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Localization of Brain 5 α -Reductase Messenger RNA in Mice Selectively Bred for High Chronic Alcohol Withdrawal Severity

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Abstract

Several lines of evidence suggest that fluctuations in endogenous levels of the γ -aminobutyric acid (GABA)ergic neurosteroid allopregnanolone (ALLO) represent one mechanism for regulation of GABAergic inhibitory tone in the brain, with an ultimate impact on behavior. Consistent with this idea, there was an inverse relationship between ALLO levels and symptoms of anxiety and depression in humans and convulsive activity in rodents during alcohol withdrawal. Our recent studies examined activity and expression of 5 α -reductase (*Srd5a1*), the rate-limiting enzyme in the biosynthesis of ALLO, during alcohol withdrawal in mice selectively bred for high chronic alcohol withdrawal (Withdrawal Seizure-Prone, WSP) and found that *Srd5a1* was down-regulated in the cortex and hippocampus over the time course of dependence and withdrawal. The purpose of the present studies was to extend these findings and more discretely map the regions of *Srd5a1* expression in mouse brain using radioactive *in situ* hybridization in WSP mice that were ethanol naïve, following exposure to 72 h ethanol vapor (dependent) or during peak withdrawal. In naïve animals, expression of *Srd5a1* was widely distributed throughout the mouse brain, with highest expression in specific regions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala. In dependent animals and during withdrawal, there was no change in *Srd5a1* expression in cortex or hippocampus, which differed from our recent findings in dissected tissues. These results suggest that local *Srd5a1* mRNA expression in WSP brain may not change in parallel with local ALLO content or withdrawal severity.

Keywords

ethanol; neurosteroid; GABA; convulsion; *in situ* hybridization

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Introduction

5 α -reductase (3-oxo-5- α -steroid 4-dehydrogenase) converts a number of delta 4, 3-keto steroids (androgens, progestins, glucocorticoids) to their 5 α -reduced metabolites. Two enzymes are responsible for 5 α -reductase activity. These 5 α -reductase isozymes are coded on two genes and are referred to as Type I (*Srd5a1*) and Type II (*Srd5a2*; e.g., Russell and Wilson, 1994). Both enzymes catalyze the same reaction, but at different pH optima and different substrate affinities. *Srd5a1* has a broad optimal pH range around 6.0 – 8.5 and $K_m = 1.0 - 5.0 \mu M$, whereas *Srd5a2* has a narrow acidic optimal pH at 5.5 and a $K_m = 0.1 - 1.0 \mu M$ (Russell and Wilson, 1994). Both are expressed in the brain, but *Srd5a2* is present only during a restricted perinatal period in rodents, while *Srd5a1* is present throughout development and adulthood (e.g., Melcangi et al., 1998). Kinetic properties and substrate specificity suggest that progesterone is actually the preferential substrate for 5 α -reductase (Anderson and Russell, 1990; Roselli and Snipes, 1984). Once the irreversible conversion of progesterone to 5 α -dihydroprogesterone takes place, the reduced progesterone metabolite can be further converted to the neurosteroid 3 α -hydroxy-5 α -pregnan-20-one (allopregnanolone; ALLO) via the enzyme 3 α -hydroxysteroid dehydrogenase (e.g., Mellon, 1994).

