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Ethanol-induced increases in neuroactive steroids in the rat brain and plasma are absent in adrenalectomized and gonadectomized rats☆

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Abstract

Peripheral administration of alcohol has been demonstrated to cause significant increases in neurosteroid levels in the brain and periphery. These findings have led to several theories suggesting a role for neurosteroids in the actions of alcohol. However, the anatomical sources of these steroids (e.g., brain or periphery) are as yet unknown. This study utilized gas chromatography/mass spectrometry (GC/MS) to assess the levels of several neuroactive steroids in plasma and brain frontal cortex 30–360 min following acute administration of alcohol (2 g/kg, i.p.). Concentrations of pregnenolone, allopregnanolone (3α-hydroxy-5α-pregnan-20-one), and allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one) were all measured. In order to determine the contribution of peripheral endocrine organs to neurosteroid responses, neuroactive steroid levels were measured in both intact and adrenalectomized/gonadectomized male Wistar rats 30 min after acute administration of alcohol. Intact animals exhibited a maximal increase of pregnenolone in plasma and frontal cortex 30 min after acute administration of alcohol. In addition, allopregnanolone levels increased, with a maximal effect observed at 60 min in plasma. However, in the adrenalectomized/gonadectomized groups treated with alcohol, no significant increases of pregnenolone, allopregnanolone, or allotetrahydrodeoxycorticosterone were found after 30 min. Thus, the alcohol-induced response was associated first with a relatively rapid increase in the first and rate-limiting step in the conversion of cholesterol to steroids, leading to increases in pregnenolone levels. This response was followed by the further secretion of the anxiolytic neuroactive steroids allopregnanolone and allotetrahydrodeoxycorticosterone, both of which appeared to be of adrenal and gonadal origin.

Keywords: Ethanol; Pregnenolone; Allopregnanolone; Allotetrahydrodeoxycorticosterone; Endocrine response

1. Introduction

It has been known for many decades that the adrenals of rats secrete steroids such as pregnenolone, progesterone, allopregnanolone, and allotetrahydrodeoxycorticosterone in response to stress (Holzbauer and Newport, 1968; Holzbauer et al., 1969, 1985). These steroids were proposed in 1992 to be termed "neuroactive steroids" in cases where they were established to be of endocrine origin, as opposed to being synthesized de novo in the nervous system (Paul and Purdy, 1992). A subset of neuroactive steroids are subsumed by the term "neurosteroid," which is now generally agreed to apply to a steroid that is biosynthesized in the nervous system from cholesterol (Baulieu et al., 2001). This distinguishes it from a steroid secreted by endocrine organs. This distinction is of importance to neuroendocrinologists because these markers distinguish between general endocrine function and paracrine function in the nervous system.

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Neuroactive steroids, some of which have been shown to potentiate γ-aminobutyric acid (GABA)-induced currents in the central nervous system at low nanomolar concentrations (including allopregnanolone and allotetrahydrodeoxycorticosterone), are relatively large C-21 secondary alcohols that share many properties in common with ethyl alcohol, including their anti-convulsant, sedative hypnotic, anxiolytic, and anesthetic properties (Morrow et al., 2001; Wang et al., 2001). These GABAergic neuroactive steroids also have been conclusively demonstrated to substitute for ethanol in drug discrimination studies (Engel and Grant, 2001). Although the precise mechanism(s) that mediate the effects of allopregnanolone and allotetrahydrodeoxycorticosterone are not fully characterized, they are believed to act at sites distinct from benzodiazepines and barbiturates on GABA receptors (Lambert et al., 2001). However, the macroelectrophysiological profile of allopregnanolone resembles barbiturates and benzodiazepines to a greater extent than ethanol (Slawecki et al., 2000).

