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Influence of neurosteroids on the development of rapid tolerance to ethanol in mice

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Abstract

Our recent study demonstrated that neurosteroids might either facilitate or block chronic tolerance to the incoordinating effects of ethanol. The present study investigated the effects of neurosteroids on the development of rapid tolerance to ethanol-induced motor impairment using the *N*-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine [(+)-MK-801] or the γ-aminobutyric acid (GABA) type A ($GABA_A$) receptor agonist muscimol. Male Swiss mice were pretreated with pregnenolone sulfate (0.03 to 0.15 mg/kg) or dehydroepiandrosterone sulfate (0.05 to 0.20 mg/kg) before administration of ethanol (1.9 or 2.25 g/kg) and tested with the rota-rod apparatus. Twenty-four hours later, all animals were re-tested with the rota-rod after receiving the same dose of ethanol. Pretreatment with pregnenolone sulfate or with dehydroepiandrosterone sulfate significantly facilitated the acquisition of tolerance. However, the administration of (+)-MK-801 reversed the stimulatory action of pregnenolone sulfate but did not affect the actions of dehydroepiandrosterone sulfate on ethanol tolerance. Pretreatment with pregnenolone sulfate or dehydroepiandrosterone sulfate prevented the inhibitory action of muscimol on tolerance development. Taken together, our results suggest that neurosteroids may stimulate the development of rapid tolerance to ethanol and that $GABA_A$ and NMDA receptor systems may be involved in these actions.

Keywords: Tolerance; Ethanol; Pregnenolone sulfate; Dehydroepiandrosterone sulfate; (+)-MK-801; Muscimol

1. Introduction

Knowledge of the phenomenon of tolerance to the effects of ethanol has helped our understanding of the underlying nature of alcohol addiction and has prompted the development of new schemes for the treatment of this disease. Tolerance to ethanol can promote an attenuation of the aversive effect of this drug with regards to its rewarding effects, usually leading to increased beverage consumption (Kalant, 1996). This relevant component of alcohol dependence has been studied with respect to its development in time. Acute tolerance is observed during the course of a single ethanol administration, whereas chronic tolerance is generally detected after several days, weeks or months of repeated ethanol administrations (Kalant and Khanna, 1990; Tabakoff, 1995). Rapid tolerance, which is usually detected between 8 and 24 h after the first injection of ethanol (Crabbe et al., 1979; Barreto et al., 1998), has been considered as a reliable predictor of chronic tolerance. This is due to the fact that similar results were obtained with rapid and chronic paradigms in studies involving ethanol and other drugs (Khanna et al., 1991, 1996).

Knowledge of the mechanisms by which alcohol acts in the central nervous system has been of crucial importance in understanding alcohol tolerance, abuse and physical dependence, and in proposing additional therapies for the treatment of alcoholism. However, research on the effects of alcohol on neurotransmission has focused mainly on the role of monoamines, opioid peptides and amino acids (Bowen et al., 1999; Tabakoff et al., 1996). Studies suggest that ethanol can stimulate the transmission mediated by γ-aminobutyric acid (GABA) and/or block the transmission mediated by glutamate (Tabakoff, 1995), and that several behavioral effects of this drug can be related to its actions at the GABA type A ($GABA_A$) receptor complex, thus, contributing to its anxiolytic, anesthetic, memory-impairing and motor-incoordinating effects (Frey et al., 1981; Givens and McMahon, 1997).