Research supported the idea for ALLO as a potent endogenous positive allosteric modulator of GABA action at GABA_A receptors (Belelli et al., 1990; Purdy et al., 1990; Rupprecht and Holsboer, 1999; Veleiro and Burton, 2009), and that endogenous *in vivo* fluctuations corresponded to levels that potentiated GABAergic inhibition electrophysiologically (Barbaccia et al., 2001; Belelli and Lambert, 2005; Paul and Purdy, 1992). Evidence also indicated that neurosteroids such as ALLO could be synthesized in the brain independent of peripheral sources (e.g., Cheney et al., 1995; Purdy et al., 1991), and that manipulation of endogenous ALLO levels in the hippocampus could alter GABA_A receptor mediated inhibition (Belelli and Herd, 2003) and seizure susceptibility (Gililand-Kaufman et al., 2008; Rhodes and Frye, 2005). A social isolation-induced decrease in brain ALLO levels, which was used as an animal model of depression in rodents, was associated with an increase in aggression and contextual fear (e.g., Dong et al., 2001; Pinna et al., 2008). Additionally, findings in humans indicated that some depressed individuals had decreased ALLO levels versus control subjects, which increased to normal levels following successful anti-depressant therapy (Uzunova et al., 1998). These findings were consistent with the idea that manipulations in endogenous ALLO levels could alter GABAergic inhibitory tone in the central nervous system (CNS), with an ultimate impact on behavior.

Acute administration of alcohol (ethanol) and ALLO share a similar pharmacological profile (see reviews by Criswell and Breese, 2005; Finn et al., 2004a; Grobin et al., 1998; Kumar et al., 2009; Morrow et al., 1999, 2001), and ethanol injection and consumption increased plasma and brain ALLO levels in rodents (e.g., Barbaccia et al., 1999; Eva et al., 2008; Finn et al., 2004b; Van Doren et al., 2000). Recent findings indicated that ethanol's acute steroidogenic effect was due in part, to increased synthesis of steroidogenic acute regulatory (StAR) protein in the adrenal gland (Boyd et al., 2010b), which was responsible for the translocation of cholesterol across the mitochondrial membrane and the initiation of steroidogenesis (Stocco, 2000). The steroidogenic effect of ethanol was shown to contribute to a delayed effect of ethanol on neuronal inhibition in medial septal band (Van Doren et al., 2000) and hippocampus (Sanna et al., 2004; Tokunaga et al., 2003) as well as to modulate sensitivity to some of ethanol's behavioral effects (discussed in Finn et al., 2004a; Gorin-Meyer et al., 2007; Kumar et al., 2009).

Chronic ethanol consumption and the induction of physical dependence reduced ethanol's acute steroidogenic effect (Boyd et al., 2010a), whereas withdrawal from chronic ethanol

decreased ALLO concentrations in rodents and humans (Cagetti et al., 2004; Finn et al., 2004a; Hill et al., 2005; Janis et al., 1998; Romeo et al., 1996; Tanchuck et al., 2009). In alcoholic patients, a decrease in GABAergic neurosteroid levels (such as ALLO and tetrahydrodeoxycorticosterone, THDOC) corresponded to an increase in subjective ratings of anxiety and depression during days 4–5 of withdrawal (considered early withdrawal phase), when compared with control subjects (Hill et al., 2005; Romeo et al., 1996, 2000). In heterogeneous rats, chronic ethanol withdrawal significantly decreased hippocampal ALLO levels and *Srd5a1* expression (Cagetti et al., 2004). In mice selectively bred for high chronic ethanol withdrawal (Withdrawal Seizure-Prone, WSP), a chronic ethanol withdrawal-induced decrease in plasma ALLO levels was associated with decreased adrenal *Srd5a1* expression, whereas the withdrawal-induced decrease in cortical ALLO levels corresponded to a decrease in cortical *Srd5a1* activity and expression (Tanchuck et al., 2009). These findings were consistent with data indicating that progesterone levels were unaltered or increased during ethanol withdrawal (Finn et al., 2004a) and pointed to a step in the steroidogenic pathway downstream of progesterone (i.e., *Srd5a1*) as contributing to the effect of chronic ethanol withdrawal on ALLO levels. Collectively, evidence indicated that down-regulation of *Srd5a1* expression contributed to the reduction in ALLO levels during ethanol withdrawal.