There are several studies demonstrating that an acute alcohol challenge can produce significant increases in neuroactive steroids in plasma and brain. Using purification of neuroactive steroids by high-performance liquid chromatography (HPLC) and subsequent measurement by radioimmunoassay (RIA), Barbaccia et al. (1999) demonstrated that 30 min after ethanol administration (1 g/kg, i.p.), there was a marked increase in allopregnanolone and allotetrahydrodeoxycorticosterone in the plasma and cerebral cortex of ethanol-naïve Sardinian ethanol-preferring and ethanol-nonpreferring rats. Similar results were found for allopregnanolone levels in plasma and cerebral cortex of male and female Sprague–Dawley rats following acute ethanol administration (Morrow et al., 1999; Van Doren et al., 2000). These authors concluded that the marked increase in allopregnanolone in brain represents an essential component of ethanol action via neurosteroid effects on the brain.

Neuroactive steroids in the brain have been hypothesized to be derived from both endocrine and centrally derived sources. In a report by Korneyev et al. (1993) using RIA of purified brain extracts from five animals, they failed to observe an increase in pregnenolone levels in the forebrain of adrenalectomized and gonadectomized rats following acute ethanol administration. However, effects on allopregnanolone and allotetrahydrodeoxycorticosterone were not reported in this study.

Because of the difficulty of accurately measuring pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone by RIA methodology, several groups subsequently developed more sensitive and specific mass spectrometric methods to measure numerous neuroactive steroids (reviewed in Alomary et al., 2001a). Using mass spectrometric methodology, we subsequently reported that several neuroactive steroids could be measured in the rat brain following acute ethanol administration using isotope dilution/negative chemical ionization with gas chromatography/mass spectrometry (GC/MS; Alomary et al., 2001b). Such GC/MS procedures have proved their reliability and specificity, as compared to RIA methodology, in numerous laboratories.

The purpose of the present study was to examine the sources of neurosteroid release following acute administration of ethanol in the male rat. Brain and plasma levels of pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone were measured following acute administration of ethanol in both intact and adrenalectomized/gonadectomized rats. The formation of pregnenolone from cholesterol is the rate-limiting step in steroid biosynthesis in the adrenals and gonads and is required for the subsequent formation of allopregnanolone and allotetrahydrodeoxycorticosterone. Therefore, our working hypothesis was that the effects of acute administration of ethanol on these neuroactive steroids would be absent in adrenalectomized/gonadectomized rats, unless these steroids were synthesized in brain. The results of the present study suggest that pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone are not synthesized in the brain following ethanol administration, but are derived from endocrine sources.

2. Materials and methods

2.1. Animals

The subjects were male Wistar rats weighing 250–350 g at the start of the experiment (Charles River Laboratory, Kingston, NY). The animals were group-housed (three per cage) with food and water available ad libitum in a temperature-controlled vivarium. They were acclimated to the handling procedure once before the test day. The lights were on a 12-h light/dark cycle, with lights on at 6 a.m. All animal care and experimental procedures were performed in strict 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 Scripps Research Institute.

The first experiment utilized intact male Wistar rats to examine neuroactive steroid levels in frontal cortex and plasma 30, 60, and 360 min post ethanol ($N=8$ per time point) or saline administration ($N=3$ per time point). The goal of this experiment was to select an optimal time point to study the levels of neuroactive steroids in brain following ethanol administration. In a second experiment, neuroactive steroid levels were measured in frontal cortex and plasma of intact ($N=12$) and adrenalectomized/gonadectomized ($N=12$) male Wistar rats 30 min after alcohol administration. This time point was chosen from the first study. These measures also were taken in an additional group of intact rats ($N=6$) that were sacrificed 30 min after saline administration. In the present study, the adrenalectomized/gonadectomized group was tested three weeks after arrival at The Scripps Research Institute, and these animals received saline instead of water for drinking. This group provided a model

in which the normal sources of peripheral steroids derived from cholesterol in male animals were removed. Testosterone levels from these animals were described in a separate publication characterizing the testosterone response to ethanol (Alomary et al., 2003).