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There is increasing evidence for the role of neurosteroids (neuroactive steroids) in the effects of ethanol (Vanover et al., 1999; VanDoren et al., 2000). These substances are synthesized at the peripheral and central levels from cholesterol and can exert actions other than as sex hormone precursors (Baulieu, 1991; McEwen, 1991; Compagnone and Mellon, 1998). Neurosteroids have distinct nongenomic actions on the central nervous system as a consequence of their effects on different neurotransmitter systems. For example, pregnenolone sulfate increases neuronal cell death induced by *N*-methyl-D-aspartate (NMDA) (Weaver et al., 1998); dehydroepiandrosterone sulfate increases free intracellular calcium consequent to the activation of NMDA receptors in the brain (Compagnone and Mellon, 1998); and dehydroepiandrosterone sulfate and pregnenolone sulfate reverse dizocilpine-induced memory impairment via σ_1 receptors (Zou et al., 2000). Moreover, tetrahydrodeoxycorticosterone, progesterone, allopregnanolone and epipregnanolone act as positive allosteric modulators of the $GABA_A$ receptor-mediated Cl^- conductance and increase $GABA_A$-mediated neuronal currents (Majewska et al., 1986; Prince and Simmonds, 1993; Schumacher and McEwen, 1989). The negative allosteric modulators of $GABA_A$ receptor currents, pregnenolone sulfate and dehydroepiandrosterone sulfate, act non-competitively to reduce the activity of $GABA_A$ and glycine receptors (Majewska et al., 1988; Majewska, 1992; Mienville and Vicini, 1989). Studies have shown that positive or negative allosteric modulators at the NMDA receptor complex bind to distinct sites on this receptor. These sites are different from the glycine, dizocilpine [(+)-MK-801], Mg^{2+} and spermine sites (Park-Chung et al., 1997). Moreover, other studies suggest that the $NMDAR2_A$ subunit of the NMDA receptor regulates the efficacy of neurosteroids (Yaghoubi et al., 1998). This evidence suggests that neurosteroids modulate actions at the NMDA and $GABA_A$ receptor systems and influence voltage-gated calcium channel currents. The behavioral effects of neurosteroids also suggest that substances with a positive influence on the $GABA_A$ receptor system show anxiolytic (Bitran et al., 1991; Wieland et al., 1991) properties, whereas those with a negative influence on this receptor system or a positive influence on the NMDA receptor system improve learning and memory (Mathis et al., 1994, 1996).

In a recent study, we have shown that neurosteroids can either block or stimulate the development of chronic tolerance to the incoordinating effect of ethanol. A positive $GABA_A$ receptor modulator (epipregnanolone) blocked, whereas the negative $GABA_A$ receptor and positive NMDA receptor modulators (pregnenolone sulfate and dehydroepiandrosterone sulfate) facilitated, the development of tolerance to ethanol in mice (Barbosa and Morato, 2000). In the present study, the influence of the neurosteroids pregnenolone sulfate and dehydroepiandrosterone sulfate on tolerance to the motor incoordination produced by ethanol was re-examined using the rapid tolerance paradigm. An attempt was also made to assess the potential underlying mechanisms mediating the influence of neurosteroids on tolerance, particularly the possible involvement of $GABA_A$ and NMDA receptors, by using the selective $GABA_A$ receptor agonist muscimol and the non-competitive NMDA receptor antagonist (+)-MK-801.

2. Materials and methods

2.1. Animals

Adult male Swiss mice from Universidade Federal de Santa Catarina's colony were used, with an age range between 2 and 2 1/2 months and weighing between 25 and 30 g. The animals were bred in-house at the university's animal house and transferred to our department's facilities at least 2 weeks prior to use, where they were housed in groups of 20 in plastic cages ($42 \times 34 \times 17$ cm) and maintained at 23 ± 1 °C under artificial illumination (lights on between 6 a.m. and 6 p.m.) with standard laboratory chow and tap water ad libitum. All experiments were conducted between 1:30 p.m. and 5:30 p.m. in order to minimize circadian influences, and all animals were naive to drug treatment and experience. All procedures were performed in accordance with the Brazilian Society of Neuroscience and Behavior animal care guidelines and European Community animal care guidelines, and were approved by our institutional ethics committee.

2.2. Drugs

Analytical grade ethanol was purchased from Merck Laboratory (Darmstadt, Germany). Dizocilpine [(+)-MK-801 hydrogen maleate], muscimol, pregnenolone sulfate (5-pregnen-3β-ol-20-one sulfate sodium), and dehydroepiandrosterone sulfate (5-androsten-3β-ol-17-one sulfate sodium) were obtained from Research Biochemicals International (Natick, USA). Ethanol, (+)-MK-801, muscimol, pregnenolone sulfate and dehydroepiandrosterone sulfate were prepared in NaCl 0.9% (saline). Ethanol was diluted to the concentration of 14% w/v. All solutions were freshly prepared and administered by the intraperitoneal route (i.p.). Volumes injected were 1 ml/kg body weight, except for ethanol, the volume of which was adjusted according to the dose used.

2.3. General procedure and selection of the doses of ethanol

Motor impairment was measured on a rota-rod apparatus (Rotamex-V-EE/85) controlled by a computational system (Columbus Instruments Computer-Counter Interface, OH, USA) where animals were trained under continuous acceleration (1 rpm/s) in 1-min sessions. Whenever

a mouse dropped off the rotating bar, it received a foot shock (0.1 mA for 2 s) and was then returned to its cage. The latency (in seconds) to fall off the rotating bar (which corresponded to the rotational velocity at which the animal dropped off the rotating bar) was taken as the performance score. Mice that did not reach a stable baseline (that is, at least 20 s) in 10 trials were discarded. The animals that had a performance between 20 and 40 s were chosen for the experiment. The percentage of animals that usually attained the desired criterion was 90–91%. After selection, experimental and control groups were matched based on both body weight and mean performance during the last training sessions on the rota-rod. With this procedure, animals had similar basal values in all groups. The baseline score was the score obtained by each animal before any treatment on one specific day (Day 1 or Day 2). The test score was the score obtained for each mouse at 30, 60 or 90 min after ethanol (or control) injections. The lowest test score obtained in each test session was used to calculate the maximum percentage of motor impairment according to the formula:

$$\text{Maximum \% of motor impairment} = [(\text{baseline score} - \text{test score}) / (\text{baseline score})] \times 100$$

