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REPLACEMENT WITH GABAergic STEROID PRECURSORS RESTORES THE ACUTE ETHANOL WITHDRAWAL PROFILE IN ADRENALECTOMY/GONADECTOMY MICE

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Abstract—The neurosteroid allopregnanolone (ALLO) is a progesterone metabolite that is one of a family of neuroactive steroids (NAS) that are potent positive allosteric modulators of γ-aminobutyric acid$_A$ (GABA$_A$) receptors. These GABAergic NAS are produced peripherally (in the adrenals and gonads) and centrally in the brain. Peripherally produced NAS modulate some effects of ethanol intoxication (e.g., anxiolytic, antidepressant, and anticonvulsant effects) in rodents. We have found that NAS also may be involved in the rebound neural hyperexcitability following a high ethanol dose. Removal of the adrenals and gonads (ADX/GDX) increased withdrawal severity following 4 g/kg ethanol, as measured by handling-induced convulsions (HICs) in male and female DBA/2J mice. NAS are produced through the metabolism of progesterone (PROG), deoxycorticosterone (DOC), or testosterone, which can be blocked with the administration of finasteride (FIN), a 5α-reductase enzyme inhibitor. The current investigation was undertaken to clarify the step(s) in the biosynthetic NAS pathway that were sufficient to restore the acute ethanol withdrawal profile in ADX/GDX mice to that seen in intact animals. Male and female DBA/2J mice underwent ADX/GDX or SHAM surgery. After recovery, separate groups of animals were administered PROG, DOC, PROG+FIN, DOC+FIN, FIN, ALLO, ganaxalone (a synthetic ALLO derivative), corticosterone, or vehicle. Animals were then administered a 4 g/kg ethanol dose and allowed to undergo withdrawal. HICs were measured for 12 h and again at 24 h. The results indicate that replacement with PROG and DOC restored the withdrawal profile in ADX/GDX animals to SHAM levels, and that this effect was blocked with co-administration of FIN. Administration of FIN alone increased the withdrawal profile in both SHAM and ADX/GDX males. These findings indicate that the increase in acute withdrawal severity after ADX/GDX may be due to the loss of GABAergic NAS, providing insight into the contribution of endogenous GABAergic NAS to ethanol withdrawal severity. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: ADX, adrenalectomy; ALLO, allopregnanolone; ANOVA, analysis of variance; AUC, area under the curve; CORT, corticosterone; DOC, deoxycorticosterone; D2, DBA/2J; EtOH, ethanol; FIN, finasteride; GABA$_A$, γ-aminobutyric acid$_A$; GAN, ganaxalone; GDX, gonadectomy; HIC, handling-induced convulsion; NAS, neuroactive steroid; NMDA, *N*-methyl-D-aspartic acid; PROG, progesterone; THDOC, 3α,5α-tetrahydrodeoxycorticosterone; VEH, vehicle; WSP, Withdrawal Seizure-Prone.

Key words: neurosteroids, alcohol, allopregnanolone, progesterone, deoxycorticosterone, finasteride.

Each year in the United States, almost 5% of the population suffers from alcohol (ethanol; EtOH) abuse or dependence as defined by the DSM IV (Grant et al., 2004). It is estimated that this disease costs the American public millions of dollars a year in lost productivity and healthcare (Mark et al., 2000). While EtOH abuse and dependence are widespread, treatment options are extremely limited (Gardner and Kosten, 2007). One reason for this may be the wide variety of mechanisms through which EtOH can exert its effects. EtOH can have direct effects at receptors, such as acetylcholine, serotonin, γ-aminobutyric acid (GABA), and *N*-methyl-D-aspartic acid (NMDA) receptors (Chastain, 2006; Davis and de Fiebre, 2006). It can also alter membrane fluidity, enzyme concentrations, as well as several other factors (Busby et al., 1999; Gurtovenko and Anwar, 2009). Additionally, acute and chronic EtOH administration can cause up and down regulation of receptors and receptor subunits (Mhatre and Ticku, 1994; Devaud et al., 1997; Matsumoto et al., 2001), and the relative contribution of any of these factors may change from acute to chronic EtOH exposure. Furthermore, withdrawal from EtOH produces severe rebound neural hyperexcitability, which also may be mediated by numerous mechanisms (Littleton, 1998; Koob, 2003). Thus, in order to provide viable treatment options for alcohol abuse and dependence, it is imperative to understand the etiology of both acute and chronic EtOH intoxication and withdrawal.

Neuroactive steroids (NAS) rapidly alter neuronal excitability through interaction with neurotransmitter-gated ion channels (Paul and Purdy, 1992; Rupprecht, 2003), and many NAS are potent allosteric agonists at the γ-aminobutyric acid$_A$ (GABA$_A$) receptor (Purdy et al., 1992; Rupprecht, 2003). NAS can be produced in the periphery (mainly the adrenals and the gonads) or *de novo* in the brain (Holzbauer et al., 1985; Mellon and Griffin, 2002a,b). The production of NAS begins with the translocation of cholesterol across the mitochondrial membrane, which is facilitated by steroidogenic acute regulatory protein or the mitochondrial benzodiazepine receptor (Papadopoulos, 1993; Stocco, 2000). Then, a cytochrome P450 enzyme converts cholesterol into pregnenolone, which is a precursor to several different steroid hormones. Further down the steroidogenic pathway, the two-step metabolism of progesterone (PROG), deoxycorticosterone (DOC) and testosterone produces NAS [3α,5α-tetrahydroprogesterone

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(allopregnanolone, ALLO), 3α,5α-tetrahydrodeoxycorticosterone (THDOC) and 3α-androstanediol, respectively] through the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (Mellon, 1994; Compagnone and Mellon, 2000). It is possible to modulate the pathways leading to NAS by using enzyme inhibitors such as finasteride (FIN; Rittmaster, 1997) or removing the peripheral sources of NAS [i.e., adrenalectomy; ADX or gonadectomy; GDX; Korneyev et al., 1993].