2.2. Drug treatment

In the first experiment, separate groups of animals were sacrificed by rapid decapitation at three time points (30, 60, and 360 min) following administration of saline ($N=3$ per time point) or alcohol ($N=8$ per time point). USP alcohol (95%) was diluted in saline (0.9% sodium chloride) and administered intraperitoneally (i.p.) as a 20% (w/v) solution at a dose of 2 g/kg. In the second experiment, intact or adrenalectomized/gonadectomized animals ($N=12$ per group) were sacrificed by rapid decapitation 30 min following administration of alcohol (2 g/kg, i.p.). A control group of intact animals also was included and was sacrificed 30 min following saline administration ($N=6$). Blood was collected from the trunk in EDTA-coated tubes, centrifuged at $1000 \times g$ for 10 min, and the plasma samples were stored at -70 °C. The entire brain was rapidly removed and the brain frontal cortex was dissected and stored at -70 °C.

2.3. Blood alcohol level determination

A 200-μl aliquot of serum was stored at -20 °C until assayed. Serum was analyzed for blood alcohol level using an Analox instrument (Lunenburg, MA).

2.4. GC/MS procedures

The steroids pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone were extracted from both plasma and brain tissue by a simple solid-phase extraction method (Vallée et al., 2000). This method was validated in terms of sensitivity, accuracy, and precision for these neuroactive steroids. Briefly, the method uses negative chemical ionization with GC/MS and involves the formation of pentafluorobenzyloxime/trimethylsilyl ether derivatives of the steroid fraction from brain or plasma extracts to enhance the mass spectrometric analysis. Mass spectra were acquired with a Finnigan TSQ-7000 mass spectrometer (Thermo Finnigan, San Jose, CA). The mass spectrometer was operated in a selective ion monitoring (SIM) mode, allowing for picograms of neurosteroids to be quantified from biological extracts (Vallée et al., 2000). The isotope dilution method was used to achieve accurate quantification. Pregnenolone-d_4 and allopregnanolone-d_4 were used as the internal standards to quantify pregnenolone and allopregnanolone, respectively, because pregnenolone-d_4 and allopregnanolone-d_4 do not have a significant amount of pregnenolone-d_0 and allopregnanolone-d_0. The quantification of allotetrahydrodeoxycorticosterone was calculated on the basis of the internal standard of allopregnanolone-d_4 in the SIM at m/z 492. The procedure was suitable for measuring concentrations of endogenous unconjugated neuroactive steroids in rat plasma and frontal cortex.

2.5. Statistical methods

Results are expressed as mean ± S.E.M. The magnitude of the effect of alcohol on the steroid level was defined as the change in concentration of the steroid in nanogram per milliliter for plasma (ng/ml) and nanogram per gram for brain tissue (ng/g). The significance of our results was determined by two-way analysis of variance, and subsequent comparisons were performed using Fisher tests ($P<0.05$).

3. Results

3.1. Effect of alcohol administration on blood alcohol levels

The time course of blood alcohol levels (in average mg/dl ± S.E.M.) following alcohol administration in intact rats