Based on the above, the purpose of the present study was to more discretely map the regions of *Srd5a1* expression in the mouse brain using radioactive *in situ* hybridization and to analyze whether *Srd5a1* mRNA expression was differentially regulated by chronic ethanol exposure and withdrawal in WSP mice. Our choice to examine only WSP (and not WSR) mice was based on the fact that the WSP mice were selectively bred for high chronic ethanol withdrawal severity (e.g., Crabbe et al., 1985) and exhibited marked reductions in plasma and brain ALLO levels during ethanol dependence and withdrawal (Tanchuck et al., 2009). Overall, we were interested in two questions: 1) Did selection for high alcohol withdrawal alter basal *Srd5a1* expression and/or the anatomical distribution versus recent findings in Swiss Webster mice (Agis-Balboa et al., 2006)? 2) Using an animal model of high alcohol withdrawal, did chronic ethanol exposure and withdrawal differentially alter *Srd5a1* expression in a manner that was consistent with results seen in dissected tissues? Thus, we reasoned that the results could provide insight on whether brain regional changes in *Srd5a1* expression (and presumably ALLO levels) modulated convulsive activity by locally regulating GABAergic tone in an animal model of high chronic ethanol withdrawal.

Materials and Methods

Subjects

Two genetically independent WSP lines have been bred for high chronic ethanol withdrawal severity following exposure to 72 h of ethanol vapor from a genetically heterogeneous stock of known composition (i.e., HS/Ibg), using within family selection (Crabbe et al., 1985). Drug naïve adult male WSP mice from the first genetic replicate were kindly provided by Dr. John Crabbe and were used in all studies. Mice were bred in the Veterinary Medical Unit at the Veterans Affairs Medical Center (Portland, OR), were group housed (4/individually ventilated polycarbonate cage), and were maintained on a 12:12 h light:dark cycle (lights on at 0600) at $23 \pm 1^\circ\text{C}$. Mice had free access to tap water and rodent chow (Labdiet 5001 rodent chow, PMI International). At the time of testing, mice were from selected generation 26 (filial generations 112 – 115), with an age range of 88 – 109 days. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and disseminated by the US National Institutes of Health (NIH) and were approved by the local Institutional Animal Care and Use Committee.

For the initial study, naïve WSP-1 male mice (n = 8) were used to map the expression of *Srd5a1* mRNA expression in brain. A second group of naïve adult male WSP-1 mice were exposed to ethanol vapor or air for 72 h to examine the effect of ethanol dependence and withdrawal on *Srd5a1* mRNA expression. Upon removal from inhalation chambers after 72 h, separate groups were euthanized immediately or 8 hrs later (i.e., Air-0 h, ethanol-0 h, ethanol-8 h; n = 6–7 animals/treatment group). For both studies, animals were deeply anesthetized with 5% isoflurane and transcardially perfused with buffered 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO.). Brains were removed, cryoprotected overnight in the same fixative containing 10% sucrose (Sigma-Aldrich, St Louis, MO.), and stored at –80°C until they were sectioned on a microtome and used for *in situ* hybridization of *Srd5a1* expression.

Chronic Ethanol Administration

Mice were exposed to ethanol vapor or air for 72 h by the Alcohol Dependence Core of the Portland Alcohol Research Center, using a standardized method for inducing physical dependence that was routinely used in the laboratory (e.g., Finn and Crabbe, 1999; Finn et al., 2006; Gililand-Kaufman et al., 2008; Tanchuck et al., 2009). Briefly, mice in the ethanol groups were initially injected with a 1.5 g/kg priming dose of ethanol (Pharmco Products, Brookfield, CT) and exposed to ethanol vapor (7–9 mg ethanol/liter air) inside the inhalation chamber. Mice in the control group were injected with saline and exposed to air in a separate inhalation chamber. All mice received daily injections (i.e., initially and 24h and 48 h later) of pyrazole hydrochloride (pyrazole, 68.1 mg/kg; Sigma-Aldrich, St. Louis, MO), an alcohol dehydrogenase inhibitor routinely used to stabilize blood ethanol concentrations (BECs) in the ethanol-exposed mice and to control for any non-specific effects of pyrazole injection in the air-exposed mice. Tail blood samples (20 µl) were taken from the ethanol-exposed mice at 24, 48 and 72 h to monitor BEC via head-space gas chromatography (Finn et al., 2007). The target BEC for chronic exposure was 1.5 mg/ml. Upon removal from the inhalation chambers at 72 h, all mice were placed into polycarbonate cages with cob bedding and taken to the laboratory where separate groups were euthanized immediately (0 h) or 8 h later. No animals exhibited spontaneous convulsions during withdrawal (prior to euthanasia), and care was taken during handling prior to euthanasia to ensure that none of the animals exhibited convulsions.

