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Acute neuroactive steroid withdrawal in withdrawal seizure-prone and withdrawal seizure-resistant mice

Matthew T. Reilly*, John C. Crabbe, Nathan R. Rustay, Deborah A. Finn

Department of Behavioral Neuroscience, Portland Alcohol Research Center, Oregon Health Sciences University, Portland, OR 97201, USA
Research Services (R&D 40), Department of Veteran Affairs Medical Center, Room G-230, Building 104, 3710 SW US Veterans Hospital Road, Portland, OR 97201, USA

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Abstract

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is an endogenously derived metabolite of progesterone, and a potent positive modulator of γ-aminobutyric acid$_A$ (GABA$_A$) receptors. A withdrawal syndrome, characterized by central nervous system (CNS) hyperexcitability, has been demonstrated following abrupt discontinuation of high progesterone levels in rats, which was due in part to altered levels of allopregnanolone. The purpose of the present study was to determine if a single administration of pregnanolone or allopregnanolone could produce an acute withdrawal response in mice selected for susceptibility (Withdrawal Seizure-Prone, WSP) or resistance (Withdrawal Seizure-Resistant, WSR) to ethanol withdrawal convulsions. WSP mice administered 75 mg/kg pregnanolone showed a significant increase in handling-induced convulsion (HIC) scores over a 25-h testing period. In contrast, HIC scores in WSR mice were negligible after acute administration of 25, 50, 75, or 100 mg/kg pregnanolone. WSP mice also showed a similar increase in HIC after withdrawal from 75 mg/kg allopregnanolone. This effect was evident at both the 10-h and 25-h overall withdrawal severity assessment. These results demonstrate that neuroactive steroids can elicit an acute withdrawal response similar to that of other positive modulators of GABA$_A$ receptors in WSP mice, supporting the notion that a common set of genes underlie acute and chronic withdrawal severity from multiple agents with depressant effects on the central nervous system.

Keywords: Neurosteroids; Allopregnanolone; Acute withdrawal; WSP; WSR; Selected lines; Ethanol

The reduced metabolites of progesterone and deoxycorticosterone are potent positive modulators of γ-aminobutyric acid$_A$ (GABA$_A$) receptors [22,23]. Exogenous administration produces anxiolytic [8,9,13,38] locomotor stimulant [24], sedative–hypnotic [25], and anticonvulsant [3,4,14,16] effects that are similar to classical GABA$_A$ receptor mimetics (e.g., ethanol, benzodiazepines, and barbiturates). Allopregnanolone (3α-hydroxy-5α-pregnan-20-one), a reduced metabolite of progesterone, interacts with GABA$_A$ receptors at a distinct recognition site, where it potentiates a GABA-stimulated chloride conductance [18,29]. Moreover, at concentrations that occur endogenously, allopregnanolone potentiates the in vitro actions of GABA, suggesting that it may be a physiologically significant neuromodulator of behaviors associated with central nervous system (CNS) excitability [23,29].

Fluctuations in brain and plasma concentrations of progesterone and its metabolite, allopregnanolone, are known to occur during stress, pregnancy, and after ethanol exposure [1,2,15,20,29,30]. The possibility exists that any CNS adaptations occurring during periods of high endogenous progesterone and allopregnanolone levels might result in CNS hyperexcitability once concentrations of progesterone or allopregnanolone are low. Consistent with this notion, several recent findings have suggested that symptoms of progesterone withdrawal may be due, in part, to decreased allopregnanolone levels [17,27,28,33]. Using a pseudo-pregnancy model, which resulted in elevated levels of progesterone, induction of withdrawal from progesterone by ovariectomy produced an exacerbation of

* Corresponding author. Research Services (R&D 40), Department of Veteran Affairs Medical Center, Room G-230, Building 104, 3710 SW US Veterans Hospital Road, Portland, OR 97201, USA. Tel.: +1-503-220-8262, ext. 56386; fax: +1-503-721-7839.

E-mail address: reillym@ohsu.edu (M.T. Reilly).

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seizures and increased anxiety in rats. These symptoms were eliminated by blocking the conversion of progesterone to allopregnanolone with indomethacin or finasteride, suggesting that withdrawal from chronic-elevated allopregnanolone concentrations contributed to the state of CNS hyperexcitability associated with progesterone withdrawal [27,28,33]. In addition, abrupt withdrawal from elevated progesterone and allopregnanolone was associated with an increase in both mRNA and peptide content for the α_4 subunit of $GABA_A$ receptors [33]. This finding lends further support to the notion that alterations in $GABA_A$ receptors contribute to the CNS hyperexcitability observed during periods of withdrawal from high endogenous progesterone and allopregnanolone.

Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) mice have been selectively bred to have severe (i.e., WSP) or mild (i.e., WSR) handling-induced convulsions (HIC) following exposure to 72 h of ethanol vapor [21]. This enhanced sensitivity of WSP mice to withdrawal induced by chronic ethanol exposure, generalized to withdrawal produced by chronic exposure to phenobarbital, diazepam, and nitrous oxide [5–7]. WSP mice also experience significant elevations in withdrawal HIC scores several hours after a single injection of various compounds with sedative effects on the CNS. This acute withdrawal response, which follows the reduction in HIC scores due to the sedative/anticonvulsant effects of the drug, is thought to represent a state of physical dependence. In contrast, acute withdrawal from CNS depressants was negligible in WSR mice [11]. These results suggest that common genes may underlie acute and chronic withdrawal from CNS depressant drugs, since WSP and WSR mice differ on responses (i.e., acute withdrawal from depressant agents) for which they were not selected [12].

