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Ethanol induction of steroidogenesis in rat adrenal and brain is dependent upon pituitary ACTH release and *de novo* adrenal StAR synthesis

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Abstract

The mechanisms of ethanol actions that produce its behavioral sequelae involve the synthesis of potent GABAergic neuroactive steroids, specifically the GABAergic metabolites of progesterone, (3 α ,5 α)-3-hydroxypregnan-20-one (3 α ,5 α -THP), and deoxycorticosterone, (3 α ,5 α)-3,21-dihydroxypregnan-20-one. We investigated the mechanisms that underlie the effect of ethanol on adrenal steroidogenesis. We found that ethanol effects on plasma pregnenolone, progesterone, 3 α ,5 α -THP and cortical 3 α ,5 α -THP are highly correlated, exhibit a threshold of 1.5 g/kg, but show no dose dependence. Ethanol increases plasma adrenocorticotrophic hormone (ACTH), adrenal steroidogenic acute regulatory protein (StAR), and adrenal StAR phosphorylation, but does not alter levels of other adrenal cholesterol transporters. The inhibition of ACTH release, *de novo* adrenal StAR synthesis or cytochrome P450 side chain cleavage activity prevents ethanol-induced increases in GABAergic steroids in plasma and brain. ACTH release and *de novo* StAR synthesis are independently regulated following ethanol administration and both are necessary, but not sufficient, for ethanol-induced elevation of plasma and brain neuroactive steroids. As GABAergic steroids contribute to ethanol actions and ethanol sensitivity, the mechanisms of this effect of ethanol may be important factors that contribute to the behavioral actions of ethanol and risk for alcohol abuse disorders.

Keywords

ACTH; adrenal; brain; ethanol; GABA; neuroactive steroid

Neuroactive steroids are endogenous allosteric modulators of GABA_A receptor function that contribute to the behavioral effects of various psychoactive drugs including ethanol (see Morrow 2007 for review). Acute ethanol administration increases plasma and brain concentrations of the GABAergic neuroactive steroids (3 α ,5 α)-3-hydroxypregnan-20-one (3 α ,5 α -THP) and (3 α ,5 α)-3,21-dihydroxypregnan-20-one (3 α ,5 α -THDOC) (Barbaccia *et al.* 1999; Morrow *et al.* 1999). These neuroactive steroids, including 3 α ,5 α -THP, are found in the periphery and central nervous system of rats, as well as humans, and finasteride inhibition of steroidogenesis inhibits some of ethanol's actions in both rats and man (Morrow 2007). Indeed, inhibition of steroid biosynthetic enzymes, or the use of adrenalectomized rodents, has demonstrated that neuroactive steroids contribute to ethanol's inhibitory actions on medial septal and hippocampal neurons (VanDoren *et al.* 2000; Tokunaga *et al.* 2003; Morrow *et al.* 2005), anxiolytic (Hirani *et al.* 2005), anti-convulsant (VanDoren *et al.* 2000), hypnotic effects (Khisti *et al.* 2003) and ethanol-induced spatial learning deficits (Matthews *et al.* 2002). As ethanol-induced elevations in neuroactive steroids contribute to ethanol actions, it is important to understand mechanisms that regulate the synthesis of these steroids.

Steroid levels fluctuate naturally and in response to various stressors and challenges. Steroidogenic organs include the adrenals, testis, ovaries, placenta, and brain. Biosynthesis of adrenal steroids is primarily initiated upon stimulation by trophic hormones and involves activation of adenylate cyclase activity resulting in increased cAMP levels and protein kinase A (PKA) activation (Brownie *et al.* 1973; Stocco *et al.* 2005). The cascade of signals that stems from trophic hormone stimulation increases cholesterol transport to the cytochrome P450 side chain cleavage (P450_{sc}) enzyme that resides on the inner mitochondrial membrane. This is the rate-limiting step in steroidogenesis (Miller 1988; Stocco 2000) and is thought to be mediated by cholesterol transport proteins such as steroidogenic acute regulatory protein (StAR) (Stocco and Clark 1996) and the mitochondrial benzodiazepine receptor (Lacapere and Papadopoulos 2003), now referred to as translocator protein (TSPO-18 kDa) (Papadopoulos *et al.* 2006). Indeed, mutations or deletions in the StAR gene disrupt steroid production causing congenital lipid adrenal hyperplasia (Lin *et al.* 1995; Miller 1997).

As multiple glands can synthesize steroids, understanding the mechanisms of steroidogenesis in adrenal and brain is critical for studying neuroactive steroids. Ethanol administration appears to mimic stress to activate the hypothalamic-pituitary-adrenal (HPA) axis and induce adrenal steroidogenesis. Indeed, previous studies have shown that ethanol-induced pituitary adrenocorticotrophic hormone (ACTH) release appears to require both corticotrophin releasing factor (CRF) and vasopressin (Lee *et al.* 2004). Stress or ethanol-induction of neuroactive steroids in plasma and brain are completely prevented by adrenalectomy, although neuroactive steroids are still detectable in the brain of adrenalectomized rats (Purdy *et al.* 1991; Khisti *et al.* 2003; O'Dell *et al.* 2004; Porcu *et al.* 2004). Interestingly, ethanol-induced steroidogenesis has been directly demonstrated in hippocampal slices *in vitro* (Sanna *et al.* 2004) and recent studies have shown that ethanol increases StAR expression in rat brain (Serra *et al.* 2006). Therefore, while several studies have shown that ethanol increases neuroactive steroids, as well as the importance of the adrenals for this effect, the adrenal mechanisms that are involved have not been elucidated, and the role of the brain in ethanol-induced steroidogenesis *in vivo* remains unclear.