Synthesis of *Srd5a1* and *Srd5a2* cDNA

Mouse specific *Srd5a1* and *Srd5a2* cDNA were cloned from mouse liver and prostate, respectively using the Clone/AMP pAMP-10 PCR cloning kit (Gibco, Invitrogen, Grand Island, NY). Primers for *Srd5a1* were forward 234–253 and reverse 570–589 (Accession No. BC115469.1), and spanned exons 1–3. Primers for *Srd5a2* were forward 121–139 and reverse 507–524 (Accession No. NM053188.2), and spanned exons 1–2.

Ribonuclease Protection Assay

The mouse *Srd5a1* and *Srd5a2* cDNAs described above were linearized with XbaI and HindIII, respectively and used to synthesize a [³²P]cRNA antisense probe by T7 RNA polymerase. An aliquot of total RNA was hybridized with gel-purified ³²P-labeled antisense probe in hybridization buffer. After hybridization overnight at 45°C, the samples were digested with ribonuclease T1. The digestion reaction was terminated and protected hybrids were phenol-chloroform extracted, precipitated with ethanol, denatured, and electrophoresed on a 5% polyacrylamide gel containing 7 M urea. The dried gel was exposed to x-ray film to generate autoradiograms. Dilutions of sense RNA were used to construct a standard curve (0.25 – 4.0 µg) for the RNase assay.

In situ Hybridization Histochemistry

Frozen perfused brains were cut into 30- μ m coronal sections, applied to positively charged slides, and dried overnight under vacuum. *In situ* hybridization was performed according to our previously published procedures (Roselli et al., 1998). Briefly, mouse-specific [33 P]-labeled cRNA probes (*Srd5a1* & *Srd5a2*) were synthesized, purified, and diluted in hybridization buffer. On the day of hybridization, the tissue sections were treated with Proteinase K, acetylated, and dehydrated. The hybridization solution with probe (1.0×10^7 dpm/ μ l) was then pipetted onto the sections (80 μ l/slide), covered with a glass coverslip, sealed and incubated for 18 h at 58°C. Following hybridization, the slides were subjected to RNase digestion and washed several times to a final stringency of $0.1 \times$ SSC at 65°C for 30 min. The slides were then dried under vacuum and exposed to Kodak Biomax MR film for 3 wk.

Several control experiments were performed to test the specificity of both the hybridization methods and the *Srd5a1* and *Srd5a2* probes. These included incubation of tissue with 32 P-labeled sense-strand probes and hybridization on sections that had been pretreated with RNase (20 μ g/ml for 30 min at 37°C). Both of these tests failed to show any specific hybridization signal (data not shown). For *Srd5a1*, the thermal stability of the probe was tested by taking adjacent serial sections that had been hybridized as described and rinsing one series each in 0.1-strength SSC at 65°C, 75°C, 85°C, and 95°C to generate a melting curve. A significant reduction in hybridization signal was observed only when the temperature of the post-hybridization wash exceeded 85°C, further suggesting that the labeling achieved was specific (expected melting temperature = 81°C; data not shown). Additional tissue controls were performed to confirm that signal for *in situ* hybridization was detectable for *Srd5a1* in liver and for *Srd5a2* in prostate (data not shown), and to demonstrate that adult brain exhibited extensive hybridization signal for *Srd5a1*, but not *Srd5a2* (Fig. 1).