Whether these same selectively bred mouse lines show differential sensitivities to acute withdrawal from allopregnanolone or other neuroactive steroids with depressant effects on the CNS has not been demonstrated. However, in a recent report there was an indication that allopregnanolone might have the potential to elicit an acute withdrawal reaction in WSP mice after a single administration [16]. In that study, both ethanol-naive WSP mice and WSP mice undergoing ethanol withdrawal were injected with allopregnanolone to determine their sensitivity to the anticonvulsant effect of this neurosteroid. WSR mice were not tested because they do not display HIC after chronic ethanol exposure. Although the purpose of that study was not to examine the effects of acute withdrawal from allopregnanolone, there was evidence to suggest a rebound hyperexcitability in ethanol-naive WSP mice, as measured by an increase over average vehicle HIC starting 18 h after an acute injection of 20 mg/kg of allopregnanolone.

Collectively, the recently demonstrated withdrawal-related symptoms following termination of high endogenous progesterone and allopregnanolone [17,27,28,33], as well as the indication of a rebound hyperexcitability following acute administration of 20 mg/kg allopregnanolone in WSP mice [16], suggest that administration of a hypnotic dose of allopregnanolone might elicit an acute withdrawal response similar to that of other sedative–hypnotic drugs. Therefore, the purpose of the present study was to determine whether acute administration of allopregnanolone or pregnanolone to WSP and WSR mice would produce an initial decrease, followed by a withdrawal-related increase in HIC, in a manner consistent with other positive modulators of $GABA_A$ receptors, such as ethanol, benzodiazepines, and barbiturates. We were also interested in determining whether WSP and WSR mice were differentially sensitive to acute neuroactive steroid withdrawal. The results from our acute pregnanolone withdrawal experiment indicated that pregnanolone did not produce a dose-dependent increase in withdrawal severity. Therefore, we also measured acute sensitivity to the hypothermic effect of pregnanolone in WSP and WSR mice to determine if the potential sedative effects of severe hypothermia at a high dose of pregnanolone (100 mg/kg) might have confounded our acute withdrawal determination.

1. Method

1.1. Subjects

Naive male WSP and WSR mice from both replicate lines (selection generation 69) were used in these experiments. Selection replication is important because if both replicate lines (e.g., WSP-1 and WSP-2, or WSR-1 and WSR-2) are found to differ on a response for which they were not selected, then this gives strong support for the existence of a genetic correlation of the trait of interest. Furthermore, the use of replicated lines in selection experiments provides a control for trait-irrelevant alleles that have become fixed by chance (genetic drift) due to inbreeding. That is, it is unlikely that the same trait-irrelevant alleles will become fixed by chance in both replicate lines [12]. All mice were 70–100 days old at time of testing, were housed four per cage, and maintained under a 12-h light/dark cycle throughout the course of the experiments. Tap water and lab chow (Purina) were freely available. All animal testing performed in these experiments was carried out in accordance with the guidelines issued for the Care and Use of Laboratory Animals by the National Institutes of Health.

1.2. Drug preparation

Allopregnanolone (synthesized by R.H. Purdy, PhD) and pregnanolone (Steraloids, Wilton, NH) were prepared in a 20% w/v solution of 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA), which was used as the vehicle. Concentrations of neurosteroid

solutions ranged from 2.5 to 10 mg/ml, and were administered to mice by intraperitoneal injection in a volume of 0.01 ml/g body weight. Solubility difficulties at higher neurosteroid concentrations were overcome by mild sonication.

1.3. Experiment 1: Acute pregnanolone withdrawal

WSP ($n = 10-12$ mice/dose and replicate) and WSR ($n = 4-8$ mice/dose and replicate) male mice from both replicate lines were randomly assigned to one of five treatment groups: 0, 25, 50, 75, or 100 mg/kg pregnanolone. A pre-drug baseline HIC score was measured 30 min prior to intraperitoneal injection of pregnanolone. At time zero, WSP and WSR mice were injected with their assigned dose of neurosteroid or vehicle. HIC was monitored every hour for 10 h beginning with hour 1 post-injection and again at hours 24 and 25. The HIC scoring scale used was modified from Goldstein [19], and has been published elsewhere [11]. Briefly, this procedure involved lifting the animal by the tail, gently turning it 180° if necessary, and observing convulsions. A score of zero indicated no convulsions. HIC scores ranging from 1 to 3 required the gentle turn to elicit a tonic or clonic convulsion, whereas convulsions elicited by merely lifting the mouse by the tail were scored as 4–6. A score of 7 indicated a spontaneous convulsion. Area under the withdrawal curve (AUC) was used as a measure of overall withdrawal severity. AUC was calculated by first calculating a difference score (each animal's neurosteroid-treated HIC score minus the average vehicle HIC score) at each time point. Similarly, for vehicle-treated animals a difference score was calculated by subtracting the average vehicle score at each time point from each animal's individual score. This difference score was then used to