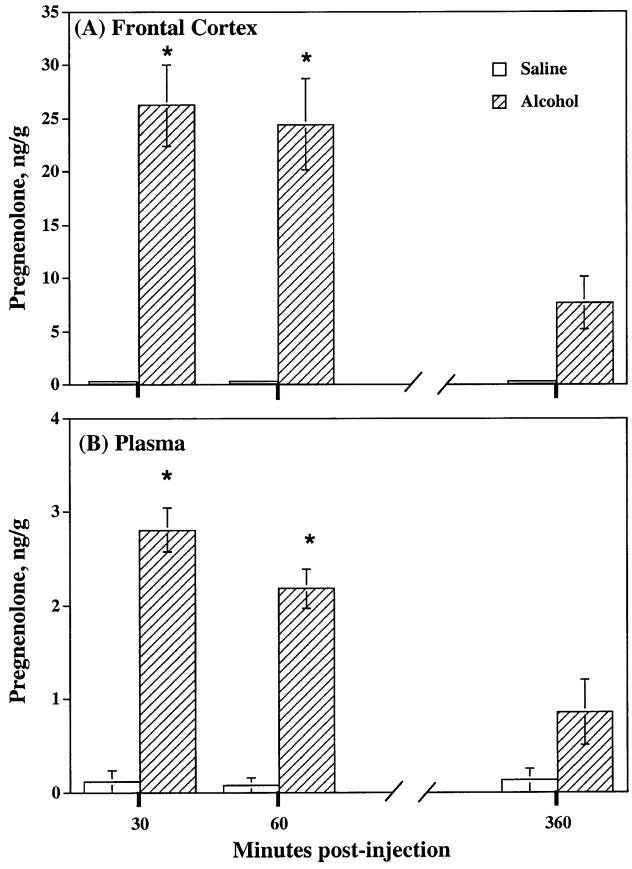

Fig. 1. Experiment 1: Pregnenolone concentration 30–360 min after saline ($N=3$ per time point) or acute alcohol administration (2 g/kg, i.p.; $N=8$ per time point) as determined by GC/MS from unconjugated steroid extracts of frontal cortex (A) and plasma (B). Asterisks (*) indicate a significant difference from saline controls ($P<0.05$).

in the first experiment demonstrated a significant time by treatment interaction, with subsequent analyses revealing that blood alcohol levels were significantly increased to 220 ± 1 at 30 min, were maintained at 232 ± 2.7 at 60 min and at 149 ± 3 at 180 min, and fell back to a baseline level of 6.8 ± 5 at 360 min. Thirty minutes after acute administration of alcohol in the second experiment, there was about a 30% lower average blood alcohol level in adrenalectomized/gonadectomized animals treated with ethanol (128 ± 6) at 30 min, as compared to the average blood alcohol level in intact rats (202 ± 6) at 30 min. The blood alcohol levels in the saline control rats were below the limit of quantification.

3.2. Effect of alcohol administration on pregnenolone levels in brain and plasma (Experiment 1)

Fig. 1 illustrates that in both cortex (A) and plasma (B) of intact animals, the concentration of pregnenolone increased following alcohol administration relative to rats that received saline. Our analyses of cortical samples revealed that the levels of pregnenolone were significantly increased at 30 and 60 min relative to saline controls ($P < 0.05$). The analyses of plasma samples revealed a significant time × treatment interaction, $F(2,28) = 4.92$, $P < 0.01$, and subsequent analyses revealed that pregnenolone levels were significantly increased at 30 and 60 min relative to saline controls ($P < 0.05$). In both cortex and plasma, the rise in the level of pregnenolone returned to control levels 360 min later.