Using an *in vivo* rat model, this study examines the steroidogenic pathway including key enzymes, signaling molecules and cholesterol transport proteins to investigate which factors are critical for ethanol-induced increases of neuroactive steroids in adrenals and brain. Moreover, by concurrently measuring both plasma and brain steroid concentrations, we

examined the importance of adrenal steroid synthesis for regulating ethanol-induced increases of neuroactive steroids in the brain.

Materials and methods

Animals

Adult male Sprague–Dawley rats weighing between 225 and 350 g were used for all experiments (Harlan, Indianapolis, IN, USA). The animals were group housed (three per cage) and maintained in standard light and dark (lights on, 7:00 _{AM} to 7:00 _{PM}) conditions with food and water available *ad libitum*. Rats were acclimated to handling for 1 week before the test day. Hypophysectomized and sham-operated animals were ordered from Harlan and experiments were carried out 2–3 weeks post-surgery. All experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina Chapel Hill.

Drug administration

Unless otherwise stated, animals were administered i.p. injections of saline or ethanol (2 g/kg, 20% v/v). The protein synthesis inhibitor cycloheximide (20 mg/mL, i.p.) was dissolved in saline and administered as a 1 mL injection either concurrently with ethanol or vehicle, or 40 min post-administration of ethanol or vehicle. The TSPO (18 kDa) agonist CB34 (15 mg/kg, i.p.) was dissolved in saline while the TSPO (18 kDa) antagonist PK11195 (1 mg/kg, i.p.) was dissolved in Tween 80, diluted with saline, and given 30 min prior to ethanol or saline. The glucocorticoid receptor activator dexamethasone 21-phosphate disodium salt (0.1 mg/kg, i.p.) was dissolved in saline and administered 90 min prior to ethanol or saline. The P450_{ssc} antagonist aminoglutethimide (10 mg/mL, i.p.) was dissolved in propylene glycol, diluted with saline and administered as a 2 mL injection 1 h prior to an ethanol or saline injection.

Tissue and protein preparations

Mitochondrial membrane fractions from adrenal glands were prepared by homogenization, low speed centrifugation in 0.32 M sucrose and centrifugation of the supernatant at 17 000 g for 30 min. The pellet (mitochondrial fraction) was resuspended in phosphate buffered saline. Individual cerebral cortices, as well as other brain regions, were homogenized directly in 2% sodium dodecyl sulfate (SDS). Protein measurement was conducted using a BCA protein assay (Thermo Fisher Scientific Inc, Rockford, IL, USA).

Western blot analysis

Adrenal mitochondrial fractions and cerebral cortical homogenate were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using Novex Tris–Glycine gels (8–16%) and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). These membranes were probed with StAR (1 : 1000) (Abcam, Cambridge, MA, USA), metastatic lymph node 64 (MLN64) (1 : 1000) (Abcam) or mitochondrial benzodiazepine receptor (called TSPO 18 kDa in this manuscript) (1 : 1000) (Trevigen, Gaithersburg, MD, USA) antibodies. Blots were subsequently exposed to a second primary antibody directed against β -actin to verify equivalent protein loading and transfer. Bands were detected by enhanced chemiluminescence (GE Life-science, Amersham, UK), apposed to x-ray films under non-saturating conditions, and analyzed by densitometric measurements using NIH Image 1.57. All comparisons were made within blots and statistical analysis was conducted using Student's *t*-test or ANOVA.

Phospho-PKA substrate immunoprecipitation analysis

Protein in the mitochondrial fraction was immunoprecipitated with phospho-PKA substrate antibody (Cell Signaling Technology Inc., Danvers, MA, USA) similar to the method previously described (Kumar *et al.* 2002). Briefly, mitochondrial protein (200 µg) was solubilized and denatured in radioimmunoprecipitation buffer (Sigma-Aldrich, St. Louis, MO, USA) with phosphatase inhibitor cocktail (Sigma-Aldrich), phenylmethylsulfonyl fluoride (1 mM), sodium fluoride (50 mM), sodium vanadate (200 µM), and EDTA (2 mM) to prevent protein degradation and dephosphorylation. Solubilized protein was centrifuged at 10 000 g and supernatant (solubilized protein) was collected. Denaturation of protein in the supernatant was confirmed by polyacrylamide gel electrophoresis.

Immunoprecipitation of phosphoproteins for the detection of phospho-StAR was performed using antibody conjugated to Dynal beads (Invitrogen) and western blot analysis of the immunoprecipitate. The optimal antibody and protein concentrations for immunoprecipitation were determined in pilot experiments to optimize the conditions. The phospho-PKA substrate specific antibody or IgG (Rockland Inc., Gilbertsville, PA, USA) was linked to Dynal beads by incubating 125 µL of Dynal beads with 100 µL of antibody (0.35 µg/µL) for 1 h at ~25°C. The solubilized receptors were mixed with antibody-linked beads in a final volume of 500 µL and incubated in an orbital shaker overnight at 4°C. The receptor-antibody-bead complex was washed three times with phosphate buffered saline, resuspended in 50 µL of 1× SDS, and boiled for 5 min. Phosphoprotein immunoprecipitates and adrenal mitochondrial fractions were analyzed by SDS-PAGE gel electrophoresis and western blotting from saline or ethanol-treated animals to examine the effects on immunoprecipitated phospho-StAR protein as well as total StAR protein.