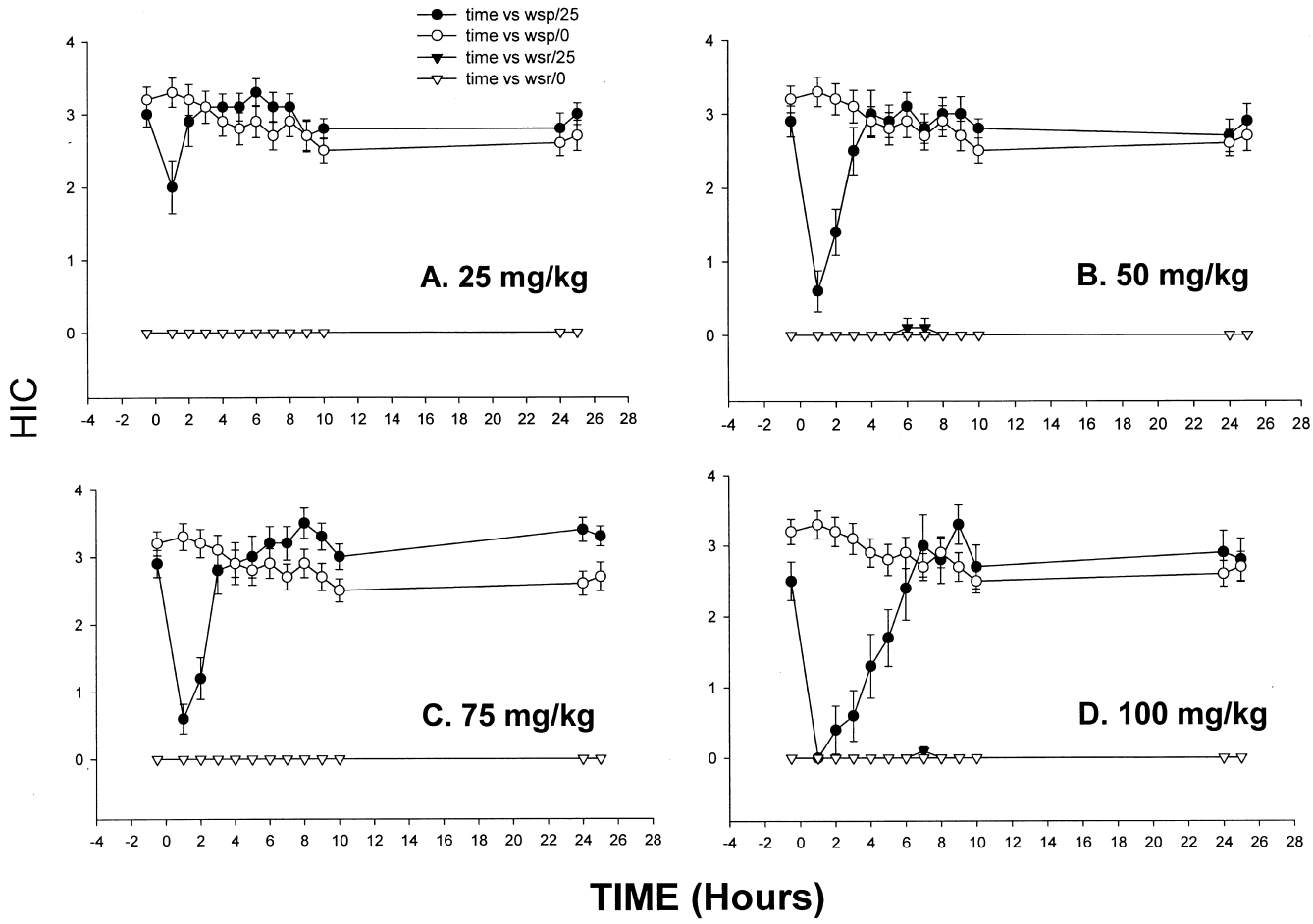

Fig. 1. Acute pregnanolone withdrawal time course in WSP and WSR mice. All doses of pregnanolone initially suppressed HIC scores in WSP mice. The HIC scores for pregnanolone-treated mice returned to vehicle-treated values at different times depending on the dose of pregnanolone administered. For example, (A) the 25 mg/kg group returned to vehicle scores at hour 3, (B) the 50 mg/kg group between hours 3 and 4, (C) 75 mg/kg group at hour 4, and (D) the 100 mg/kg group between hours 6 and 7. There was a rebound hyperexcitability (i.e., an increase in average HIC scores over average vehicle HIC scores) observed for WSP mice injected with the 75 mg/kg dose of pregnanolone (C). There was no effect of pregnanolone for any of the doses administered to WSR mice throughout the entire 25 h time course. Symbols for pregnanolone-treated WSR mice appear beneath symbols for vehicle-treated WSR mice. Values represent mean ± S.E.M. for 10–12 mice per dose and replicate (WSP), and 4–8 mice per dose and replicate (WSR).