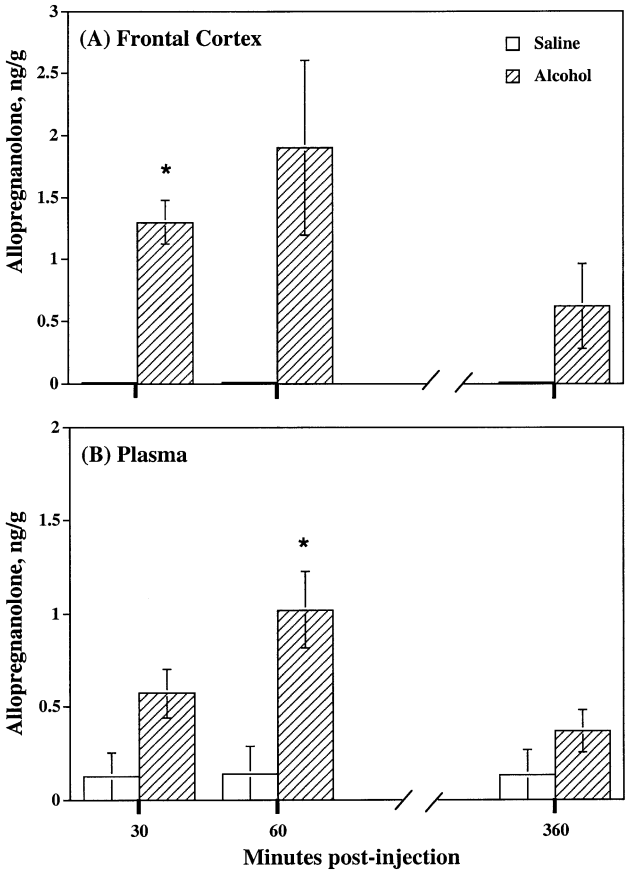

Fig. 2. Experiment 1: Allopregnanolone concentration 30–360 min after saline ($N = 3$ per time point) or acute alcohol administration (2 g/kg, i.p.; $N = 8$ per time point) as determined by GC/MS from unconjugated steroid extracts of frontal cortex (A) and plasma (B). Asterisks (*) indicate a significant difference from saline controls ($P < 0.05$).

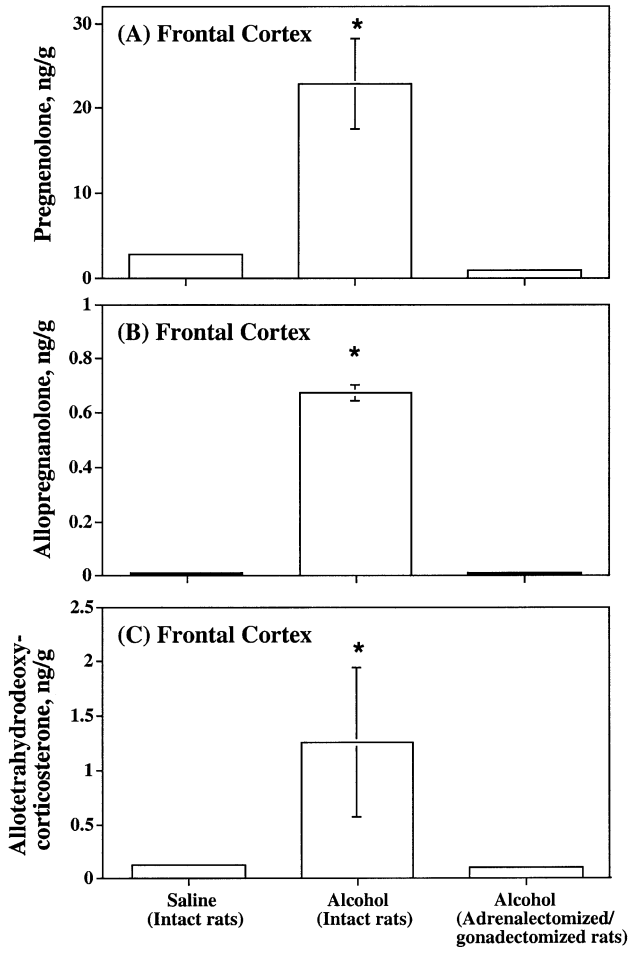

Fig. 3. Experiment 2: Pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone concentrations in the frontal cortex of intact male Wistar rats 30 min after saline ($N = 6$) or acute alcohol administration (2 g/kg, i.p.; $N = 12$). These measures were also taken in adrenalectomized/gonadectomized male Wistar rats 30 min after administration of the same dose of alcohol ($N = 12$). Asterisks (*) indicate a significant difference from saline controls ($P < 0.05$).

3.3. Effect of alcohol administration on allopregnanolone levels in brain and plasma (Experiment 1)

Fig. 2 illustrates that in both cortex (A) and plasma (B) of intact animals, the concentration of allopregnanolone increased following alcohol administration relative to rats that received saline. Following alcohol administration, the levels of allopregnanolone were significantly increased at 30 min in frontal cortex and at 60 min relative to rats that received saline ($P < 0.05$).

A comparison of the relative ratios of pregnenolone and allopregnanolone in plasma at 30 min (see Figs. 1 and 2) revealed that pregnenolone is initially formed at a more rapid rate than allopregnanolone. This is anticipated from the metabolic sequence of conversion from cholesterol to pregnenolone, oxidation to progesterone, and two successive reductive steps to allopregnanolone. The conversion of progesterone to allotetrahydrodeoxycorticosterone first involves the formation of 11-deoxycorticosterone followed by two similar reductive steps to allotetrahydrodeoxycorticosterone.