Radioimmunoassay of neuroactive steroid 3α,5α-THP

Radioimmunoassays (RIAs) were conducted as previously described (Janis *et al.* 1998). Briefly, brain samples were weighed and suspended in 2.5 mL of 0.3 N NaOH, homogenized with a sonic dismembrator, and extracted three times with 3 mL aliquots of 10% ethyl acetate in heptane (v/v). Extraction recovery was monitored by the addition of 2000 cpm of [³H]3α,5α-THP. The brain extracts were purified using solid phase silica columns (Burdick and Jackson, Muskegon, MI, USA) and subsequently dried. Samples were reconstituted and assayed in duplicate by the addition of [³H]3α,5α-THP and anti-3α,5α-THP polyclonal sheep antibody (Obtained from Dr. Robert Purdy, Scripps Research Institute). Total binding was determined in the absence of unlabeled 3α,5α-THP and non-specific binding was determined in the absence of antibody. The antibody binding reaction was allowed to equilibrate for 2 h and cold dextran-coated charcoal was used to separate bound from unbound steroid. Bound radioactivity was determined by liquid scintillation spectroscopy. Steroid levels in the samples were extrapolated from a concurrently run standard curve and corrected for their respective extraction efficiencies. The inter-assay coefficient of variation was 9.1% and the intra-assay coefficient of variation is 2.2%. The 3α,5α-THP antibody has minimal cross-reactivity with other circulating steroids (Janis *et al.* 1998). 3α,5α-THP antiserum cross-reacts with progesterone < 3%, 3α,5β-THP 6.6%, 3β,5α-THP 2.8%, 3β,5β-THP 0.5%, 5α-pregnan-3α,20α-diol 0.1% and 5α-pregnan-3,20-dione 3.5%. The antiserum cross-reacts with 3α-hydroxy-4-pregnen-20-one > 100%. This steroid is also a potent modulator of GABA_A receptors (Morrow *et al.* 1990) and has been found at comparable levels to 3α,5α-THP in serum (Wiebe *et al.* 1994), but at markedly lower levels than 3α,5α-THP in cerebral cortex (Griffin and Mellon 2001).

RIA of steroid precursor pregnenolone

Pregnenolone levels were measured by RIA as previously described (Porcu *et al.* 2006). Briefly, pregnenolone was extracted from 250 µL of plasma with 2 mL of diethyl ether three

times. Extraction recovery was monitored by the addition of 1000 cpm of [^3H]pregnenolone. Samples were reconstituted and assayed in duplicate by the addition of [^3H]pregnenolone and anti-pregnenolone antibody (MP Biomedicals, Orangeburg, NY, USA). Total binding was determined in the absence of unlabeled pregnenolone and non-specific binding was determined in the absence of antibody. The antibody binding reaction was allowed to equilibrate for a minimum of 4 h and cold dextran coated charcoal was used to separate bound from unbound steroid. Bound radioactivity was determined by liquid scintillation spectroscopy. Steroid levels in the samples were extrapolated from a concurrently run standard curve and corrected for their respective extraction efficiencies. The intra-assay and inter-assay coefficients of variation were 7.71% and 5.93% respectively. The antiserum cross-reacts with $3\alpha,5\alpha$ -THP 16%, $3\alpha,5\beta$ -THP 5.9%, progesterone 3.1%, $3\alpha,5\alpha$ -THDOC 1.1%. All of the following steroids had less than 1% cross-reactivity: 5α -dihydroprogesterone, 17α -hydroxyprogesterone, deoxycorticosterone, cortisol, 11-deoxycortisol, corticosterone, androsterone, 5α -dihydrotestosterone, cholesterol, 17β -estradiol, estrone and estriol.

RIA of progesterone and ACTH

Plasma progesterone and ACTH levels were measured using RIA kits according to the manufacturer's instructions (MP Biomedicals, Costa Mesa, CA, USA). Total binding was determined in the absence of unlabeled progesterone or ACTH and non-specific binding was determined in the absence of antibody. Steroid levels in the samples were extrapolated from a concurrently run standard curve. The intra-assay and inter-assay coefficients of variation for progesterone are 3.6% and 6.7% and for ACTH are 4.1% and 3.9%, respectively.

Data analysis

Results are expressed as mean $\pm$ SEM. Steroid levels are expressed as ng/g for brain tissue and ng/mL for plasma. Western blot data are normalized to β -actin signals from the same blot and expressed as % control values taken from each blot. Significance was determined by ANOVA followed by *post-hoc* Newman-Keuls or Bonferroni test or the Student's *t*-test as appropriate. Analyses were performed using the software GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA).

Results

First, we investigated the threshold and dose effects of ethanol-induced increases in plasma pregnenolone and progesterone as well as plasma and cerebral cortical $3\alpha,5\alpha$ -THP. Ethanol (0.5–3.0 g/kg) increases plasma levels of $3\alpha,5\alpha$ -THP and its precursors, pregnenolone and progesterone, at doses of 1.5 g/kg and above compared to saline controls (Fig. 1a–c). For plasma pregnenolone, the 0.5 g/kg group was excluded from the analysis as only one value was detectable [$F(5,14) = 26.54$, $p < 0.0001$; Fig. 1a]. There were also significant differences between groups for plasma progesterone [$F(6,21) = 30.32$, $p < 0.0001$; Fig. 1b], and $3\alpha,5\alpha$ -THP; although all $3\alpha,5\alpha$ -THP levels in the 0.5 g/kg group were below the limit of detection [$F(5,13) = 48.67$, $p < 0.0001$; Fig. 1c]. Interestingly, there does not appear to be a dose dependent effect of ethanol on plasma pregnenolone, progesterone or $3\alpha,5\alpha$ -THP as the 1.5 g/kg dose elicited a maximal response that was not increased by greater doses of ethanol. In cerebral cortex, 1.5 g/kg ethanol was also the lowest dose that increased $3\alpha,5\alpha$ -THP levels [$F(5,15) = 7.23$, $p = 0.0013$; Fig. 1d] demonstrating the same threshold response as plasma steroid levels. Ethanol-induced increases in plasma pregnenolone were highly correlated with plasma progesterone levels, and plasma progesterone was highly correlated with plasma $3\alpha,5\alpha$ -THP levels ($r = 0.75$ and 0.86 respectively, $p < 0.03$, Spearman correlation). Furthermore, plasma $3\alpha,5\alpha$ -THP levels were highly correlated with cerebral cortical $3\alpha,5\alpha$ -THP levels ($r = 1$, $p = 0.0014$, Spearman correlation). Thus, in subsequent

experiments, either pregnenolone or progesterone was measured in plasma while $3\alpha,5\alpha$ -THP was measured in cerebral cortex.