On Day 1, four groups of trained mice received one of the following doses of ethanol: 1.9, 2.0, 2.25 or 3.0 g/kg, in order to select one dose of ethanol that produced rapid tolerance and another dose that did not produce rapid tolerance to the motor-incoordinating effect induced by ethanol. For each dose of ethanol, parallel control groups received the corresponding volume of saline. At 30, 60 and 90 min after ethanol or saline injection, performance was evaluated on the rota-rod. Mice were then returned to their home cages. On Day 2, all groups including controls received ethanol at the same dose that they received on the day before and were re-tested on the rota-rod as on the previous day to evaluate rapid tolerance.

2.4. Possible facilitation of tolerance by pregnenolone sulfate and dehydroepiandrosterone sulfate: influence of (+)-MK-801

Four groups of 20 trained mice were pretreated with one of the four doses of pregnenolone sulfate (0.03, 0.05, 0.08 or 0.15 mg/kg) and another four groups received saline (control groups). After 30 min, each group was further divided into two subgroups that received ethanol (1.9 g/kg) or saline, respectively. This dose of ethanol was used since it did not cause tolerance per se. Thus, four groups of 10 animals were used for each dose of pregnenolone sulfate. All animals were tested on the rota-rod at 30, 60 and 90 min following the last injection and then returned to their home cages. Twenty-four hours later, all animals received ethanol (1.9 g/kg) and were submitted to the test 30, 60 and 90 min later. In other groups of rats, a similar procedure was followed except that dehydroepiandrosterone sulfate (0.05, 0.10, 0.15 or 0.20 mg/kg) and the respective control solution were administered. In order to verify whether (+)-MK-801 could interfere with the effects of pregnenolone sulfate and of dehydroepiandrosterone sulfate on tolerance to ethanol, another set of experiments was carried out. On Day 1, one group of mice was treated with (+)-MK-801 (0.06 mg/kg) and another group received saline. After 15 min, half of the animals from each group received pregnenolone sulfate (0.08 mg/kg) and the remaining animals received saline. After 30 min, each subgroup was further divided into two subgroups that received ethanol (1.9 g/kg) or saline. Therefore, eight subgroups of mice were formed. After 30, 60 and 90 min, they were again tested on the rota-rod as in the previous experiment. On Day 2, all groups including controls received ethanol and were tested on the rota-rod 30 min later in order to evaluate rapid tolerance. Another group of mice was used to study the influence of pregnenolone sulfate on the blockade of rapid tolerance to ethanol (2.25 g/kg) produced by (+)-MK-801 (0.06 mg/kg). The influence of (+)-MK-801 (0.06 mg/kg) on the effects of dehydroepiandrosterone sulfate (0.15 mg/kg) on the development of rapid tolerance was also investigated using a similar procedure with the dose of 1.9 g/kg ethanol.

2.5. Effect of pregnenolone sulfate and dehydroepiandrosterone sulfate on the impairment of rapid tolerance to ethanol produced by muscimol

On Day 1, two groups of mice were treated with pregnenolone sulfate (0.08 mg/kg) or saline, and after 15 min, each group received muscimol (0.6 mg/kg) or saline, respectively. Thirty minutes later, each group was again divided into two, and each subgroup received ethanol (2.25 g/kg) or saline. After 30, 60 and 90 min, they were tested on the rota-rod. On Day 2, all groups including controls received ethanol and were tested on the rota-rod 30 min later in order to evaluate rapid tolerance. In other groups of animals, a similar procedure was followed except that dehydroepiandrosterone sulfate at a dose of 0.15 mg/kg was used.

