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Effect of epipregnanolone and pregnenolone sulfate on chronic tolerance to ethanol

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Abstract

The aim of the present study was to investigate the influence of neurosteroids on the development of tolerance to ethanol. Male Swiss mice were injected daily with the positive allosteric modulator of the gamma amino butyric acid-A ($GABA_A$) receptor epipregnanolone (5β-pregnan-3β-ol-20-one; 0.15 mg/kg ip) or pregnenolone sulfate (5-pregnen-3β-ol-20-one sulfate sodium; 0.08 mg/kg ip) — considered a negative allosteric modulator of this receptor and/or positive allosteric modulator of the *N*-methyl-D-aspartate (NMDA) receptor — 30 min before ethanol (2.5 g/kg ip). They were tested on the rota-rod apparatus, under continuous acceleration (1rpm/s), at 30, 60 and 90 min after ethanol injections for 5 days. The results showed that tolerance to the motor incoordinating effect of ethanol occurred on the fifth day of treatment when this effect was blocked by pretreatment with epipregnanolone. On the other hand, ethanol tolerance was enhanced by pretreatment with pregnenolone sulfate from the second to the fifth days of treatment. Taken together, our results suggest that neurosteroids can either stimulate or block the development of chronic tolerance to ethanol. Moreover, since neurosteroids can interact with $GABA_A$ or NMDA receptor systems, our results suggest the involvement of these systems in the actions of neurosteroids upon ethanol tolerance.

Keywords: Tolerance; Ethanol; Neurosteroids; Pregnenolone sulfate; Epipregnanolone

1. Introduction

Neurosteroids are steroids that can be synthesized de novo in the central nervous system. In addition to their well-known genomic action, several steroids also exhibit non-genomic effects by binding at ligand-gated ion channels and changing neuronal excitability [4,43]. Some neurosteroids such as progesterone, allopregnanolone, pregnanolone and epipregnanolone act as positive allosteric modulators of the $GABA_A$ receptor [4,38,54,58], whereas pregnenolone sulfate and dehydroepiandrosterone sulfate act as negative allosteric modulators of this receptor [39,49]. Moreover, it has been shown that pregnenolone sulfate acts also as a positive allosteric modulator of the NMDA receptor in in vitro studies [7,17,39,49,68]. Several studies suggest that sulfated neurosteroids can facilitate learning and memory [13,14,40–42]. Furthermore, it has been demonstrated that chronically administered neurosteroids prevent the development of tolerance to the antinociceptive effect of morphine and to the effect of benzodiazepines on locomotion [55,56].

For many years, ethanol research has paid much attention to the study of the phenomenon of acquired tolerance, which is defined as an adaptive process of organisms, where the effects of a drug decrease after a period in which it is administered repeatedly [35,62]. One reason for this continuous interest is the hypothesis that tolerance to ethanol is one of the factors associated with dependence on this drug [9,22,24,34,60]. The most commonly studied form of tolerance is chronic tolerance which occurs gradually after days, weeks or months of exposure [22,23], and which usually includes both dispositional and functional components. There are studies showing also the development of crossed tolerance between ethanol and other sedative hypnotic drugs, such as barbiturates and benzodiazepines [27,28]. Several studies have demonstrated that the acquisition of tolerance to the effects of ethanol presents many properties similar to learning and memory processes [5,10,21,36,50].

Ethanol interferes with the central nervous system, affecting membrane and ion channel functions, or several

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neurotransmission systems, such as acetylcholine, monoamines, aminoacids, neuropeptides and neuroactive steroids [6,61,66]. It has been demonstrated that ethanol can also block glutamatergic neurotransmission and stimulate GABAergic neurotransmission [53,61,63]. The effect of ethanol on $GABA_A$ receptors can contribute to its anxiolytic and sedative actions [1,2,12] and the impairment of learning and memory [15,64,67]. Evidence has appeared of the involvement of NMDA receptor in the actions of ethanol and the participation of this system in the development of tolerance to ethanol [3,25,26,29–33,59,69]. Studies have demonstrated that the administration of non-competitive NMDA-receptor antagonists blocks the development of rapid tolerance to the hypothermia and incoordination produced by ethanol [25,32,33], as well as the chronic tolerance to motor incoordinating effect of ethanol [29,31,69].