Data Analysis

The optical densities from autoradiographic images of *in situ* hybridization were measured using Image J (version 1.43) software obtained from the NIH. The specific hybridization signal was obtained by measuring the optical density (arbitrary units) in standardized sampling windows from specific brain regions and subtracting the background optical density – defined on each section as an adjacent area that did not contain specific signal. For each brain region, three sections per animal were analyzed, and the mean specific hybridization optical density value from each animal was calculated. A two-way analysis of variance was used to test for differences in optical density between treatment groups and across brain regions, with brain region as a repeated measures factor and a Greenhouse-Geisser correction (SPSS, version 19; Chicago, IL). Significance was set at $p \leq 0.05$.

Results

RNA Protection Analysis of Total RNA from Mouse Tissues

A 32 P-labeled cRNA probe synthesized from the *Srd5a1* insert was used to examine the distribution and integrity of the protected RNA fragment in dissected mouse tissues. The *Srd5a1* probe protected a single 354-base RNA fragment in the mouse liver, prostate and hypothalamus, all positive control tissues (Fig. 2). The labeled probe also detected increasing concentrations of sense RNA. These results confirmed that the cRNA probe used for *in situ* hybridization recognized a single and specific *Srd5a1* RNA transcript in the mouse brain.

Neuroanatomical distribution of *Srd5a1* mRNA expression in the mouse brain

Expression of *Srd5a1* mRNA was examined by *in situ* hybridization histochemistry, and it was found to be widely distributed throughout the mouse brain (Fig. 3). The highest expression was observed in specific regions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala (Table 1). Within the cerebral cortex, strong signal for *Srd5a1* was observed in layers II – V of the motor and somatosensory cortex and in the cingulate cortex. Hybridization signal was also highest in the anterodorsal and parafascicular thalamus, medial habenula, hippocampal CA2, CA3 regions and the dentate gyrus. Intermediate signal intensities were observed in midline structures extending through the septum, hypothalamus, and brainstem, as well as the reticular thalamic nucleus. The medial, cortical, central and basolateral nuclei of the amygdala all exhibited substantial expression of *Srd5a1*.

Expression of *Srd5a1* in dependent animals and during ethanol withdrawal

Following exposure to 72 h ethanol vapor, mean BEC upon removal from the inhalation chamber was 1.65 mg/ml, which represented high intoxication. For comparative purposes, mean BEC was 1.10 mg/ml in WSP mice that were used for the examination of *Srd5a1* expression and activity in dissected tissues (Tanchuck et al., 2009). In the present study, expression of *Srd5a1* was examined in dependent animals (i.e., 0 h; animals intoxicated; BEC = 1.65 mg/ml) and during withdrawal (i.e., 8 h; BEC = 0 mg/ml). The 8 h time point was based on evidence that WSP mice exhibited peak withdrawal severity, measured by an increase in handling induced convulsions, at 8 h after 72 h of ethanol vapor exposure (e.g., Crabbe et al., 1985; Finn and Crabbe, 1999). Peak withdrawal also corresponded to a time when it had been shown that ALLO levels were significantly lower than in air-exposed controls (Tanchuck et al., 2009).

Srd5a1 mRNA expression was evaluated in 6 brain regions that exhibited intermediate to high signal (see Fig. 4). The initial analyses were conducted across all three treatments. Expression of *Srd5a1* varied significantly by brain region [$F(2,8,41.96) = 78.17, p < 0.001$], but was not significantly altered by treatment [$F(2,15) = 0.89$]. There was a trend for an interaction between brain region and treatment [$F(5.59,41.96) = 1.76, p = 0.135$]. Since our *a priori* hypothesis was that *Srd5a1* would be lower during withdrawal than in controls, separate analyses were conducted across these two treatment groups (air control and withdrawal). The results were similar, in that *Srd5a1* expression was significantly influenced by brain region [$F(5,55) = 54.59, p < 0.001$] but not by withdrawal [$F(1,11) = 2.14, p = 0.17$]. There was a trend for an interaction between treatment and brain region [$F(5,55) = 2.27, p = 0.06$]. Overall, the results indicated that there were no significant changes in *Srd5a1* expression at 8 h after ethanol exposure (i.e., withdrawal), when compared to either air-exposed controls or ethanol dependent animals that were euthanized at the 0 h time point (Fig. 4).