2.6. Blood ethanol assay

Groups of animals were pretreated with saline, pregnenolone sulfate (0.08 mg/kg), dehydroepiandrosterone sulfate (0.15 mg/kg) or (+)-MK-801 (0.06 mg/kg) 30 min before administration of 1.9 g/kg of ethanol ($N = 20$). Other groups were pretreated with saline or muscimol (0.60 mg/kg) 30 min before the administration of ethanol (2.25 mg/kg) ($N = 8$). Blood samples were collected from animals by direct tail puncture 30 min after ethanol administration. Blood ethanol concentration was evaluated enzy-

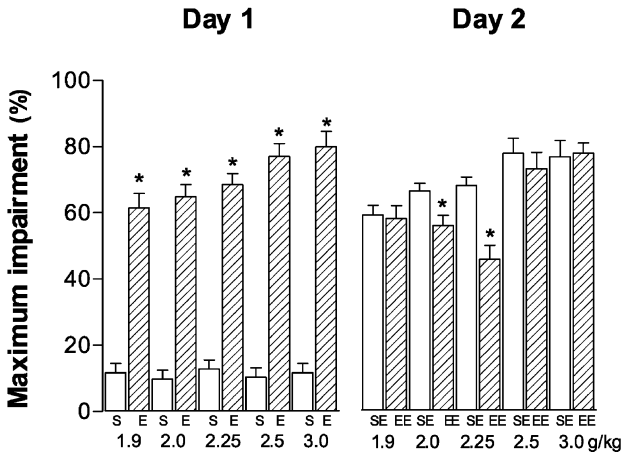

Fig. 1. Development of rapid tolerance to motor impairment induced by different doses of ethanol in mice tested on the rota-rod. On Day 1, the control group received saline and the other group received ethanol (E; 1.9, 2.0, 2.25, 2.5 or 3.0 g/kg i.p.). Animals were tested 30 min after ethanol or saline injection. Rapid tolerance to ethanol was assessed on Day 2 when all the groups were treated with ethanol and were tested again. The results shown are means ± S.E.M. for 10 animals per group, and $^{*}P < 0.05$ compared to respective control (Tukey test).

matically based on the conversion of ethanol to acetaldehyde by the action of alcohol dehydrogenase (Poklis and Mackell, 1982).

2.7. Data analysis

Statistica® for Windows 4.5 (Statsoft, Tulsa, OK, USA) software was used to perform the statistical analysis. Data for the maximum percentage of motor impairment were analyzed using a multifactorial analysis of variance (ANOVA) with pretreatment, treatment and days as the independent variables. Post hoc comparisons were performed using Tukey test. Values of $P < 0.05$ were considered significant. Figures were drawn using GraphPad Prism® 1.03 (GraphPad Software, San Diego, CA, www.graphpad.com). Experimental data are presented as means ± standard error of mean (S.E.M.).

3. Results

3.1. Selection of the doses of ethanol

Ethanol induced dose-dependent motor impairment on Day 1 (Fig. 1). On Day 2, animals that had received ethanol at doses of 2.0 or 2.25 g/kg were tolerant to the motor impairment induced by the drug [$F(9,90) = 302.313$, $P < 0.0001$]. The doses of 2.25 and 1.9 g/kg of ethanol were selected for the next experiments (one dose that produces rapid tolerance and one dose that does not).