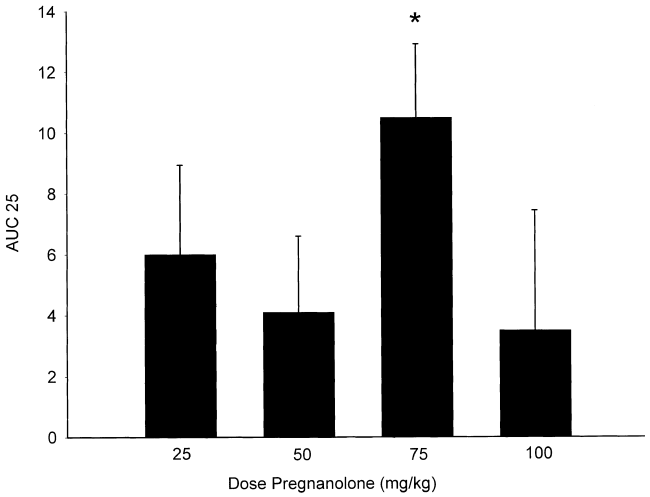

Fig. 2. Area under the 25-h withdrawal curve (AUC 25) for WSP mice administered 25, 50, 75, and 100 mg/kg pregnanolone. Values represent mean ± S.E.M. for WSP mice depicted in Fig. 1. AUC for vehicle-treated mice was 0.0 ± 2.7, and is not shown. AUC 25 was significantly different for WSP mice injected with 75 mg/kg pregnanolone (* $P < .04$ vs. vehicle).

calculate AUC by summing these scores, beginning with the time point at which average HIC scores for neurosteroid-treated animals exceeded the average vehicle-treated HIC scores. Thus, time points at which neurosteroid-treated HIC scores were below vehicle-treated HIC scores were not considered in the calculation for AUC. AUC was calculated for HIC data encompassing 10 (AUC 10) and 25 h (AUC 25) of testing.

1.4. Experiment 2: Acute allopregnanolone withdrawal

WSP mice ($n = 10$ mice/dose and replicate) from both replicate lines were randomly assigned to receive either 0 or 75 mg/kg of allopregnanolone. WSR mice were not tested in this experiment because they were found to be nearly insensitive to all doses of pregnanolone in the previous experiment. The procedure for withdrawal severity testing was the same as that described above. At 1 and 8 h post-injection, separate groups of WSP mice ($n = 4$ mice/dose and replicate) were euthanized by decapitation and trunk blood was collected for later determination of allopregnanolone plasma concentrations using radioimmunoassay (RIA).

Plasma levels of allopregnanolone were measured by RIA as described [14,16]. Briefly, [^{3}H]allopregnanolone (54 Ci/mmol; NEN, Boston, MA), and a polyclonal antiserum (gift from CoCensys, Irvine, CA) with minimal cross-reactivity to other related steroids (e.g., testosterone and progesterone) [14] were used in the RIA. Ether-extracted plasma samples were reconstituted in assay buffer and run with a standard curve (20–5000 pg). Counts per minute were normalized and fit to a least-squares fit regression equation produced by log–logit transformation of the standards. Mass of samples were calculated by interpolation of the standards and corrected for extraction efficiency.

1.5. Experiment 3: Pregnanolone-induced hypothermia

WSP and WSR mice from both replicate lines ($n = 8$ mice/dose/line and replicate) were housed and tested at 22 ± 1°C. On test day, all mice were weighed and randomly assigned to one of four treatment conditions: 0, 50, 75, or 100 mg/kg pregnanolone. Mice were placed in individual, ventilated Plexiglas containers (8 × 17 × 8 cm) for 30 min prior to baseline rectal temperature measurement with a 0.5-mm diameter probe inserted 2.5 cm. Temperatures were displayed on a Sensortek TH-8 digital thermometer and recorded after 5 s. All mice were then injected (intraperitoneally) with their assigned dose of pregnanolone or vehicle and returned to the Plexiglas container. Rectal temperatures were taken at 30 min post-injection and again at hours 1, 2, and 4.

1.6. Statistical analysis

Data are expressed and presented as mean ± S.E.M. Analysis of variance (ANOVA) was performed on data from WSP mice to assess dose and replicate line (i.e., WSP-1 vs. WSP-2) effects on the dependent variable AUC (Experiments 1 and 2). Because WSR mice consistently had scores of zero using the HIC measure, data for WSR mice were not analyzed for Experiment 1. ANOVA was performed on data from WSP and WSR mice to assess dose and replicate line effects on the dependent variable rectal body temperature (Experiment 3). In all experiments, there was no main effect of genetic replicate, nor was there an interaction with selected line. Therefore, data are presented collapsed across replicate lines. Post-hoc analyses were performed for significant main effects and interactions at $P < .05$.

