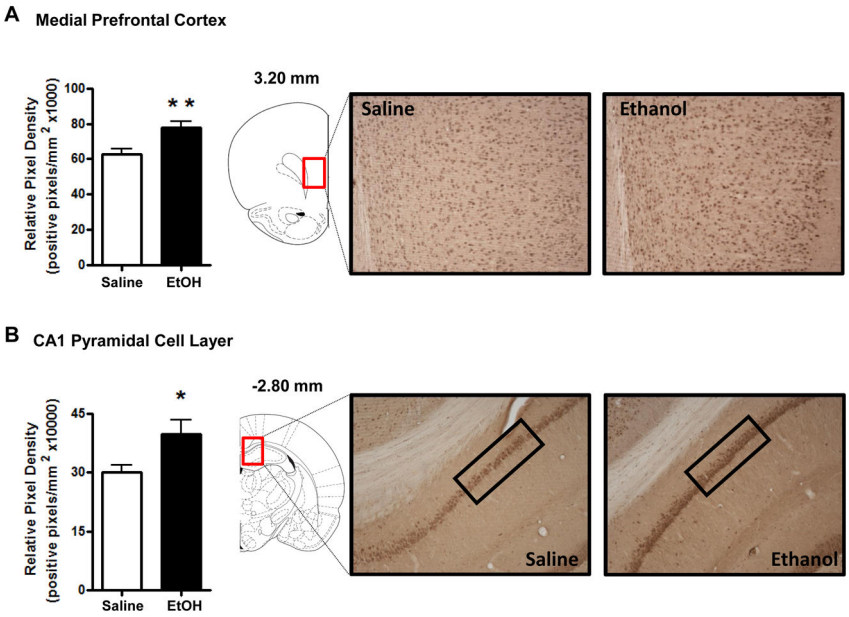

Figure 1.

Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the mPFC and pyramidal cell layer of the CA1 hippocampus. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the mPFC compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the mPFC(3.20 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration.(B) Ethanol administration increased 3α,5α-THP immunoreactivity in the pyramidal cell layer of the CA1 hippocampus compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in CA1 pyramidal cells (highlighted in rectangle, −2.80 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ± SEM. * $p < 0.05$, ** $p < 0.01$ compared to saline administration. Medial prefrontal cortex (mPFC), ethanol (EtOH), Cornu Ammonis area 1 (CA1).

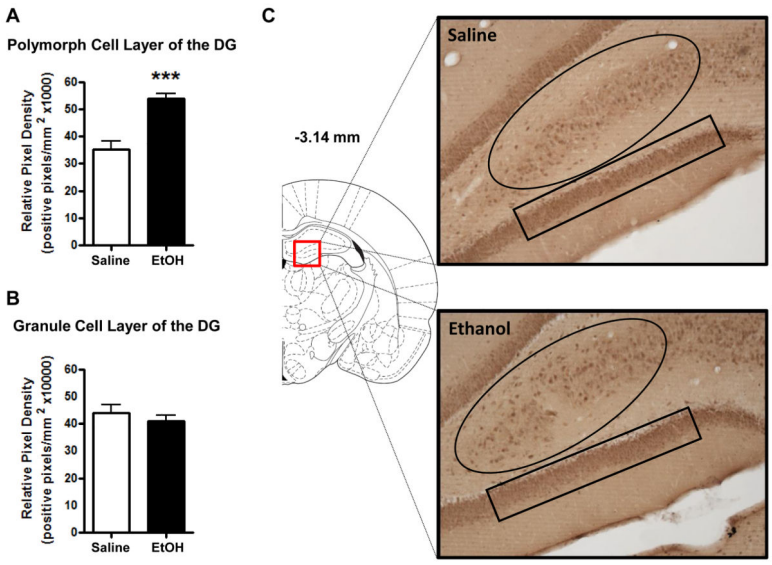

Figure 2.

Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the polymorph and granule cell layers of the DG. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the polymorph cell layer compared to saline controls. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the granule cell layer compared to saline controls. (C) Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the polymorph cell layer (oval) and granule cell layer (rectangle) of the DG (−3.14 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ±SEM. *** $p < 0.001$ compared to saline administration. Dentate gyrus (DG), ethanol (EtOH).

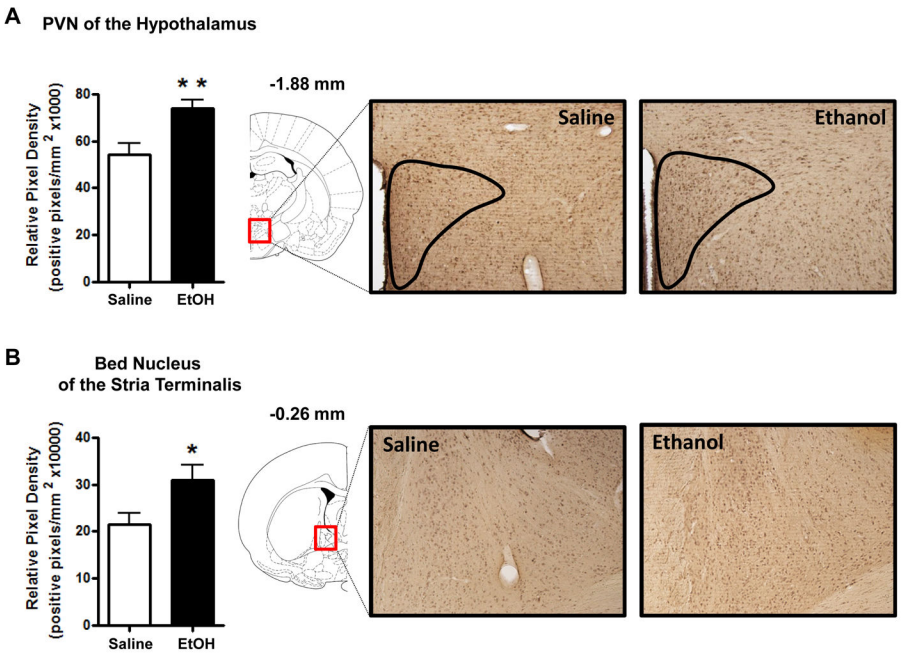

Figure 3.

Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in the PVN of the hypothalamus and BNST. (A) Ethanol administration increased 3α,5α-THP immunoreactivity in the PVN compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the PVN (−1.88 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. (B) Ethanol administration also increased 3α, 5α-THP immunoreactivity in the BNST compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in BNST (−0.26 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ±SEM. * $p < 0.05$, ** $p < 0.01$ compared to saline administration. Paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BNST), ethanol (EtOH).

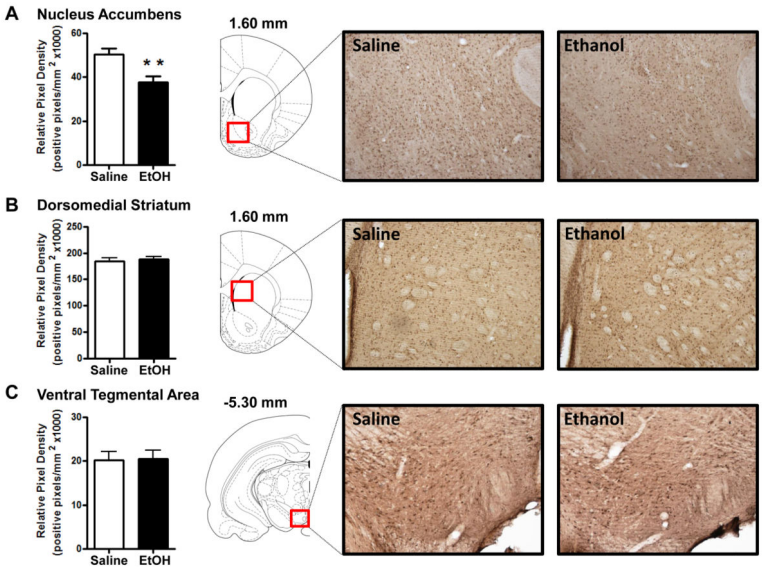

Figure 4.

Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in NAc "shore" (core/shell border), DMS, and VTA. (A) Ethanol administration reduced 3α,5α-THP immunoreactivity in the NAc "shore" compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in NAc "shore" (1.60 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the DMS compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in DMS (1.60 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. (C) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the VTA compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the VTA (−5.30 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ±SEM. ** $p < 0.01$ compared to saline administration. Nucleus accumbens (NAc), dorsomedial striatum (DMS), ventral tegmental area (VTA), ethanol (EtOH).

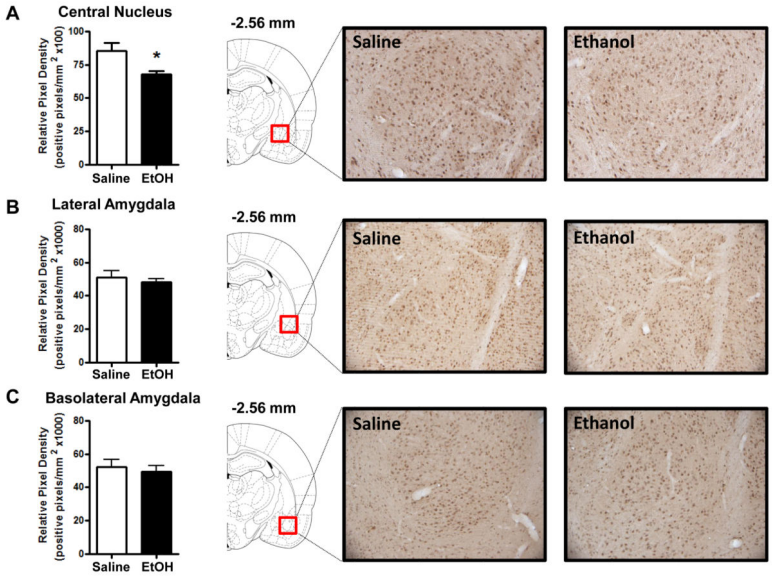

Figure 5.

Effect of acute ethanol administration (2g/kg, i.p.) on 3α,5α-THP immunoreactivity in CeA, lateral amygdala, and basolateral amygdala. (A) Ethanol administration reduced 3α,5α-THP immunoreactivity in the CeA compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in CeA (−2.56 mm relative to bregma) following saline (n=8) or ethanol (n=8) administration. (B) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the lateral amygdala compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the lateral amygdala (−2.56 mm relative to bregma) following saline (n=8) or ethanol (n=7) administration. (C) Ethanol administration did not alter 3α,5α-THP immunoreactivity in the basolateral amygdala compared to saline controls. Representative photomicrographs (10x) of 3α,5α-THP immunoreactivity in the basolateral amygdala (−2.56 mm relative to bregma) following saline (n=7) or ethanol (n=7) administration. Ethanol (2g/kg, i.p.) or saline were administered 60 min prior to tissue fixation and collection. Data are expressed as mean positive pixels/mm^2 ±SEM. * $p < 0.05$ compared to saline administration. Central nucleus of the amygdala (CeA), ethanol (EtOH).