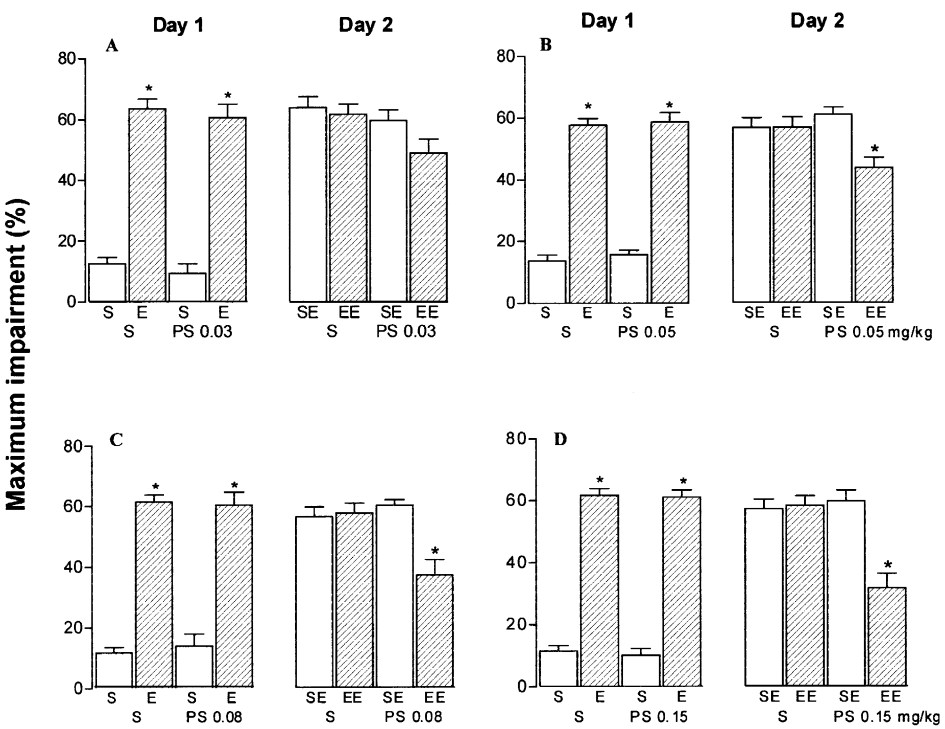

Fig. 2. Effect of pregnenolone sulfate on the development of rapid tolerance to ethanol. On Day 1, eight groups received saline (S) and another eight groups received pregnenolone sulfate (PS; 0.03, 0.05, 0.08 or 0.15 mg/kg i.p.) 30 min before saline or ethanol (E; 1.9 g/kg i.p.). Rapid tolerance to ethanol was assessed on Day 2 when all the groups were treated with ethanol (1.9 g/kg i.p.). Group SE received saline on Day 1 and ethanol on Day 2, whereas the EE group received ethanol on both days. Results shown are means ± S.E.M. for 10 animals per group, and $^{*}P < 0.05$ compared to respective control (Tukey test).

3.2. Possible facilitation of tolerance by pregnenolone sulfate and dehydroepiandrosterone sulfate: influence of (+)-MK-801

Those groups pretreated with pregnenolone sulfate (0.05, 0.08 or 0.15 mg/kg) before ethanol on Day 1 showed a significant reduction of motor impairment, suggesting a facilitation of the development of rapid tolerance on Day 2 (Fig. 2). However, the motor-incoordinating effect of ethanol was not reduced in those control groups treated with ethanol on both days, confirming that the dose of 1.9 g/kg of ethanol does not produce rapid tolerance when given alone (Fig. 2). Three-way ANOVA revealed a significant effect of treatment (Fig. 2A: $F(1,36) = 33.012$, $P < 0.0001$; Fig. 2B: $F(1,36) = 71.082$, $P < 0.0001$; Fig. 2C: $F(1,36) = 46.478$, $P < 0.0001$; Fig. 2D: $F(1,36) = 81.853$, $P < 0.0001$); pretreatment (Fig. 2D: $F(1,36) = 10.045$, $P < 0.0031$); and treatment day (Fig. 2A: $F(1,36) = 67.173$, $P < 0.0001$; Fig. 2B: $F(1,36) = 55.601$, $P < 0.0001$; Fig. 2C: $F(1,36) = 57.412$, $P < 0.0001$; Fig. 2D: $F(1,36) = 54.354$, $P < 0.0001$). Moreover, the pretreatment × treatment × day interactions were significant (Fig. 2B: $F(1,36) = 22.713$, $P < 0.0001$; Fig. 2C: $F(1,36) = 5.988$, $P < 0.0194$; Fig. 2D: $F(1,36) = 12.157$, $P < 0.0013$). The post hoc analysis confirmed that pregnenolone sulfate (at 0.05, 0.08 and 0.15 mg/kg) facilitated the development of rapid tolerance (Tukey test). The lowest dose of pregnenolone sulfate (0.03 mg/kg) did not facilitate rapid tolerance (Fig. 2A, Day 2).

Similarly, the groups pretreated with dehydroepiandrosterone sulfate (0.10, 0.15 or 0.20 mg/kg) before ethanol on Day 1 showed facilitation of rapid tolerance evaluated on Day 2, whereas motor impairment was not reduced in the control groups treated with ethanol on both days (Fig. 3). The three-way ANOVA revealed an effect of treatment (Fig. 3A: $F(1,36) = 274.325$, $P < 0.0001$; Fig. 3B: $F(1,36) = 53.840$, $P < 0.0001$; Fig. 3C: $F(1,36) = 65.803$, $P < 0.0001$; Fig. 3D: $F(1,36) = 66.997$, $P < 0.0001$); pretreatment (Fig. 3B: $F(1,36) = 4.587$, $P < 0.039$); and treatment day (Fig. 3A: $F(1,36) = 89.487$, $P < 0.0001$; Fig. 3B: $F(1,6) = 104.280$, $P < 0.0001$; Fig. 3C: $F(1,36) = 49.904$, $P < 0.0001$; Fig. 3D: $F(1,36) = 52.164$, $P < 0.0001$) and significant pretreatment × treatment × day interactions (Fig. 3C: $F(1,36) = 13.032$, $P < 0.0009$; Fig. 3D: $F(1,36) = 14.691$, $P < 0.0004$). The post hoc analysis indicated that dehydroepiandrosterone sulfate at 0.10, 0.15 and 0.20 mg/kg facilitated the development of rapid tolerance (Tukey test). The dose of 0.05 mg/kg of dehydroepiandrosterone sulfate did not facilitate rapid tolerance (Fig. 3A, Day 2).