There are some studies showing that neuroactive steroids can influence the effects of ethanol. For instance, neurosteroids play an important role in functions affected by ethanol, such as anxiety [46], reinforcing and discriminating stimulus mechanisms [16,19]. They also have a role to play in the disruptive effect of ethanol on memory [47]. However, to our knowledge, there is a paucity of behavioral studies showing the effects of neurosteroids on ethanol tolerance. In the present study, the influence of the neurosteroids epipregnanolone — a positive modulator of $GABA_A$ receptor [38,58] — and pregnenolone sulfate — a negative $GABA_A$ and positive NMDA receptor modulator [7,39] — on chronic tolerance to the motor incoordination induced by ethanol was examined in mice.

2. Methods

2.1. Animals

Adult male Swiss mice 2.5 months of age, weighing 30–35 g, were used. Animals were housed in groups of 20 in plastic cages, and were maintained at $23 \pm 1^{\circ}C$ under artificial illumination (lights on between 6:00 A.M. and 6:00 P.M.) with free access to standard laboratory chow and tap water.

2.2. Drugs

Analytical grade ethanol purchased from Merck Laboratory (Rio de Janeiro, Brazil), pregnenolone sulfate (5-pregnen-3β-ol-20-one sulfate sodium), from Research Biochemicals International (Natick, USA), and epipregnanolone (5β-pregnan-3β-ol-20-one), from Sigma (St. Louis, USA), were used. Ethanol and pregnenolone sulfate were prepared in NaCl 0.9% (saline). Ethanol was diluted to the concentration of 14% w/v. Epipregnanolone was dissolved in a solution of 1% polyoxyethylenesorbitan monooleate (Tween 80; Sigma) in saline. All solutions were freshly prepared and administered by the intraperitoneal route (ip). All volumes injected were of 1 ml/kg body weight except for ethanol that was adjusted according to the dose used. The dose range of neurosteroids we used was based on our preliminary experiments and on the similarity to the doses previously shown to affect memory in mice [47].

2.3. Rota-rod apparatus

Motor impairment was measured on rota-rod apparatus (Rotamex-V-EE/85) controlled by a computational system (Columbus Instruments Computer–Counter Interface, USA). Animals were trained under continuous acceleration (1 rpm/s) in 1-min sessions. Whenever an animal dropped off the rotating bar, it received a foot shock (0.3 mA for 2 s) and was then returned to its cage. The rotational velocity at which the animal dropped off the rotating bar was taken as the performance score. Animals that did not reach a stable baseline (at least 20 rpm) in 10 trials were discarded. The animals that presented performances between 20 and 40 rpm were selected for the experiment. About 90% of animals usually reach the criterion. After selection, experimental and control groups ($N = 10$) were matched based on both their body weight and mean performance during the last training sessions on the rota-rod. With this procedure, animals presented similar basal values in all groups.

2.4. Statistical analysis

The difference between the baseline and maximum impairment scores provided the maximum percentage of motor impairment induced by ethanol. Data were analyzed using two-way ANOVA and two-way ANOVA for repeated measures, according to the experimental protocol, with Statistica for Windows, 4.5 (Statsoft) software. Post hoc comparisons were performed using the Tukey's test. Values of $P < .05$ were considered significant. Figures were drawn using GraphPad Prism 1.03, GraphPad Software. All experimental data are presented as the mean ± S.E.M.