Following acute stress, ACTH is released from the pituitary to stimulate steroid production from the adrenals. To determine the role of ACTH in ethanol-mediated steroidogenesis, we investigated the effects of hypophysectomy and dexamethasone in adult rats. Ethanol (2 g/kg) increased plasma ACTH, plasma progesterone and cerebral cortical $3\alpha,5\alpha$ -THP levels in intact animals (Fig. 2). Hypophysectomized animals did not exhibit any changes in plasma ACTH, plasma progesterone, or cerebral cortical $3\alpha,5\alpha$ -THP levels (Fig. 2). There was a main effect of both ethanol [$F(1,14) = 50.18, p < 0.0001$] and hypophysectomy on ACTH levels [$F(1,14) = 58.06, p < 0.0001$] as well as an interaction between ethanol and hypophysectomy [$F(1,14) = 50.58, p < 0.0001$] (Fig. 2a). Ethanol also increased plasma progesterone levels of intact animals 36-fold (Fig. 2b). There was a main effect of both ethanol [$F(1,14) = 228.8, p < 0.0001$] and hypophysectomy [$F(1,14) = 249.3, p < 0.0001$] as well as an interaction between ethanol and hypophysectomy [$F(1,14) = 233.7, p < 0.0001$] (Fig. 2b). Furthermore, ethanol increased cerebral cortical $3\alpha,5\alpha$ -THP levels of intact animals ninefold (Fig. 2c). There was a main effect of both ethanol [$F(1,12) = 34.56, p < 0.0001$] and hypophysectomy [$F(1,12) = 23.68, p = 0.0004$] as well as an interaction between ethanol and hypophysectomy [$F(1,12) = 34.96, p < 0.0001$].

Pretreatment with the synthetic steroid dexamethasone also prevented ethanol-induced increases in plasma ACTH, plasma progesterone, and cerebral cortical and hippocampal $3\alpha,5\alpha$ -THP. There were significant differences between groups for plasma ACTH [$F(3,19) = 28.52, p < 0.0001$; Fig. 3a], plasma progesterone [$F(3,19) = 110.5, p < 0.0001$; Fig. 3b], and cerebral cortical and hippocampal $3\alpha,5\alpha$ -THP levels [$F(3,19) = 9.07, p = 0.001$; Fig. 3c]. However, the statistical analysis for brain $3\alpha,5\alpha$ -THP levels only compares the dexamethasone + ethanol group to the ethanol group as most of the saline values were below the limit of detection. In contrast, dexamethasone enhanced ethanol-induced increases in adrenal StAR protein expression by 65% [$F(2,43) = 26.61, p < 0.0001$; Fig. 3d]. Together, these results suggest the importance of ACTH for ethanol-mediated steroidogenesis.

In order to evaluate the importance of *de novo* StAR protein synthesis in ethanol-induced steroidogenesis we investigated the time course and effects of ethanol dose on adrenal StAR expression. Similar to the plasma steroid levels seen in Fig. 1, there was a threshold of 1.5 g/kg ethanol required to elicit increases in plasma ACTH levels [$F(6,21) = 42.87, p < 0.0001$; Fig. 4a] as well as adrenal StAR expression (Fig. 4b). To investigate the temporal effects of ethanol, rats were administered ethanol (2 g/kg) and plasma progesterone levels were measured along with adrenal StAR protein levels at various time points. Adrenal StAR protein (32 kDa) levels were elevated as early as 20 min and remained elevated across the 2-h testing period (Fig. 4c) corresponding with increased steroid levels [$F(5,17) = 27.92, p < 0.0001$; Fig. 4d]. The maximal steroidogenic response occurred at 60 min corresponding to previous data examining the temporal response of progesterone in plasma and $3\alpha,5\alpha$ -THP in cerebral cortex (VanDoren *et al.* 2000). No effect of ethanol on StAR (32 kDa) expression was observed in cerebral cortex or any brain region tested (Table 1). The 37 kDa form of StAR was not detected by the antibody under any condition in adrenal mitochondria or brain homogenates.

We also examined the effect of ethanol on other cholesterol transporters that may be involved in steroidogenesis. Rats were administered 2 g/kg dose of ethanol and TSPO (18 kDa) and MLN64 protein levels were measured 60 min later in adrenal and cerebral cortical fractions. Ethanol administration decreased expression of the TSPO (18 kDa) monomer in the cerebral cortex (Table 1), but did not alter TSPO (18 kDa) or MLN64 levels in the adrenal (Fig. S1a and b) or MLN64 in brain (Table 1).

To determine if ethanol altered the activity of TSPO (18 kDa), we investigated the effect of the antagonist, PK11195, on ethanol-mediated steroidogenesis by measuring effects on plasma progesterone. PK11195 (1 mg/kg) had no effect on ethanol-induced increases in steroid levels, but a selective high affinity ligand of TSPO (18 kDa), CB34 (15 mg/kg), increased plasma steroid levels similar to ethanol [$F(4,12) = 168.3, p < 0.0001$; Fig. S1c]. Higher doses of PK11195 (5 and 10 mg/kg) also had no effect on ethanol-induced increases in steroid levels (data not shown).