2. Results

2.1. Experiment 1: Acute pregnanolone withdrawal

All doses of pregnanolone initially suppressed HIC in WSP mice, consistent with its previously reported anticonvulsant effect (Fig. 1) [24]. In contrast, pregnanolone did not significantly alter HIC scores in WSR mice during the entire withdrawal time course when compared to vehicle-treated control animals (Fig. 1). Average HIC scores for WSP mice injected with pregnanolone returned to basal levels (i.e., average vehicle HIC scores) between hours 2 to 7, depending on the dose of pregnanolone administered (Fig. 1). HIC scores for WSP mice injected with 75 mg/kg continued to rise over vehicle HIC scores peaking at hour 8, and remained above average vehicle HIC scores throughout the entire

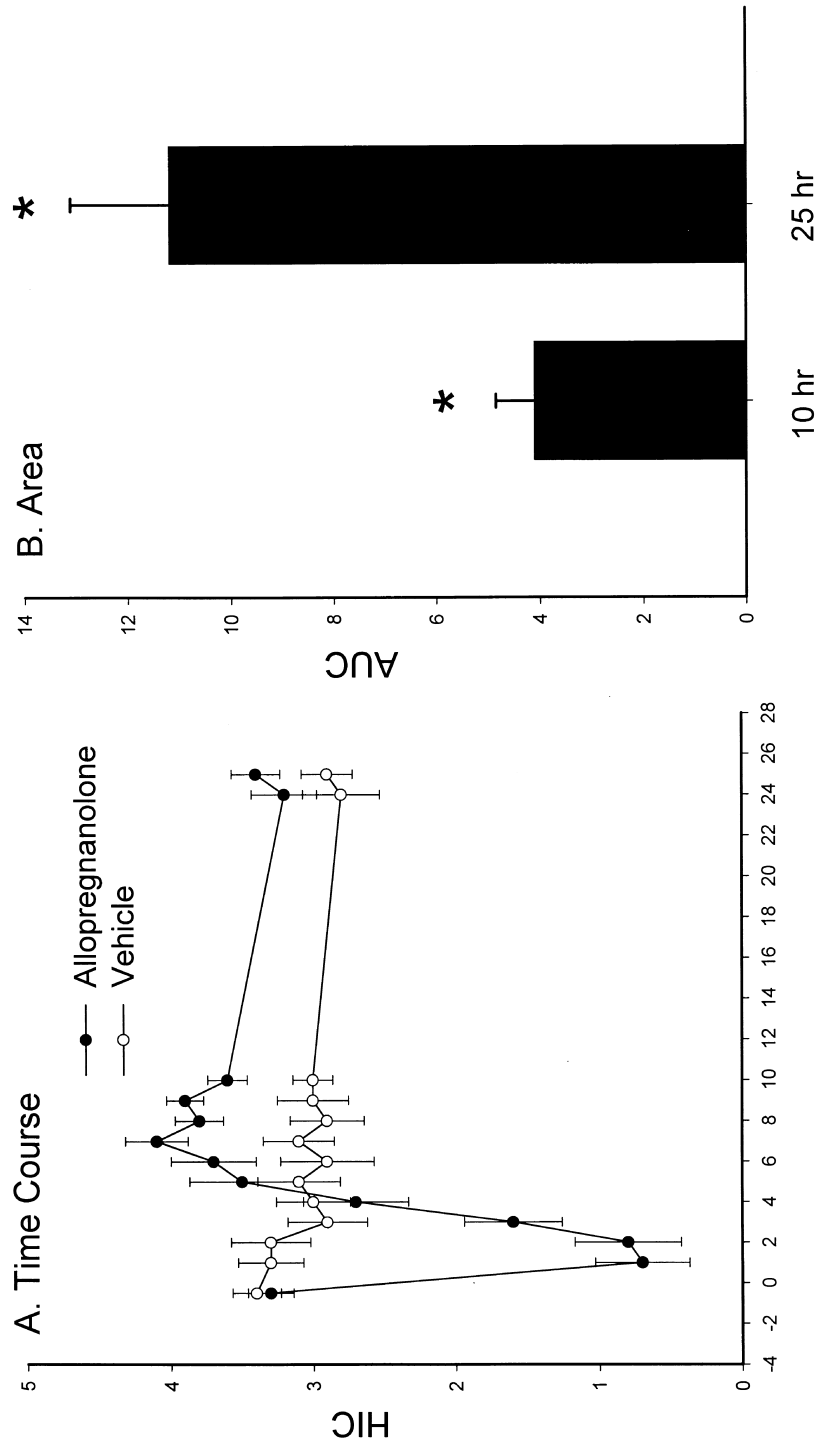

Fig. 3. (A) Acute allopregnanolone withdrawal time course in WSP mice. Allopregnanolone initially suppressed HIC scores. A return to vehicle HIC scores for allopregnanolone-treated mice occurred between hours 4 and 5, and continued to rise over baseline values peaking at hour 7. (B) AUC analysis for the withdrawal curves in allopregnanolone-injected WSP mice were significant (* $P < .0001$ vs. vehicle). AUC for vehicle-treated mice was 0.00 ± 0.51 (AUC 10) and 0.0 ± 2.3 (AUC 25) (not shown). Values represent the mean ± S.E.M. for 10 mice per dose and replicate.