Discussion

In general, the results of the present study suggested that selection for high chronic ethanol withdrawal severity was not associated with marked differences in basal brain regional distribution of *Srd5a1* expression, when compared with the anatomical localization of *Srd5a1* in Swiss Webster mouse (Agis-Balboa et al., 2006) or Long-Evans rat brains (Lauber and Lichtensteiger, 1996). Our results confirmed that *Srd5a1* was distributed within specific forebrain and midbrain circuits and was particularly abundant in cortical, thalamic and amygdaloid regions. Agis-Balboa et al. (2006) previously demonstrated that *Srd5a1* colocalized to neurons and not glia in adult mice. These investigators also found that neurosteroidogenic enzymes were highly expressed in principal GABAergic and

glutamatergic output neurons and absent from GABAergic interneurons. With this in mind, the pattern of expression of *Srd5a1* transcripts identified in the present study closely corresponded with the pattern of expression of the GABA_A receptor subunits $\alpha 1$ and $\beta 2$, which could comprise synaptic GABA_A receptors, as well as thalamic expression of the $\alpha 4$ subunit, which could comprise fast-acting tonic (extrasynaptic) GABA_A receptors (e.g., Heldt and Ressler, 2007; Jia et al., 2007; Wisden et al., 1992; see review by Farrant and Nusser, 2005). Thus, our results provide further support for the idea that ALLO could be synthesized by principal output neurons to modulate GABA action at postsynaptic, phasic and tonic GABA_A receptors.

A surprising finding was the lack of significant changes in the *in situ* hybridization signal for *Srd5a1* mRNA expression in the discrete corticolimbic nuclei that were analyzed in dependent animals and during withdrawal in the present study. Since our recent work in dissected tissues reported a non-significant but 55% decrease in hippocampal *Srd5a1* expression and a significant and 75% decrease in cortical *Srd5a1* expression in WSP mice during withdrawal, we have considered several possible explanations for the different results between the two studies. First, BEC was approximately 50% higher in the present versus previous study. While ethanol exposure was closer to the optimal level that produced BECs of 1.5 mg/ml in the current study (1.65 mg/ml) than in the recent work conducted in dissected tissue (1.10 mg/ml), WSP mice in the Tanchuck study with lower ethanol exposure did respond to alcohol dependence and withdrawal with a decrease in plasma and cortical ALLO concentrations that appeared to be associated with reductions in *Srd5a1* activity and mRNA expression in dissected tissue (Tanchuck et al. 2009). It also was unlikely that differences in BECs contributed to the dissimilarities between the two studies in the effects of withdrawal on *Srd5a1* expression, since previous work (Finn and Crabbe, 1999; Terdal and Crabbe, 1994) indicated that withdrawal severity in WSP mice was not markedly different following ethanol vapor exposures that yielded BECs ranging from 1.0 – 2.0 mg/ml. Second, another difference between the two studies was that the PCR primers utilized in the earlier study targeted different areas of the *Srd5a1* gene than the cDNA probe utilized in the current study. However, this methodological difference would only have presented a confound if alternative splicing was possible, and there was no evidence for alternative splicing of the *Srd5a1* gene (Russell and Wilson, 1994). Third, given that the 5 α -reductase isozymes were reported to have long half-lives (20 – 30 hrs; Russell and Wilson, 1994), we believe that it was unlikely that 8 hr withdrawal time point would have differentially altered *Srd5a1* half-life between the two studies. Finally, two inherent difficulties with directly comparing the two experimental approaches involve the heterogeneous composition of dissected tissue and the fact that treatment differences likely represented an average effect of both regulation and tissue dilution. Moreover, as recently demonstrated in a social isolation model, changes in *Srd5a1* expression could be limited to specific cell populations in discrete brain regions, such as the pyramidal neurons in layer V of the frontal cortex or CA3 hippocampus (Agis-Balboa et al., 2007). However, even if this level of tissue specificity was achieved, the current anatomical approach could not address the possibility that enzyme activity was regulated independently of mRNA expression. Indeed, our previous study hinted at this in cortex of the WSR mouse strain, where a discordance between *Srd5a1* mRNA expression and enzyme activity was observed (Tanchuck et al. 2009). Future studies will need to address these issues by examining the effect of alcohol dependence and withdrawal on *Srd5a1* protein levels in conjunction with mRNA expression.