A previous study suggested that only newly formed StAR is active and supports steroidogenesis (Artemenko *et al.* 2001). In order to determine if ethanol-induced elevation of neuroactive steroids is dependent upon *de novo* StAR synthesis, we tested the effect of the general protein synthesis inhibitor cycloheximide on ethanol-induced steroidogenesis. Simultaneous administration of cycloheximide with a 2 g/kg dose of ethanol, 60 min before killing, prevented the ethanol-induced increases in StAR protein and plasma progesterone levels without affecting ethanol-induced increases in plasma ACTH. There was a significant difference between groups for StAR protein [$F(3,24) = 15.29, p < 0.0001$; Fig. 5a], plasma ACTH levels [$F(3,17) = 36.31, p < 0.0001$; Fig. 5b] and plasma progesterone levels [$F(3,17) = 230, p < 0.0001$; Fig. 5c]. Ethanol-induced increases in cortical and hippocampal 3 α ,5 α -THP levels were no longer detectable after simultaneous administration of cycloheximide (data not shown). In another experiment, cycloheximide was administered 40 min following ethanol but 20 min prior to killing. This strategy more clearly prevented *de novo* StAR synthesis, and cycloheximide decreased ethanol-induced StAR protein expression and reduced steroid levels by 60%. There was a significant difference between groups for adrenal StAR protein expression [$F(2,20) = 6.36, p = 0.0073$; Fig. S2a], plasma progesterone levels [$F(3,20) = 205.8, p < 0.0001$; Fig. S2b] and cerebral cortical and hippocampal 3 α ,5 α -THP levels [$F(4,20) = 19.23, p < 0.0001$; Fig. S2c]. In addition to blocking the elevation in plasma steroids, cycloheximide inhibition also attenuated 3 α ,5 α -THP increases in the cerebral cortex and the hippocampus.

Steroidogenic acute regulatory protein phosphorylation by PKA has been shown to be important for steroidogenesis (Arakane *et al.* 1997; Jo *et al.* 2005). In order to determine if ethanol alters phosphorylation of StAR, we examined phosphorylated StAR levels by immunoprecipitation with a phospho-PKA substrate antibody. First, the specificity of the phospho-PKA substrate antibody for phosphorylated proteins was confirmed by SDS-PAGE following incubation of adrenal fractions with Lambda phosphatase to dephosphorylate proteins (see Fig. S3a). In addition, after solubilization and denaturation in radioimmunoprecipitation buffer, SDS-PAGE analysis confirmed that StAR protein was not bound to any nonspecific proteins that may be immunoprecipitated by the phospho-PKA substrate antibody (Fig. S3b). Furthermore, no band for StAR peptide was visible via western blot from adrenal fractions immunoprecipitated with rabbit IgG signifying that immunoprecipitation with phospho-PKA substrate antibody is specific (Fig. S3c). Following confirmation of the antibody specificity, adrenal mitochondrial fractions were immunoprecipitated with the phospho-PKA substrate specific antibody, separated by SDS-PAGE, and probed for StAR. Ethanol administration to rats (2 g/kg) increased phosphorylation of adrenal StAR 5.7-fold compared to saline treated rats (Fig. 6). Furthermore, in the same adrenal fraction, ethanol-induced increases in total StAR increased 2.1-fold indicating that ethanol-mediated increases of StAR phosphorylation exceed its effect on StAR protein synthesis ($p = 0.0095$, Student's *t*-test).

Steroidogenic acute regulatory protein is important for steroidogenesis, but the only enzyme known to convert cholesterol to pregnenolone is the P450_{scc} enzyme (Miller 2007b). Therefore, inhibition of this enzyme should prevent the formation of pregnenolone and neuroactive steroids irrespective of StAR protein levels. Aminoglutethimide was used to

inhibit the P450_{scc} enzyme and blocked ethanol-induced increases in plasma steroid levels by 58% [$F(2,29) = 39.83$, $p < 0.0001$; Fig. S4a]; although there was a slight increase in steroid levels from the inhibitor alone. Aminoglutethimide had no effect on ethanol-induced increases in adrenal StAR protein levels (Fig. S4b).

Discussion

While numerous studies have established that acute ethanol administration increases neuroactive steroids in plasma and brain, it has not been clear where ethanol acts to increase steroidogenesis. Because previous studies showed that adrenal integrity was required for ethanol-induced steroidogenesis in plasma and brain, the current study examined key steps in the adrenal steroidogenic pathway to attempt to determine which factors are required for ethanol-induced increases in neuroactive steroids.

The results indicate that both pituitary-derived ACTH and *de novo* StAR synthesis in the adrenals are required for ethanol-induced increases in circulating and cerebral cortical levels of 3 α ,5 α -THP, as well as circulating levels of pregnenolone and progesterone. Ethanol also increases deoxycorticosterone (Khisti *et al.* 2005) and 3 α ,5 α -THDOC levels (Barbaccia *et al.* 1999) in plasma and brain and these effects are likely dependent on the same mechanisms as deoxycorticosterone is derived from progesterone. Further, we show that ethanol increases adrenal StAR phosphorylation, which has previously been shown to enhance steroidogenesis in COS-1 and Leydig cells (Arakane *et al.* 1997; Jo *et al.* 2005). Although cholesterol transport to the inner mitochondrial membrane is the rate-limiting step in steroidogenesis, the ability of ethanol to increase StAR protein levels, independent of ACTH, appears to be necessary, but not sufficient for ethanol-induced steroidogenesis. Hypophysectomy and dexamethasone both prevented ethanol-induced increases in neuroactive steroids, even while StAR protein was enhanced by ethanol. Ethanol-induced increases in brain 3 α ,5 α -THP levels were also prevented by hypophysectomy and dexamethasone inhibition of ACTH as well as cycloheximide inhibition of adrenal StAR synthesis. Ethanol-induced increases in ACTH are independent of StAR formation as cycloheximide inhibition of *de novo* StAR synthesis does not alter the effect of ethanol on ACTH. Likewise, ethanol-induced increases in StAR synthesis are independent of ACTH, as dexamethasone inhibition of ACTH does not block the effects of ethanol on StAR synthesis. However, it appears that ethanol-induced increases in GABAergic neuroactive steroids in both plasma and brain are dependent upon pituitary activation to release ACTH, and independent of *de novo* StAR formation to promote adrenal steroidogenesis.