3. Procedure and results

3.1. Experiment 1: Effect of epipregnanolone on the development of tolerance to ethanol-induced motor impairment

After taking their basal values, 40 trained mice were divided into two groups. On day 1, groups were pretreated with saline or epipregnanolone 0.15 mg/kg, respectively. Thirty minutes later, each group was further divided in two subgroups to receive saline or ethanol (2.5 g/kg), respectively. Thus, there were four groups of 10 mice. After 30 min, they were tested on the rota-rod in the same way as in the training period. The measurements were repeated at 60 and 90 min. This procedure was repeated on days 2, 3 and 4.

On day 5, all animals received only ethanol (2.5 g/kg), and 30, 60 and 90 min later, were tested on the rota-rod to evaluate tolerance.

The results of this experiment are shown in Fig. 1. In all experiments, the average basal value in the rota-rod test was 27.0 ± 2.9 rpm. Motor impairment produced by ethanol showed a decline throughout successive days of treatment (group E + E). This effect was significant on the fifth day of the experiment. ANOVA with repeated measures revealed a significant effect of treatment days [$F(4,144) = 87{,}919$, $P < .0001$]. The post hoc analysis showed a significant difference between the values obtained on the first and the fifth days in animals treated only with ethanol, but not in animals pretreated with epipregnanolone (Tukey's test).

On the fifth day, ANOVA showed an effect of ethanol treatment [$F(1,36) = 28.412$, $P < .0001$), of epipregnanolone pretreatment [$F(1,36) = 12.251$, $P < .0016$] and a significant factor interaction [$F(1,36) = 7.895$, $P < .0092$]. Groups pretreated with epipregnanolone (0.15 mg/kg) before ethanol on days 1 to 4 did not show any change in their performance, including on day 5, suggesting that this drug blocked tolerance. The post hoc analysis did not indicate any difference between the values obtained on days 1 and 5 (group EPI + E), suggesting that epipregnanolone (0.15 mg/kg) blocked the development of tolerance (Tukey's test).

3.2. Experiment 2: Effect of pregnenolone sulfate on the development of tolerance to ethanol-induced motor impairment

On day 1, four groups of trained mice were pretreated with saline or pregnenolone sulfate (0.08 mg/kg). Thirty minutes later, two groups of saline pretreated mice and two groups of pregnenolone sulfate pretreated mice received ethanol (2.5 g/kg), and the other groups received saline. After 30, 60 and 90 min, they were tested on the rota-rod.

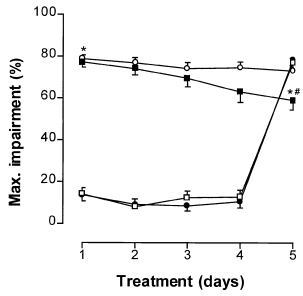

Fig. 1. Effect of epipregnanolone on the development of chronic tolerance to ethanol. Two groups received saline (S) and two other groups received epipregnanolone (EPI; 0.15 mg/kg, ip), 30 min before saline or ethanol (E; 2.5 g/kg, ip), on days 1, 2, 3 and 4. Chronic tolerance to ethanol was assessed on day 5, when all the groups were treated with ethanol (2.5 g/kg, ip). Symbol (□) represents the SS group (S + S), (■) the SE group (S + E), (●) the EPIS group (EPI + S) and (○) the EPIE group (EPI + E). Results shown are means ± S.E.M. of 10 animals per group. * $P < .05$ compared to respective control and #$P < .05$ compared with days 1 and 2 (of the SE group) (Tukey's test).

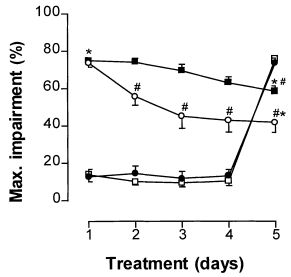

Fig. 2. Effect of pregnenolone sulfate on the development of chronic tolerance to ethanol. Two groups received saline (S) and two other groups received pregnenolone sulfate (PS; 0.08 mg/kg, ip), 30 min before saline or ethanol (E; 2.5 g/kg, ip), on days 1, 2, 3 and 4. Chronic tolerance to ethanol was assessed on day 5, when all the groups were treated with ethanol (2.5 g/kg, ip). Symbol (□) represents the SS group (S + S), (■) the SE group (S + E), (●) the PSS group (PSS + S) and (○) the PSE group (PS + E). Results shown are means ± S.E.M. of 10 animals per group. * $P < .05$ compared to respective control; #$P < .05$ compared to respective control or with day 1 (Tukey's test).