The previous administration of (+)-MK-801 blocked the effects of pregnenolone sulfate on the development of

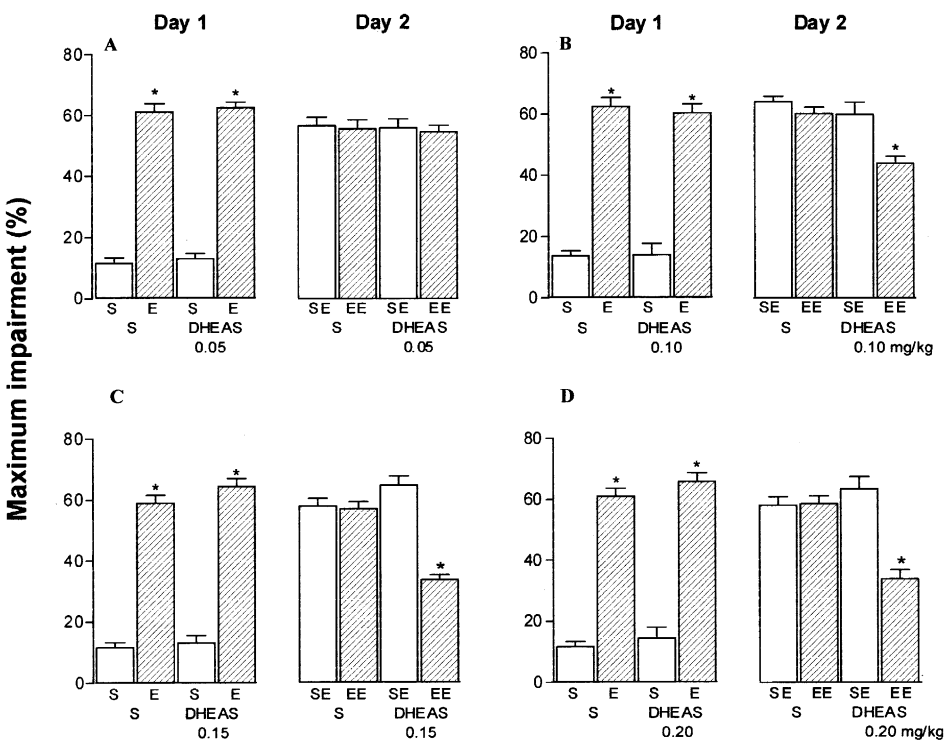

Fig. 3. Effect of dehydroepiandrosterone sulfate on the development of rapid tolerance to ethanol. On Day 1, eight groups received saline (S) and another eight groups received dehydroepiandrosterone sulfate (DHEAS; 0.05, 0.10, 0.15 or 0.20 mg/kg i.p.) 30 min before saline or ethanol (E; 1.9 g/kg i.p.). Rapid tolerance to ethanol was assessed on Day 2 when all the groups were treated with ethanol (1.9 g/kg i.p.). Group SE received saline on Day 1 and ethanol on Day 2, whereas the EE group received ethanol on both days. Results shown are means ± S.E.M. for 10 animals per group, and $^{*}P < 0.05$ compared to respective control (Tukey test).