The findings of the present study suggest that ethanol-induced increases in neuroactive steroids result from stimulation of the HPA axis, as well as effects on the adrenal independent of HPA axis activity. Indeed, others have shown that ethanol activates the HPA axis (Ogilvie and Rivier 1997) and increases ACTH levels (Rivier *et al.* 1996), an effect that is dependent upon CRF and vasopressin (Lee *et al.* 2004). However, ACTH signaling is critical for adrenal steroidogenesis as CRF replacement does not reverse the effect of dexamethasone on corticosterone synthesis, but ACTH administration does (Cole *et al.* 2000). Further, in humans a hypothalamic lesion does not prevent an adrenal response to ethanol, but subjects with a pituitary adenoma have no adrenal response to ethanol even though the patients have a normal response to exogenous ACTH (Jenkins and Connolly 1968). Although CRF is clearly critical in coordinating HPA axis responses (Sarnyai *et al.* 2001), and ethanol may activate signaling upstream of ACTH, CRF does not appear to stimulate adrenal steroidogenesis without the presence of ACTH. Therefore, irrespective of how ethanol acts to increase ACTH, this increase is critical for steroidogenesis.

The use of hypophysectomized animals further demonstrated the significance of the pituitary and ACTH signaling on ethanol-induced increases in adrenal steroidogenesis. One drawback of using hypophysectomized animals is that the adrenals are atrophied due to lack of stimulation. However, the results in hypophysectomized rats are consistent with the effects of dexamethasone, suggesting that ACTH signaling is needed for ethanol-induced steroidogenesis. Furthermore, hypophysectomy and dexamethasone administration not only inhibited ethanol-induced increases in plasma neuroactive steroids, but also similarly blocked the elevation of cerebral cortical and hippocampal neuroactive steroid levels. Many studies have examined the effects of ethanol on ACTH, but the present study demonstrates that ACTH is necessary, but not sufficient, for ethanol-induced steroidogenesis.

While ACTH stimulation of the adrenal is important for steroidogenesis, cholesterol transport to P450_{scc} is the rate-limiting step (Miller 1988; Stocco 2000). We investigated the effects of ethanol on the cholesterol transport proteins StAR, TSPO (18 kDa), and MLN64 in adrenal and brain. While StAR is initially synthesized as a 37 kDa pre-protein, we were only able to measure the processed 32 kDa mitochondrial form. Although the 37 kDa form can mediate cholesterol uptake in non-steroidogenic COS-1 cells, it is barely detectable in steroidogenic cells (Artemenko *et al.* 2001), and its processed form may be the major cholesterol transporter (for review Jefcoate 2002). We found that ethanol-induced increases in adrenal StAR protein expression corresponded with increases in plasma steroid levels. In addition to the temporal association, we looked for a dose-dependent response of StAR and steroid levels. Rather than a dose response, we found a threshold effect where ethanol induction of adrenal StAR protein required a dose greater than 1.0 g/kg ethanol. Interestingly, this finding agrees with studies in humans where blood alcohol levels less than 0.1 mg% (comparable to a 1.0 g/kg injection), did not increase plasma steroid levels (Jenkins and Connolly 1968; Waltman *et al.* 1993; Holdstock *et al.* 2006; Pierucci-Lagha *et al.* 2006). Indeed, rodent studies show similar results, where ethanol increases 3 α ,5 α -THP at doses $\geq$ 1.3 g/kg (VanDoren *et al.* 2000), although others observe effects at 1.0 g/kg (Barbaccia *et al.* 1999; Serra *et al.* 2003).

The lack of dose dependency between 1.5 and 3.0 g/kg ethanol is consistent with prior studies (VanDoren *et al.* 2000). A similar lack of dose dependency was reported for ethanol-induced elevation of ACTH and corticosterone levels in rats that self-administer ethanol (Richardson *et al.* 2008). The lack of dose dependency may indicate that a threshold dose of ethanol is required for effects on both pituitary and adrenal activation. Prior studies have shown that ethanol-induced CRF and vasopressin release are required for the elevation of ACTH, however the ethanol dose response was not studied (Lee *et al.* 2004). Further studies are needed to determine the mechanisms that underlie ethanol activation of StAR and CRF synthesis.

Both the temporal and dose relationship of StAR with steroid levels following ethanol administration suggest the importance of StAR protein in mediating ethanol-induced steroidogenesis. MLN64 was examined in this study because it has been shown to have StAR-like activity in cells (Watari *et al.* 1997; Bose *et al.* 2000), but its expression in adrenals and brain was not changed by ethanol. It has been suggested that MLN64 plays a role in the placenta, where StAR is absent but steroidogenesis still occurs. Perhaps a more intriguing example is TSPO (18 kDa), formally known as the mitochondrial benzodiazepine receptor. It has been suggested that StAR and TSPO (18 kDa) may work together to promote steroidogenesis (Miller 2007a; Papadopoulos *et al.* 2007). In the present study, we did not detect any change in adrenal TSPO (18 kDa) expression following ethanol administration; possibly because it is already highly expressed in steroidogenic cells. However, TSPO (18 kDa) agonists have been shown to increase steroid levels (Serra *et al.* 1999; Rupprecht *et al.* 2009) and the present study also found an increase comparable to that seen following

ethanol administration. Further, we found no effect of the TSPO (18 kDa) antagonist PK11195 (1, 5, 10 mg/kg) on ethanol-induced increases in neuroactive steroids, suggesting that ethanol does not alter TSPO (18 kDa) activity. These doses have previously been shown to prevent TSPO (18 kDa)-mediated anxiolysis (Hirani *et al.* 2005; Rupprecht *et al.* 2009). Thus, while TSPO (18 kDa) is clearly capable of promoting steroidogenesis, it does not appear to be necessary for ethanol-induced steroidogenesis. Taken together, these studies also suggest, albeit indirectly, that StAR is the cholesterol transporter involved in ethanol-induced steroidogenesis.