Mice were then returned to their home cages. Day 1 procedure was repeated on days 2, 3 and 4. On day 5, all animals received ethanol, and 30, 60 and 90 min later were tested on the rota-rod to evaluate tolerance.

The results of this experiment are shown in Fig. 2. On the fifth day of the experiment, motor impairment was significantly reduced in both groups that received ethanol on days 1–4. Two-way ANOVA showed an effect of ethanol treatment [$F(1,36) = 44.511$, $P < .0001$] on the fifth day, and the post hoc analysis indicated the development of tolerance to ethanol ($p < .05$, Tukey's test). The groups pretreated with pregnenolone sulfate (0.08 mg/kg) before ethanol on days 1–4 showed facilitation of tolerance evaluated on day 5. ANOVA revealed the effect of pregnenolone sulfate pretreatment ($F(1,36) = 5.291$, $P < .0273$) and a significant factor interaction [$F(1,36) = 5.052$, $P < .0308$]. ANOVA for repeated measures revealed the effect of treatment days [$F(4,144) = 52{,}328$, $P < .0001$] and the post hoc analysis indicated differences between values obtained on days 1 and 5 (group PS + E), confirming that pregnenolone sulfate (0.08 mg/kg) facilitates the development of tolerance (Tukey's test).

4. Discussion

The results of the present study showed that the development of chronic tolerance to the motor incoordination produced by ethanol in mice occurred after 5 days of treatment, and this process was significantly facilitated by concomitant treatment with the neurosteroid pregnenolone sulfate. Conversely, chronic treatment with epipregnanolone for 4 days significantly blocked the development of tolerance to ethanol, an effect which was more evident on the fifth day of the experiment. To our knowledge, this is the first behavioral study showing that neurosteroids affect

ethanol tolerance, although previous studies have shown that chronic administration of progesterone and pregnenolone sulfate prevented the development of morphine and benzodiazepines tolerance and reduced withdrawal symptoms in mice chronically treated with morphine and benzodiazepines [55,56]. In spite of the fact that we did not measure ethanol or neurosteroid blood concentrations, the effects of pregnenolone and epipregnanolone observed here appear to be pharmacodynamic rather than pharmacokinetic, since data from the literature shows that these drugs, at the same dosage range we used, do not interfere with ethanol metabolism [45].

It is important to note that the doses of neurosteroids chosen for the study are within the dose ranges found to affect memory in mice [47], yet did not interfere with the motor coordination of the animals throughout the testing days, and no residual treatment effect on the animals' behavior was observed on day 5.