Nevertheless, evidence in rodents and humans suggested that there was an association between *Srd5a1* genotype and ethanol withdrawal severity (Bergeson et al., 2003; Crabbe, 1998) or alcohol dependence (Milivojevic et al., 2011), respectively. Comparison of allele frequencies for single nucleotide polymorphisms (SNPs) in the coding regions of the *Srd5a1* (*SRD5A1*) and 3 α -HSD (*AKR1C3*2*) genes between alcohol dependent and control subjects

revealed that the minor alleles for both genes were more frequent in control subjects. The significant interaction of genotype for *SRD5A1* and *AKR1C3*2* revealed that the protective effect of the minor allele at each marker for risk of alcohol dependence was conditional of the genotype of the second marker (Milivojevic et al., 2011). In other words, risk for alcohol dependence was reduced by 50% in individuals homozygous for both minor alleles. This study provided indirect evidence for a relationship between neurosteroid biosynthetic enzymes (and presumably neurosteroid levels) and alcohol dependence in humans.

With regard to data in rodents, initial gene mapping studies determined that there was a significant correlation between ethanol withdrawal severity and a region of murine chromosome 13 where *Srd5a1* mapped (Crabbe, 1998; Jenkins et al., 1991). In a subsequent study (Bergeson et al., 2003), an epistatic interaction between the chromosomal 13 region where *Srd5a1* was located and a region on chromosome 11 that contained several genes for GABA_A receptor subunits ($\alpha 1$, $\alpha 6$, $\gamma 2$) suggested that an interaction between *Srd5a1* and GABA_A receptor subunit genes might occur. Consistent with this idea, ethanol withdrawal significantly reduced endogenous ALLO levels (Tanchuck et al., 2009) as well as the sensitivity of GABA_A receptors to ALLO, measured biochemically or behaviorally following systemic or microinjection (Finn et al., 2006; Gililand-Kaufman et al., 2008), in WSP mice. Thus, it was possible that the modulatory effects of ALLO on ethanol withdrawal severity in WSP mice reflected a balance between local concentration of ALLO at GABA_A receptors and the concomitant change in sensitivity of the GABA_A receptors to ALLO that were occurring during ethanol withdrawal. Taken in conjunction with the present findings, the reduction in GABA_A receptor sensitivity likely had a greater impact on ethanol withdrawal severity in WSP mice than alterations in endogenous ALLO levels.

In conclusion, the similar basal brain regional distribution of *Srd5a1* expression in WSP (present study) and Swiss Webster mice (Agis-Balboa et al., 2006) suggested that local ALLO could be synthesized by principal output neurons to modulate GABAergic neurotransmission. The fact that local *Srd5a1* mRNA expression did not change in parallel with withdrawal severity in WSP mice was consistent with the idea that the sensitivity of GABA_A receptors to fluctuations in ALLO levels was more important than altered local ALLO synthesis for modulating ethanol withdrawal severity in WSP mice. Nonetheless, the recent association between neurosteroid biosynthetic enzyme genotype (*SRD5A1* and *AKR1C3*2*) and alcohol dependence in humans (Milivojevic et al., 2011) emphasized the importance of future examinations to dissect the relationship between neurosteroid biosynthesis, GABA_A receptor sensitivity, and alcohol dependence.