rapid tolerance to ethanol (1.9 g/kg) evaluated on Day 2. ANOVA revealed an effect of the treatment with ethanol (Fig. 4: $F(1,72) = 171.391$, $P < 0.01$), pretreatment with (+)-MK-801 (Fig. 4: $F(1,72) = 27.541$, $P < 0.001$) and treatment day (Fig. 4: $F(1,72) = 117.799$, $P < 0.0001$). The post hoc analysis indicated that pretreatment with (+)-MK-801 blocked the stimulating effect of pregnenolone sulfate on the development of rapid tolerance (Tukey test). Moreover, pregnenolone sulfate prevented the effect of (+)-MK-801 on the development of tolerance to ethanol (2.25 g/kg) (Fig. 5). ANOVA revealed an effect of the treatment with ethanol (Fig. 5: $F(1,72) = 156.221$, $P < 0.0001$), pretreatment with (+)-MK-801 (Fig. 5: $F(1,72) = 6.593$, $P < 0.02$), treatment with pregnenolone sulfate (Fig. 5: $F(1,72) = 30.595$, $P < 0.0001$) and treatment day (Fig. 5: $F(1,72) = 156.221$, $P < 0.0001$). There was a significant pretreatment × treatment × day interaction (Fig. 5: $F(1,72) = 6.574$, $P < 0.02$). Statistical comparison of the effects of ethanol (2.25 g/kg) in the absence and the presence of pregnenolone sulfate and dehydroepiandrosterone sulfate on Day 2 showed no significant differences (Fig. 5, $P = 0.08$). However, (+)-MK-801 did not block the facilitation of tolerance induced by dehydroepiandrosterone sulfate. ANOVA revealed an effect of the treatment with ethanol (Fig. 6: $F(1,72) = 82.241$, $P < 0.001$) and treatment day (Fig. 6: $F(1,72) = 58.633$, $P < 0.001$). Post hoc comparison (Tukey test) of the effects of ethanol (1.9 g/kg) in the absence and presence of (+)-MK-801 on Day 1 revealed no significant differences (Fig. 4, $P = 0.061$ and Fig. 6, $P = 0.564$).

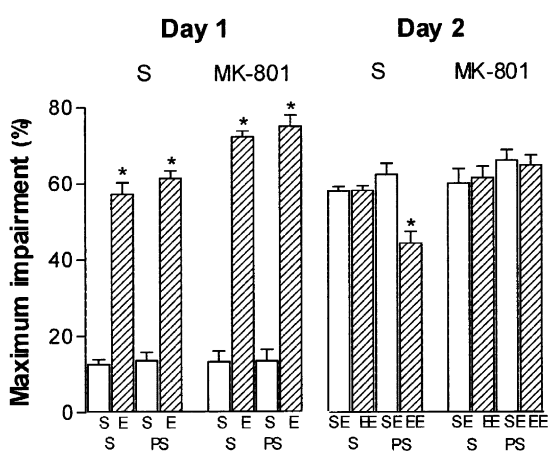

Fig. 4. Effect of pretreatment with (+)-MK-801 on the enhancement of rapid tolerance to ethanol produced by pregnenolone sulfate. On Day 1, four groups received saline (S) and another four groups received (+)-MK-801 (0.06 mg/kg i.p.), and after 15 min, each group received pregnenolone sulfate (PS; 0.08 mg/kg i.p.) or saline, respectively. Thirty minutes later, each group was again divided in order to receive ethanol (E; 1.9 g/kg i.p.) or saline. After 24 h (Day 2), all mice were injected with ethanol (1.9 g/kg). Results shown are means ± S.E.M. for 10 animals per group, and * $P < 0.05$ compared to respective control (Tukey test).

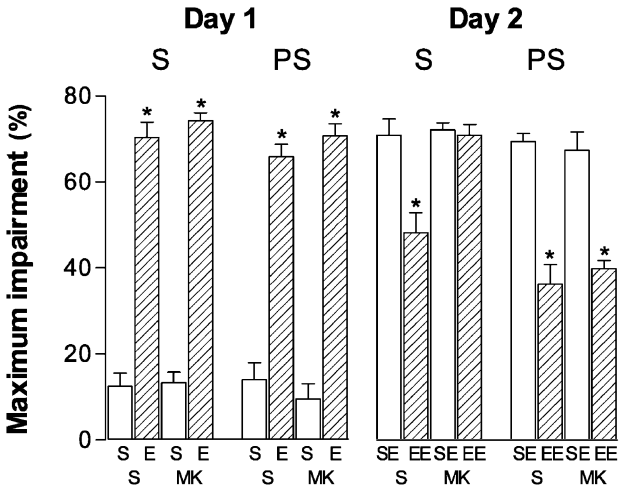

Fig. 5. Effect of treatment with pregnenolone sulfate (PS) on the blockade of rapid tolerance to ethanol produced by (+)-MK-801. On Day 1, four groups received saline (S) and another four groups received PS (0.08 mg/kg i.p.), and after 15 min, each group received (+)-MK-801 (0.06 mg/kg i.p.) or saline, respectively. Thirty minutes later, each group was again divided in order to receive ethanol (E; 2.25 g/kg i.p.) or saline. After 24 h (Day 2), all mice were injected with ethanol (2.25 g/kg). Results shown are means ± S.E.M. for 10 animals per group, and * $P < 0.05$ compared to respective control (Tukey test).