As mentioned in the Introduction, neurosteroids influence $GABA_A$- and NMDA-receptor neurotransmission. For example, pregnenolone sulfate has been shown to reduce the GABA-induced activation of $GABA_A$ receptors, as well as the frequency of opening of chloride channel associated with $GABA_A$ receptor [38,49], and also, to specifically increase the NMDA-induced activation of hippocampal [7] and spinal cord neurons [37]. Conversely, epipregnanolone exhibits a weak-positive influence on the $GABA_A$ system [54]. On the other hand, it is known that neurosteroids interact with ethanol. Positive $GABA_A$ modulators such as allopregnanolone and pregnanolone increase the ethanol-induced motor impairment and sleeping-time in mice [65,66]. The fact that these neurosteroids, but not the negative $GABA_A$ receptor modulators pregnenolone sulfate and dehydroepiandrosterone sulfate, substituted for ethanol in discriminative stimulus tests in rats, suggest that positive $GABA_A$ modulators and ethanol give rise to similar discriminative cues [6]. Therefore, ethanol and some neurosteroids may exhibit similar actions associated with the $GABA_A$ receptor system, which could explain the chronic interactions between these drugs. Chronic ethanol treatment has been associated with reduced sensitivity to $GABA_A$ receptor-mediated responses in the central nervous system and with augmented NMDA function [9,52,57]. Likewise, recent studies have been shown that chronic treatment with the $GABA_A$ positive modulator allopregnanolone produced down-regulation of $GABA_A$ receptor binding in cultured mammalian cortical neurons, and decreased pentobarbital and allopregnanolone potentiation of GABA-induced currents in whole cortical cells [70]. Based on these evidences, obtained using either ethanol or neurosteroid alone, one could hypothesize that repeated daily treatment with the positive $GABA_A$ receptor modulator epipregnanolone, would enhance the down-regulation of the $GABA_A$ receptor system by ethanol, thus increasing tolerance to the later drug. Conversely, chronic treatment with pregnenolone sulfate, a negative and positive modulator of $GABA_A$ and NMDA receptors, respectively, could produce adaptive changes opposite to those induced by epipregnanolone, resulting in blockade of ethanol tolerance. Notwithstanding, however, we actually observed the contrary, i.e. ethanol-induced tolerance was blocked by epipregnanolone and facilitated by pregnenolone sulfate. Thus, it appears that given in association with ethanol, epipregnanolone may counteract the down regulation of $GABA_A$ receptors by ethanol, whereas pregnenolone sulfate exerts the opposite effect. In line with this view, the ability of allopregnanolone to enhance the density of $GABA_A$ receptor-like binding sites, as evidenced by binding of [^{3}H] flunitrazepam and [^{3}H] muscimol, has been found to be potentiated in cerebellum taken from rats treated with ethanol for 6 days, as compared to controls animals [44].

Manipulations of the $GABA_A$ and NMDA receptors seem to result in changes in the development of ethanol tolerance. For instance, the acute exposure to the benzodiazepine antagonist flumazenil was able to attenuate behavioral and biochemical expression of ethanol tolerance [8], whereas a single injection of Ro19-4603, a benzodiazepine inverse agonist, prevented the depressant effects of ethanol and tolerance [20]. Results obtained in our and other laboratories have shown that ethanol tolerance is inhibited by drugs that impair memory, such as the competitive NMDA antagonists dizocilpine and ketamine or the nitric oxide synthase inhibitors in a dose-dependent way, either in rats and mice [3,25,29,31–33,50]. Additionally, NMDA antagonists blocked rapid tolerance to ethanol as well as cross-tolerance between alcohol and chlordiazepoxide in rats [30]. Conversely, tolerance is stimulated by drugs that improve learning, such as D-cycloserine [26]. Therefore, an alternative explanation for our results is the influence of neurosteroids on other adaptive processes related to synaptic plasticity. For instance, dehydroepiandrosterone sulfate has been shown to modulate synaptic transmission in the CA1 region of the rat hippocampus [48]. Moreover, neurosteroids can either facilitate or impair learning and memory, as pregnenolone sulfate has been shown to increase, while epipregnanolone decreases the percentage of correct responses in mice submitted to the win-shift paradigm [47]. Further, pregnenolone sulfate reverses the impairment of performance induced by NMDA receptor antagonists in rodents [40,41]. Considering these data, together with the fact that the NMDA and $GABA_A$ receptor systems are involved in learning and memory processes [11,18,51], it is conceivable that our results might be a consequence of influence of neurosteroids on the learning mechanisms underlying tolerance to ethanol.

In conclusion, our results suggest that pregnenolone sulfate and epipregnanolone influence the development of tolerance to ethanol motor incoordination in opposite ways. Studies using $GABA_A$ and NMDA receptor agonists and antagonists are in course to verify the influence of these systems on the interaction between neurosteroids and ethanol tolerance.

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