25-h testing period. In support of this conclusion, there was a significant main effect of dose of pregnanolone on AUC for the entire 25-h period [$F(4,107)=2.54$, $P<.04$] (Fig. 2). Post-hoc analysis (Bonferroni) showed that WSP mice injected with 75 mg/kg of pregnanolone produced a significant increase in HIC ($P<.004$). In contrast, AUC analysis for the 10-h withdrawal curve showed no dose-related differences in HIC scores (not shown). Nonetheless, injection of 75 mg/kg pregnanolone significantly increased HIC scores in WSP mice, demonstrating a rebound CNS hyperexcitability.

2.2. Experiment 2: Acute allopregnanolone withdrawal

In order to determine whether allopregnanolone would produce an increase in HIC scores similar to that seen following injection of pregnanolone, WSP mice were injected with vehicle or 75 mg/kg of allopregnanolone. Allopregnanolone initially suppressed HIC, consistent with its anticonvulsant effect [4,14,16] (Fig. 3A). HIC scores for allopregnanolone-treated mice returned to vehicle HIC scores between hours 4 and 5, and continued to rise over vehicle HIC scores, peaking at hour 8 (Fig. 3A). There was a significant main effect of dose on AUC for both the 10-h [$F(1,38)=30.4$, $P<.0001$] and 25-h [$F(1,38)=34.6$, $P<.0001$] withdrawal curves (Fig. 3B). Thus, WSP mice injected with 75 mg/kg of allopregnanolone showed an intensification of overall withdrawal severity that was evident at both the early (10 h) and late (25 h) withdrawal assessments.

Plasma concentrations of allopregnanolone at 1 and 8 h post-injection are shown in Table 1. Injection of 75 mg/kg allopregnanolone increased plasma levels to micromolar concentrations at the first hour post-injection [$F(1,6)=74.9$, $P<.0001$]. At the 8-h measurement, plasma allopregnanolone returned to near basal levels, but these values remained significantly greater than vehicle [$F(1,6)=51.4$, $P<.0004$)].

2.3. Experiment 3: Pregnanolone-induced hypothermia

The results of experiment one indicated that pregnanolone did not produce a dose-dependent increase in acute withdrawal severity in WSP mice. We hypothesized that a potential sedative effect associated with severe hypothermia following injection of the highest dose of pregnanolone (100 mg/kg) may have confounded our acute withdrawal determination at this dose [24]. Therefore, the effect of pregnanolone on rectal body temperature was assessed in separate groups of WSP and WSR mice. Baseline rectal temperatures did not differ between WSP (38.3 ± 0.1°C) and WSR (38.0 ± 0.1°C) mice. All doses of pregnanolone initially suppressed rectal body temperatures in WSP and WSR mice, consistent with its hypothermic effect [24]. As predicted, the most severe hypothermia was observed in WSP and WSR mice injected with 100 mg/kg pregnanolone, where body temperatures decreased to 30.7 ± 0.6°C and 30.2 ± 0.6°C, respectively. ANOVA detected significant main effects of line [$F(1,105)=8.10$, $P<.005$], dose [$F(3,105)=74.91$, $P<.001$], and time [$F(4,420)=203.84$, $P<.001$]. There was a significant interaction between dose and time [$F(12,420)=46.73$,

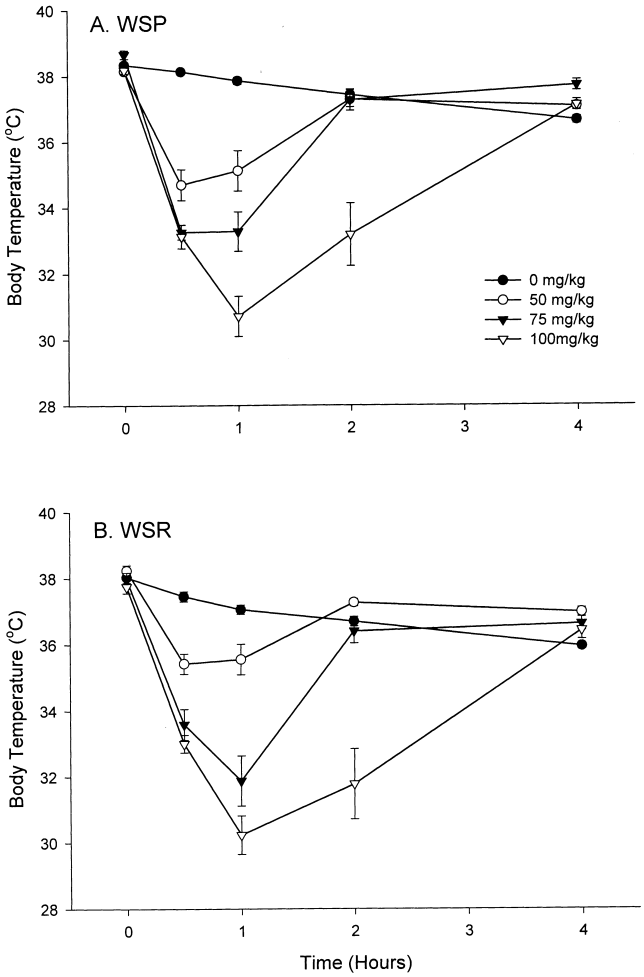

Fig. 4. Pregnanolone-induced hypothermia in (A) WSP and (B) WSR mice. Pregnanolone produced a dose-dependent hypothermia in both WSP and WSR mice. Values represent the mean ± S.E.M. for eight mice per dose, line, and replicate.