A convincing role for StAR's importance in steroidogenesis is also evident in other animal and human studies. Generation of a StAR knockout mouse showed the absence of StAR to be lethal (Caron *et al.* 1997) and humans with mutations in the StAR gene have congenital lipid adrenal hyperplasia (Miller 1997). Thus, we investigated the importance of StAR for ethanol-induced steroidogenesis in more detail. If ethanol is increasing steroid levels by increasing cholesterol transfer to the P450_{scc}, then inhibition of *de novo* StAR synthesis, a cholesterol transport protein, should diminish this ethanol effect. In fact, administration of cycloheximide concurrently with ethanol completely inhibited ethanol-induced increases in StAR protein expression and both plasma and brain steroid levels. It is important to note that cycloheximide is a general protein synthesis inhibitor so other proteins could be inhibited and potentially affect steroid synthesis. To minimize inhibition of other proteins, we took advantage of the fact that StAR is rapidly synthesized and administered cycloheximide 40 min post-ethanol administration (20 min prior to killing). This experiment showed that inhibition of *de novo* StAR synthesis arrested steroid production. As steroids were increased by ethanol prior to cycloheximide administration, steroid levels were not completely blocked, but are decreased as preexisting levels are metabolized. Nevertheless, inhibition of *de novo* StAR synthesis dramatically affects steroid levels and strongly supports its role as a major component of increases in ethanol-induced neuroactive steroid levels. Furthermore, synthetic hydroxycholesterols, which can cross the membrane freely, stimulate steroid synthesis in cells where StAR has been inhibited (Kim *et al.* 1997) providing further support for the essential role of cholesterol transport in steroidogenesis.

While ethanol's ability to increase adrenal StAR protein is important for ethanol mediated steroidogenesis, StAR phosphorylation is critical for full steroidogenic activity of StAR (Arakane *et al.* 1997). In the present study, ethanol not only increased adrenal StAR protein expression but also increased StAR phosphorylation. While the present study cannot definitively conclude that this is PKA phosphorylation, a point mutation of the PKA phosphorylation site diminishes StAR activity (Arakane *et al.* 1997). Furthermore, experiments in Leydig cells demonstrated that protein kinase C activation can elicit increases in StAR protein expression, but protein kinase C does not increase steroid levels or phosphorylate StAR. However, addition of a cAMP analogue increases StAR phosphorylation and steroid levels (Jo *et al.* 2005). Studies to determine if StAR phosphorylation is required for ethanol steroidogenesis *in vivo* are underway.

Although cholesterol transport is the rate-limiting step in steroidogenesis, steroid synthesis is still dependent upon the conversion of cholesterol to pregnenolone and subsequent metabolism to neuroactive metabolites. To date, the only known enzyme capable of converting cholesterol to pregnenolone is the P450_{scc} enzyme (Miller 2007c). In the present study, inhibition of P450_{scc} abolished ethanol-induced increases in steroid levels without having any effect on increases in StAR expression. This suggests that cholesterol was still being transported to the inner mitochondrial membrane and may account for the low level of steroid formation as cholesterol accumulated. Cholesterol accumulates at the inner mitochondrial membrane following P450_{scc} inhibition and at the outer mitochondrial membrane following cycloheximide administration (Privalle *et al.* 1983). We therefore

conclude that ethanol enhancement of cholesterol transport from the outer to inner mitochondrial membrane is necessary for its effect on steroidogenesis.

There is no doubt that the brain is a steroidogenic organ, but the results of the present study suggest that the increased neuroactive steroid levels measured by RIA after acute ethanol administration are dependent upon adrenal steroidogenesis. Other work suggests that ethanol elevates StAR in brain (Serra *et al.* 2006) and increases steroidogenesis in hippocampal slices (Sanna *et al.* 2004) in the absence of adrenal-derived precursors. An explanation for these discrepancies is unclear. Ethanol-mediated steroidogenesis in hippocampal slices was conducted in adolescent rats whereas our studies were conducted in adult rats. However, it should be pointed out that in experiments where we inhibited peripheral steroid synthesis, the RIA may not be sensitive enough to detect localized changes in brain steroidogenesis. Further studies are needed to address these questions.

Neuroactive steroids are lipophilic and capable of crossing the blood brain barrier. While the neuroactive metabolites can be synthesized peripherally and travel to the brain, it is also likely that adrenal precursors contribute to the central levels of GABAergic steroids (Kraulis *et al.* 1975; Wang *et al.* 1997; Khisti *et al.* 2005). These precursors can be synthesized in the adrenals and metabolized to neuroactive metabolites in the brain with their regional distribution dependent upon steroidogenic enzyme expression (Mellon and Deschepper 1993; Li *et al.* 1997).

Basal StAR protein expression (32 kDa) was observed in multiple brain regions, but we found no evidence for ethanol-induced increases in cortex or hippocampus, possibly because ethanol-induced increases in neuroactive steroids are dependent upon adrenal biosynthesis. Blockade of *de novo* adrenal StAR synthesis with cycloheximide, and ACTH production with dexamethasone both show that inhibition of adrenal steroid biosynthesis prevents ethanol-induced increases in brain 3 α ,5 α -THP levels. Prior adrenalectomy experiments further emphasize the importance of peripheral steroidogenesis for controlling brain steroid levels. This concept is not unique to ethanol as adrenalectomized animals subjected to stress, gamma-hydroxybutyric acid, ethanol, nicotine, morphine, olanzapine or clozapine administration fail to show increases in cortical levels of the potent GABAergic neuroactive steroids (Purdy *et al.* 1991; Khisti *et al.* 2003; Marx *et al.* 2003; O'Dell *et al.* 2004; Porcu *et al.* 2004; Concas *et al.* 2006). Thus, an interesting dynamic exists where the brain is dependent upon the periphery for global increases in neuroactive steroids, yet, ultimately the control of GABAergic neuroactive steroids is dependent upon HPA axis activation.