3.4. Effect of adrenalectomy/gonadectomy on ethanol-induced neuroactive steroid levels in brain and plasma (Experiment 2)

Fig. 3 illustrates that in the frontal cortex of intact rats, pregnenolone (A), allopregnanolone (B), and allotetrahydrodeoxycorticosterone (C) were all significantly increased 30 min following acute administration of alcohol relative to intact rats that received saline ($P < 0.05$). The mean level of pregnenolone in this experiment was similar to that of the first experiment (see Fig. 1). However, the mean level of allopregnanolone in this experiment was only about half of that found in the first experiment (see Fig. 2A). Such variations in allopregnanolone levels in the brains of different groups of stressed animals are not uncommon (Barbaccia et al., 2001). The ability of ethanol to increase pregnenolone, allopregnanolone, or allotetrahydrodeoxycorticosterone in either plasma or cortex also was absent in adrenalectomized/gonadectomized rats (see Fig. 3).

4. Discussion

As expected from previous studies, a marked increase in brain and plasma levels of pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone was found following acute administration of ethanol in the present study. However, significant levels of these neuroactive steroids were not detected in brain and plasma of adrenalectomized/gonadectomized animals following acute ethanol. These results clearly implicate the adrenals and gonads, rather than the brain, as the source of these neuroactive steroids following ethanol administration.

The present study is focused on an animal model of acute ethanol administration in humans, where an individual's initial response to alcohol produces an increase in plasma cortisol levels within 30 min of alcohol administration (1.1 ml/kg; Schuckit et al., 1987; Schuckit, 1998). In rats, acute administration of ethanol is well recognized to produce a stress response involving the secretion of steroids by the adrenal glands. The present results using GC/MS are in agreement with the previous studies of Barbaccia et al. (1996, 2001) using RIA for neuroactive steroid measurement. They demonstrated that the acute stressor effects of foot shock and CO_2 inhalation on the levels of pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone in male Sprague–Dawley rats was prevented in adrenalectomized/gonadectomized animals, as judged by the failure to detect brain levels of these neuroactive steroids after these stressors. The present results further imply that the adrenals and gonads of male rodents respond to acute administration of ethanol through a rapid secretion of the neuroactive steroid pregnenolone, as well as secretion of the GABAergic positive-modulatory steroids allopregnanolone and allotetrahydrodeoxycorticosterone formed from pregnenolone in the adrenal cortex (Mellon and Vaudry, 2001).

The significantly decreased blood alcohol levels in adrenalectomized/gonadectomized animals treated with ethanol compared to controls are consistent with previous studies showing that castration increases the activity of alcohol dehydrogenase in the liver (Cicero and Badger, 1977; Mezey et al., 1980). Previous work from our laboratory has shown that the kinetics of blood ethanol in male rats 30 min following administration of 1.5 g/kg of ethanol corresponds to an average blood alcohol level of about 125 mg/dl (Morse et al., 2000). It also has been established that administration of 1.5 g/kg of ethanol leads to only a modest reduction of allopregnanolone in the cerebral cortex, as compared to 2 g/kg of ethanol (Morrow et al., 2001). Therefore, we believe that the 30% reduced blood alcohol level in adrenalectomized/gonadectomized animals is not an explanation for the failure to detect significant levels of neuroactive steroids in these animals.

The much higher ratio of frontal cortex/plasma (ng/g divided by ng/ml) concentrations of pregnenolone, as compared to allopregnanolone, is anticipated from the presence of the high-affinity, low-capacity binding protein for pregnenolone in brain, which has been characterized in microtubules from rodent and calf brain tissue (Murakami et al., 2000). Conversely, no similar binding protein for allopregnanolone or allotetrahydrodeoxycorticosterone have been reported in brain.