Table 1
Plasma allopregnanolone concentrations in WSP mice

Treatment group	Hour post-injection	Allopregnanolone (ng/ml)
Vehicle	1	2.96 ± 0.40
Allopregnanolone	1	1001 ± 115 **
Vehicle	8	5.09 ± 0.80
Allopregnanolone	8	33.5 ± 3.89 *

Plasma concentration of allopregnanolone administered at a dose of 75 mg/kg reached approximately 3 μM at 1 h post-injection, and had dropped to approximately a 100 nM concentration at hour 8 post-injection. Values represent the mean ± S.E.M. for four mice per dose and replicate line.

* $P<.004$ vs. respective vehicle-injected mice.

** $P<.0001$ vs. respective vehicle-injected mice.

$P < .001$], indicating that the recovery of body temperature to baseline values differed for the various doses of pregnanolone, whereas the line difference in average body temperature was due to an overall difference of 0.5°C between WSP and WSR mice. Rectal temperatures in WSP and WSR mice injected with 50 or 75 mg/kg pregnanolone recovered to vehicle-treated temperatures by hour 2 post-injection (Fig. 4A and B). In contrast, rectal temperatures in WSP and WSR mice injected with 100 mg/kg pregnanolone took longer (i.e., 4 h) to return to vehicle-treated temperatures. There was no significant interaction between selected line and dose of pregnanolone or time. Therefore, the lack of significant interaction indicates that the dose-dependent hypothermia following injection of pregnanolone was similar in WSP and WSR mice.

3. Discussion

The present findings demonstrate that both allopregnanolone and pregnanolone can elicit an acute withdrawal-related increase in HIC after a single administration of a hypnotic dose in WSP mice. In contrast, pregnanolone had a negligible effect on HIC in WSR mice after acute administration of either 25, 50, 75, or 100 mg/kg pregnanolone. This result is not surprising because WSR mice fail to show an acute withdrawal-related increase in HIC after administration of other agents that depress the CNS [11]. A similar acute withdrawal response was found following injection of 75 mg/kg allopregnanolone in WSP mice, with a significant increase in both the 10-h and 25-h AUC determination. Therefore, allopregnanolone appeared to produce a more robust increase in HIC in WSP mice than that seen with pregnanolone. This effect is likely due to differences in the relative potencies at $GABA_A$ receptors for these two agents [18].

Selected lines are useful for determining whether there is a genetic correlation between the selected phenotype and another experimental phenotype under investigation [12]. We found that WSP and WSR mice, which were selected for chronic ethanol withdrawal severity, also differ on their response to acute neurosteroid withdrawal severity. Both replicate lines of WSP and WSR mice differed in acute neurosteroid withdrawal severity, providing very strong evidence that acute neurosteroid withdrawal is a correlated response to selection. Thus, it can be concluded that to some degree, an overlap of the mechanisms controlling chronic ethanol withdrawal severity and acute neurosteroid withdrawal severity exists. In addition, this result adds further support to the finding that a common set of genes controls withdrawal severity from multiple CNS depressant agents [11]. It is attractive to speculate, based on our results, that individuals with a genetic predisposition to severe ethanol withdrawal severity might also be more sensitive to fluctuating endogenous levels of neurosteroids, and thus augment the expression of ethanol withdrawal hyperexcitability. Consistent with this notion, there was a 50% reduction in endogenous allopregnanolone levels in ethanol-dependent WSP mice, which persisted during peak withdrawal. In contrast, the chronic ethanol-related decrease in endogenous allopregnanolone levels was transient in WSR mice [15]. Human studies also agree with this hypothesis in that lower levels of allopregnanolone were found during an early withdrawal phase in alcoholic patients compared to non-alcoholic subjects. Furthermore, these lower levels of endogenous allopregnanolone were observed in alcoholics, when anxiety and depression scores were high [31]. Additional support for the interaction of neurosteroids and ethanol is provided by a recent study showing that acute ethanol administration elevates cerebral cortical allopregnanolone levels in a dose- and time-dependent manner to physiologically relevant concentrations in rats [37]. Cerebral cortical allopregnanolone levels were correlated with ethanol-induced sleep time, and the anticonvulsant effect of an acute ethanol injection was reversed by pretreatment with the neurosteroid biosynthesis inhibitor, finasteride. While the time course for the ethanol-induced increases in endogenous allopregnanolone concentrations does not correspond with the onset of ethanol's actions, it is possible that a sustained elevation in allopregnanolone levels may alter sensitivity to some of ethanol's pharmacological effects.