As neuroactive steroids have been postulated to affect ethanol sensitivity and the risk for alcoholism, it is important to understand how ethanol affects neuroactive steroid synthesis. Chronic ethanol administration elicits ethanol tolerance in both rodents and humans. While studies in humans are limited, ethanol dependent rodents do not exhibit increased neuroactive steroid levels (Janis *et al.* 1998) and have a blunted response to ethanol challenge (Morrow *et al.* 2001; Khisti *et al.* 2005). Furthermore, adrenalectomy, which reduces peripheral and central neuroactive steroids, increases ethanol withdrawal severity in mice (Gililand and Finn 2007). Thus, the present study demonstrates the importance of adrenal StAR protein and plasma ACTH in regulating ethanol-induced increases in neuroactive steroids. Moreover, the results of this study will also be utilized as a comparison for the effects of chronic ethanol exposure to examine ethanol-induced alterations in steroidogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

3α,5α-THDOC	(3 α ,5 α)-3,21-dihydroxypregnan-20-one
3α,5α-THP	(3 α ,5 α)-3-hydroxypregnan-20-one
ACTH	adrenocorticotrophic hormone
CHX	cycloheximide
CRF	corticotrophin releasing factor
EtOH	ethanol
HPA	hypothalamic-pituitary-adrenal
MLN64	metastatic lymph node 64
P450scc	P450 side chain cleavage
PKA	protein kinase A
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
StAR	steroidogenic acute regulatory protein
TSPO (18 kDa)	translocator protein 18 kDa

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Fig. 1.

Threshold for ethanol-induced increases in plasma steroids. Ethanol was administered at varying doses and plasma and cerebral cortex were collected after 60 min to measure steroid levels. (a) Plasma pregnenolone, (b) plasma progesterone, (c) plasma 3 α ,5 α -THP and (d) cerebral cortical 3 α ,5 α -THP. * $p < 0.001$ and ** $p < 0.05$ compared to saline control (one-way ANOVA followed by Newman-Keuls test), $n = 4-5$ for each group in duplicate.

Fig. 2.

Hypophysectomy abolishes ethanol-induced increases in ACTH release as well as plasma and brain steroid levels. (a) Plasma ACTH, (b) plasma progesterone and (c) cerebral cortical 3 α ,5 α -THP levels following hypophysectomy compared to sham-operated controls. * $p < 0.001$ compared to all groups (two-way ANOVA followed by Bonferroni post-tests), $n = 8-9$ in duplicate.

Fig. 3.

Dexamethasone inhibits ACTH release as well as ethanol-induced increases in steroid levels. Effect of dexamethasone (0.1 mg/kg, i.p.) for 90 min on the ethanol-induced increase in (a) plasma ACTH, (b) plasma progesterone, (c) 3α,5α-THP in the cerebral cortex and hippocampus and (d) adrenal StAR protein expression. Representative blot shown. StAR protein was normalized to β-actin and presented as % control. * $p < 0.001$ compared to control, ** $p < 0.01$ compared to control, # $p < 0.001$ compared to ethanol (EtOH) (ANOVA, followed by Newman-Keuls test), $n = 6$ in duplicate.

Fig. 4.

Ethanol exhibits a threshold for plasma ACTH and adrenal StAR induction, and StAR expression and plasma progesterone levels are rapidly increased. Rats were administered various doses of ethanol and (a) plasma ACTH and (b) adrenal StAR protein expression were measured. A separate group of rats was administered ethanol and blood was collected at varying time points. (c) Time course of StAR protein induction. (d) Time course of plasma progesterone induction. * $p < 0.01$ compared to control, # $p < 0.01$ compared to 1.5 g/kg ethanol, and @ $p < 0.05$ compared to 2.5 g/kg ethanol (Student's t -test or ANOVA followed by Newman-Keuls test where appropriate), $n = 4-6$ for each group in duplicate.

Fig. 5.

Simultaneous administration of cycloheximide (CHX) with ethanol (EtOH) prevents ethanol-induced increases in StAR protein, as well as plasma and brain steroid levels, but does not affect ethanol-induced ACTH. Cycloheximide (20 mg/mL, i.p.) was administered with ethanol (2 g/kg, i.p.) and tissue was collected 60 min later. (a) Adrenal StAR protein expression was measured by western blot analysis as shown in a representative blot. StAR was normalized to β -actin and presented as % control values. (b) Plasma ACTH and (c) plasma progesterone levels. * $p < 0.001$ compared to control and # $p < 0.001$ compared to ethanol (ANOVA followed by Newman–Keuls test), $n = 6$ in duplicate.

Fig. 6.

Acute ethanol administration increases phosphorylation of adrenal StAR protein. Rats were administered ethanol (2 g/kg, i.p.) and tissues were collected 60 min later. Adrenal fractions were immunoprecipitated with phospho-PKA substrate antibody, run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with StAR. Results are reported as fold increase of phospho-StAR (EtOH vs. saline) compared to fold increase of total StAR (EtOH vs. saline) * $p < 0.01$ (Student's t -test), $n = 4$.

Table 1

Ethanol decreases TSPO (18 kDa) expression in cerebral cortex but does not alter StAR or MLN64 expression

	StAR (32 kDa)	TSPO (18 kDa)	MLN64 (53 kDa)
Saline	100 ± 4.95	100 ± 5.88	100 ± 18.34
EtoH	101.5 ± 7.27	75.37 ± 4.79*	108.5 ± 26.63

Tissues were collected 60 min post-ethanol administration. Data represent mean values ± SEM and are normalized to β-actin and presented as % control values from each western blot.

* $p < 0.01$ compared to saline control (Student's t -test), $n = 8-16$.