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Figure 1. Brain tissue control

Comparison of the expression of *Srd5a1* and *Srd5a2* with the thionin stained brain section illustrates that only *Srd5a1* is expressed in adult brain.

Figure 2.

RNase protection assay showing the distribution of *Srd5a1* in adult mouse liver, prostate (Prost) and hypothalamus (HYP) as detected by a ^{32}P -labeled cRNA probe. The UP (undigested probe) lane illustrated the migration of the *Srd5a1* (424 nt) cRNA probes used in the assay. The DP (digested probe) lane illustrated that probe incubated with tRNA was completely digested by RNase (negative control). A standard curve was constructed using 62.5–4000 fg of sense RNA. Sense RNA included 40 bases of vector resulting in a 354-nt protected band. The arrow indicated the band corresponding to protected *Srd5a1*. Each tissue lane represented 25 μg of total RNA.

Figure 3.

Digitized autoradiogram of ^{32}P *in situ* hybridization, demonstrating the rostral to caudal expression of *Srd5a1* mRNA in the cingulate cortex (Cg); medial septum (MS); vertical limb of the diagonal band (VDB); piriform cortex (Pir); lateral septum (LS); bed nucleus of the stria terminalis (BST); medial preoptic area (MPA); paraventricular thalamic nucleus (PVA); medial preoptic nucleus (MPN); retrosplenial cortex (RSG); anterior hypothalamic area (AHA); hippocampal CA1 & CA3 regions; dentate gyrus (DG); medial habenula nucleus (MHb); basal amygdala (BA); central amygdala (CeA); cortical amygdala (CoA); medial amygdala (MeA); dorsal medial hypothalamus (DMH); ventral medial hypothalamus (VMH); parafascicular thalamic nucleus (PF); posterior hypothalamic nucleus (PH); periaqueductal gray (PAG); ventral tegmental area (VTA); and substantia nigra (SN).

Figure 4. Optical density measurements for *Srd5a1* expression in specific brain regions in male WSP mice exposed to air or alcohol for 72 h

The 8 h withdrawal time point corresponded to when WSP mice exhibited peak levels of handling induced convulsions after 72 h of alcohol vapor exposure and when plasma ALLO levels were significantly lower than in air-exposed controls. The present results showed that local *Srd5a1* mRNA expression, measured as the optical density (arbitrary units) of the *in situ* hybridization autoradiogram, did not change in parallel with withdrawal severity, suggesting that GABA_A receptor sensitivity to ALLO rather than altered local ALLO synthesis was important for modulating withdrawal in WSP mice.

Table 1Relative intensity of *Srd5a1* mRNA expression in the mouse brain.

Brain Area	Relative intensity of hybridization signal
Cortex	
Cingulate cortex	+++
Motor cortex	++
Retrosplenial cortex	+++
Somatosensory cortex	++
Piriform cortex	+++
Medial septum	++
Lateral septum	++
Ventral diagonal band	++
Thalamus	
Anterodorsal n.	+++
Paraventricular n.	++
Periventricular n.	++
Parafascicular n.	+++
Reticular n.	++
Medial habenula	+++
Zona inserta	+
Hypothalamus	
Medial preoptic area	+
Anterior hypothalamic area	+
Dorsomedial n.	+
Ventromedial n.	++
Arcuate n.	+
Posterior n.	++
Hippocampus	
CA1	++
CA2	+++
CA3	+++
Dentate gyrus	+++
Amygdala	
Basomedial n.	++
Central n.	++
Cortical n.	++
Medial n.	++
Medial mammillary n.	++
Ventral tegmental area	++
Periaquiductal gray	++

The pluses provide an indication of the relative intensity of the hybridization signal as judged by optical density units ranging from 0.05 – 0.2 (+) low; 0.2 – 0.4 (++) medium; > 0.4 (+++) high.