In animals, NAS administration produces anxiolytic, antidepressant, anticonvulsant and sedative effects (Gasior et al., 1999), consistent with their GABAergic properties. These behavioral effects of NAS are mediated via the $GABA_A$ receptor. Recent evidence indicates that NAS bind in a specific pocket between the α and β subunits, allowing chloride to flux into the cell (Hosie et al., 2009). There are many distinct subunits (Olsen and Sieghart, 2009) and the subunit composition can fluctuate in response to environmental and physiological changes (Smith et al., 2007). While subunit composition of $GABA_A$ receptors may contribute to sensitivity of the receptor to modulation by NAS (Belelli and Lambert, 2005), manipulation of local endogenous GABAergic NAS levels also can alter $GABA_A$ receptor-mediated inhibition (Belelli and Herd, 2003).

Many of the behavioral effects of EtOH intoxication and NAS administration overlap. In fact, research over the past several decades has shown that some of EtOH's behavioral consequences may be modulated by an increase in NAS production (Kumar et al., 2009). Acute EtOH administration increases the production of both plasma and brain concentrations of ALLO and THDOC (Barbaccia et al., 1999; VanDoren et al., 2000; Finn et al., 2004). It has been shown that the increases in these NAS contribute to the delayed actions of EtOH on neuronal inhibition in medial septal band (VanDoren et al., 2000) and hippocampus (Sanna et al., 2004). That is, EtOH has been shown to have a direct and indirect effect on $GABA_A$ receptor-mediated inhibition, with the indirect effect being due to steroidogenesis (Sanna et al., 2004). Additional research has shown that NAS also contribute to some behavioral effects of EtOH, such as the anxiolytic (Hirani et al., 2005), antidepressant (Hirani et al., 2002), anticonvulsant and sedative/hypnotic effects (VanDoren et al., 2000). Based on these findings, it is likely that NAS may alter sensitivity to, or the duration of, some behavioral effects of EtOH.

Recent work in our laboratory has also shown that the rebound neuronal hyperexcitability seen during withdrawal from a 4 g/kg acute dose of EtOH may be mediated by peripherally produced NAS (Gililland and Finn, 2007). Male DBA/2J (D2) mice that had their adrenals removed had increased withdrawal severity (as measured by handling-induced convulsions; HICs), while female D2 mice had increased withdrawal severity when both their adrenals and gonads were removed. The results of these experiments suggested that an endogenous PROG- or DOC-derived anticonvulsant NAS was an important contributor to the neuronal rebound hyperexcitability seen during withdrawal from an acute, high dose of EtOH in intact animals (i.e., since removal of an anticonvulsant steroid with ADX/GDX would increase withdrawal). Given that removal of the gonads did not alter acute EtOH withdrawal in male mice, we reasoned that testosterone and its GABAergic derivatives may exhibit minimal contributions to the acute EtOH withdrawal profile in intact animals. However, in the current experiments we chose to use animals from both sexes that had both ADX and GDX surgery to insure total removal of the main sources of peripheral NAS production.

In order to further characterize this response, the purpose of the present studies was to identify specific steps along the NAS biosynthetic pathway that were necessary or sufficient to modulate neuronal rebound hyperexcitability following a high dose of EtOH (i.e., acute EtOH withdrawal). In order to fully explore this idea, we felt it was imperative that several arms along the NAS biosynthetic pathway and several steps within each arm were tested in our paradigm. We have chosen to investigate both the PROG and DOC arm of the NAS biosynthetic pathway because of previous data from our laboratory (discussed in the previous paragraph) indicate these two arms may be involved in EtOH withdrawal. The strategy was two-fold: (1) to administer NAS (ALLO, ganaxalone (GAN) and corticosterone (CORT)) or their precursors (PROG and DOC) and determine the effect on acute EtOH withdrawal in ADX/GDX animals and (2) to determine whether metabolism of NAS precursors was necessary for the modulatory effect on acute EtOH withdrawal in ADX/GDX animals through the use of FIN. We hypothesized that replacing ADX/GDX animals with PROG or DOC, or their 5α-reduced metabolites would restore acute withdrawal severity back to the levels seen in intact animals and that co-administering FIN would abolish these effects.

EXPERIMENTAL PROCEDURES

Subjects

Drug naive D2 male and female mice were purchased from Jackson West Laboratories (Davis, CA, USA) and were 8–12 weeks old at the time of experiment. Animals were group housed (four/cage, separated by sex) and were allowed free access to rodent chow (Labdiet 5001 rodent diet; PMI International, Richmond, IN, USA) and water. Mice were maintained on a 12 h (6 AM to 6 PM) light/dark cycle in polycarbonate cages (Thorens, Hazleton, PA, USA) in a room kept at 21±2 °C with humidity control. Mice were allowed to acclimate to the facility for at least 1 week before any experimental manipulations were undertaken. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the USA National Institutes of Health and were approved by the local Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering, including the use of post-operative analgesics.

Procedure

All animals were assigned to one of two groups: ADX/GDX surgeries (in which both the adrenals and the gonads were removed; surgery detailed below) or SHAM surgery (in which no organs were removed). After the appropriate surgery was performed, animals were allowed to recover for 7–14 days. Once recovered, animals were assigned to one of nine treatment groups (outlined below in drug section and in Table 1). Due to the large number of

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