It is puzzling that the 100 mg/kg dose of pregnanolone was no more effective in eliciting an exacerbation of the HIC response in WSP mice than either the 25 or 50 mg/kg doses. Based on the results of Melchior and Allen [24], we hypothesized that the potential sedative effect of severe hypothermia at high doses of pregnanolone (e.g., 100 mg/kg) might be suppressing HIC, making it more difficult to detect an acute withdrawal-related increase in HIC. When body temperatures were measured following injection of the 100 mg/kg dose of pregnanolone, both WSP and WSR mice were severely hypothermic. In addition, mice injected with this dose took longer to recover to vehicle-treated body temperatures compared to mice injected with either 50 or 75 mg/kg pregnanolone. These results are consistent with the results of Melchior and Allen [24] in which they observed a similar dose-dependent reduction in body temperature after administration of 12.5, 50, and 100 mg/kg pregnanolone in mice. However, since body temperature had recovered to baseline values by 4 h post-injection following the 100 mg/kg dose of pregnanolone, a dose-dependent difference in hypothermia cannot explain the lesser acute withdrawal HIC response of WSP mice to 100 vs. 75 mg/kg pregnanolone.

The lack of dose-dependence for acute pregnanolone withdrawal is consistent with recent findings with other sedative–hypnotic drugs. Using the acute withdrawal paradigm employed in the current paper, we have shown that Swiss Webster mice do not differ significantly in the acute withdrawal response following doses of 3.5, 4.0, 4.5, and 5.0 g/kg ethanol [26]. All doses elicited significant acute withdrawal, but neither the peak nor the duration of with-

drawal was dose-dependent. Rather, animals treated with higher doses showed longer initial suppression of HIC, and achieved peak withdrawal later, than animals treated with lower doses. A similar test of multiple diazepam doses showed an inverted U dose–response in withdrawal severity [26]. We have no explanation for this lack of dose-dependence, but it is not unique to neuroactive steroids and appears to reflect some intrinsic physiological characteristic of the acute withdrawal HIC.

In contrast to the acute pregnanolone withdrawal results, where WSR mice show no withdrawal-related increase in HIC scores, pregnanolone produced a similar dose-dependent hypothermia in both WSP and WSR mice. This finding indicates that sensitivity to pregnanolone-induced hypothermia is not a correlated response to selection and agrees with previous studies examining ethanol-induced hypothermia in WSP and WSR mice, in which the acute hypothermic response to several doses of ethanol was similar in both lines [10]. Collectively, these results suggest that independent genetic factors affect sensitivity to hypothermia and acute or chronic withdrawal severity.

The mechanisms by which pregnanolone and allopregnanolone produce a withdrawal-related increase in CNS hyperexcitability is not fully understood, but there is evidence to suggest that it is mediated in part by $GABA_A$ receptors. A functional uncoupling of the binding domains for GABA, barbiturates, benzodiazepines, and neurosteroids has been noted following chronic administration of allopregnanolone, in vitro [35]. This suggests that CNS hyperexcitability associated with allopregnanolone withdrawal might be related to a functional reduction in the activity of $GABA_A$ receptors. Further support for this idea comes from the observed decrease in efficacy of benzodiazepines and neurosteroids in potentiating $GABA_A$ receptor-mediated Cl^- flux following prolonged treatment with allopregnanolone in vitro [36]. In addition, $GABA_A$ receptor subunits have been shown to be affected by chronic allopregnanolone treatment. Yu et al. [34] showed that chronic allopregnanolone treatment down-regulated α_2, α_3, β_2, and β_3 mRNA content in mammalian cortical neurons. The down-regulation of these subunits may correspond to the uncoupling and decreased efficacy in binding of $GABA_A$ modulatory agents [35,36]. Recent in vivo studies have shown that insensitivity to the sedative effects of lorazepam was paralleled by an increase in both mRNA and peptide content for the $GABA_A$ receptor α_4 subunit in rats undergoing progesterone withdrawal [33]. Conversely, suppression of the $GABA_A$ receptor α_4 subunit by antisense treatment prevented withdrawal symptoms associated with progesterone withdrawal [32].

The doses of allopregnanolone and pregnanolone used in the present study may not result in concentrations of these neurosteroids achieved endogenously. In other studies, CNS hyperexcitability was observed in rats during withdrawal from either chronic low doses of progesterone (5 mg/kg, $2\times$ day, 1 week), continuous progesterone release capsules (3 weeks), or after chronic elevations in endogenous progesterone levels in female rats by injection of pregnant mare gonadotropin serum for 11 days (i.e., pseudo-pregnancy) [17,27,28]. In our study, a single high dose of allopregnanolone (e.g., 75 mg/kg) was required to elicit a withdrawal-related increase in HIC scores in WSP mice. Importantly, this result is consistent with studies using chronic steroid treatment paradigms. However, studies assessing "acute withdrawal" generally require the administration of higher doses than those used in "chronic withdrawal" paradigms.

In summary, our data demonstrate that the neuroactive steroids pregnanolone and allopregnanolone have the ability to elicit an acute withdrawal response after a single administration in WSP mice. This effect is similar to that observed in WSP mice following a single administration of other positive modulators of $GABA_A$ receptors [5,11]. Our results support the notion that common genes underlie withdrawal severity from multiple depressant agents [11]. Thus, some of the genes that affect ethanol withdrawal severity in WSP and WSR mice also appear to contribute to the effects of acute neuroactive steroid withdrawal. This finding has important implications in our understanding of withdrawal-related symptomatology associated with chronic ethanol use.

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