The mean level of allopregnanolone in brain 30 min after acute ethanol administration in the present study was similar to that found in our laboratory using GC/MS analysis of frontal cortex obtained after 10 min of swim stress in male Wistar rats (1.3 ng/g; Vallée et al., 2000). This level of allopregnanolone in cortex is lower than previous reports that used HPLC combined with RIA to measure allopregnanolone in cortex after similar treatment of male rats with acute ethanol (Morrow et al., 1999; Van Doren et al., 2000). The anti-allopregnanolone antisera used in such measurements by RIA (Finn and Gee, 1994) is known to cross-react with a variety of structurally related steroids and other lipids which are difficult to separate by HPLC. Immunoassays measuring low concentrations of steroids in complex biological matrices are highly variable (Herold and Fitzgerald, 2003). The 15 m GC column used to separate the pentafluorobenzyloxime/trimethylsilyl ether derivatives in our methodology has excellent chromatographic properties

which, coupled with specific ion monitoring by MS, gives the specificity required for confidence of the analysis of the complex mixture of steroids found in mammalian tissues.

The rate-limiting step in the synthesis of steroids (including neuroactive steroids), in peripheral endocrine organs, is the transfer of cholesterol from the cytosol to the inner mitochondrial membrane that is catalyzed by the steroidogenic acute regulatory protein (StAR; reviewed in Stocco, 2000). Both StAR and its mRNA also are expressed within glia and neurons of the rodent brain (King et al., 2002), which is consistent with the hypothesis that StAR also is involved in the de novo formation of neurosteroids. StAR mRNA levels in the frontal cortex and several other brain regions have recently been shown to be significantly increased after the administration of acute alcohol, and also found to be maintained in adrenalectomized/gonadectomized animals (Kim et al., 2003). Therefore, our failure to find any significant concentrations of the neurosteroids pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone in the brain of adrenalectomized/gonadectomized animals is probably not due to the absence or marked reduction of StAR in brain. We believe that these results are due to the inability of any significant amount of circulating adrenocorticotropic hormone (ACTH) to cross the blood–brain barrier (reviewed in Koob, 1987), and thereby activate StAR. This is in agreement with the known failure of peripherally administered ACTH to cause the excessive grooming in rodents that is seen after intraventricular injection of ACTH (reviewed in Akil and Watson, 1983).

Some endocrine responses to acute administration of ethanol seen in the present study most likely only generalize to other strains of male rodents, and males of other species whose adrenals also secrete corticosterone in response to stress. However, it is unlikely that these results are applicable to human and non-human primate males, whose adrenals secrete cortisol in response to stress. The important reason this is true is the presence of the 17-hydroxyl group in cortisol and its metabolites (as opposed to corticosterone and deoxycorticosterone) that markedly reduce any significant potentiation of $GABA_A$ receptors by the 3α-hydroxy-5α- and -5β-reduced metabolites of cortisol and cortisone (Purdy et al., 1990; Hawkinson et al., 1994). The finding of the secretion of progesterone (the precursor of allopregnanolone) by the rat testes in response to stress is well known (Rivier and Vale, 1981). On the other hand, the ovaries of female primates and non-human primates secrete allopregnanolone (as well as its anxiolytic epimer pregnanolone) in the luteal phase of the menstrual cycle (Schmidt et al., 1994). Therefore, it is not surprising that female adolescent humans showed a dramatic increase in their circulating levels of allopregnanolone after acute alcohol intoxication, presumably of ovarian origin (Torres and Ortega, 2003).

In summary, this report demonstrates that acute administration of alcohol, in intact male rats, produces an increase in circulating and brain levels of pregnenolone, allopregnanolone, and allotetrahydrodeoxycorticosterone, and that these effects are abolished in adrenalectomized/gonadectomized animals. We are unaware of any evidence that increases in endogenous ACTH stimulates neurosteroid formation in brain. Therefore, we conclude that the increases in these neuroactive steroids following acute ethanol are of peripheral origin rather than being formed in the brain itself. Collectively, our findings suggest that acute administration of alcohol in male rats may not be an appropriate animal model for studying the role of neuroactive steroids in humans following acute ethanol ingestion[1].

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