3.3. The effect of pretreatment with pregnenolone sulfate and dehydroepiandrosterone sulfate on the impairment of rapid tolerance to ethanol produced by muscimol

The results for the effects of pregnenolone sulfate on the influence of muscimol on the development of rapid tolerance to ethanol are depicted in Fig. 7. Motor impairment was significantly reduced in all control groups that received ethanol on both days of the experiment, indicating that 2.25 g/kg of ethanol induced rapid tolerance as revealed by ANOVA (day effect: $F(1,72) = 140.993$, $P < 0.0001$; Fig. 7) and confirmed by the post hoc test. ANOVA also showed that muscimol significantly interfered with the development of rapid tolerance ($F(1,72) = 21.010$, $P < 0.0001$), and that pretreatment with pregnenolone sulfate (0.08 mg/kg) before muscimol (0.60 mg/kg) on Day 1 significantly reversed the effect of muscimol ($F(1,72) = 21.416$, $P < 0.0001$). ANOVA also revealed a significant pretreatment 1 × pretreatment 2 × treatment × day interaction ($F(1,72) = 4.559$, $P < 0.0361$). The post hoc analysis suggested that pretreatment with pregnenolone sulfate interfered with the blockade by muscimol in the development of rapid tolerance to ethanol (Tukey test).

As in the previous experiment, the groups pretreated with dehydroepiandrosterone sulfate (0.15 mg/kg) before treatment with muscimol (0.60 mg/kg) on Day 1 showed a blockade of rapid tolerance to muscimol evaluated on Day 2. The overall ANOVA revealed a significant effect of treatment with ethanol (Fig. 8: $F(1,72) = 148.901$, $P < 0.0001$), treatment with dehydroepiandrosterone sulfate

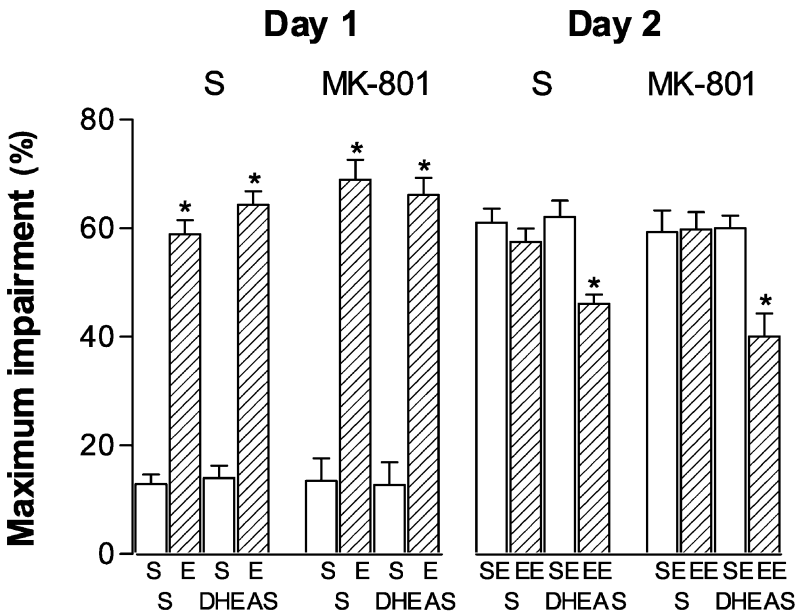

Fig. 6. Effect of pretreatment with (+)-MK-801 on the enhancement of rapid tolerance to ethanol produced by dehydroepiandrosterone sulfate. On Day 1, four groups received saline (S) and another four groups received (+)-MK-801 (0.06 mg/kg i.p.), and after 15 min, each group received dehydroepiandrosterone sulfate (DHEAS; 0.15 mg/kg i.p.) or saline, respectively. Thirty minutes later, each group was again divided in order to receive ethanol (E; 1.9 g/kg i.p.) or saline. After 24 h (Day 2), all mice were injected with ethanol (1.9 g/kg). Results shown are means ± S.E.M. for 10 animals per group, and * $P < 0.05$ compared to respective control (Tukey test).

(Fig. 8: $F(1,72) = 14.849$, $P < 0.0002$), treatment with muscimol (Fig. 8: $F(1,72) = 27.205$, $P < 0.0001$), and treatment day (Fig. 8: $F(1,72) = 210.941$, $P < 0.0001$). An interaction among these factors was found (Fig. 8: $F(1,72) = 7.093$, $P < 0.0095$). Statistical comparison of the effects of ethanol in the absence and the presence of pregnenolone sulfate and dehydroepiandrosterone sulfate on Day 2 showed no differences (Fig. 7, $P = 0.390$ and Fig. 8, $P = 0.139$). Moreover, the post hoc analysis indi-

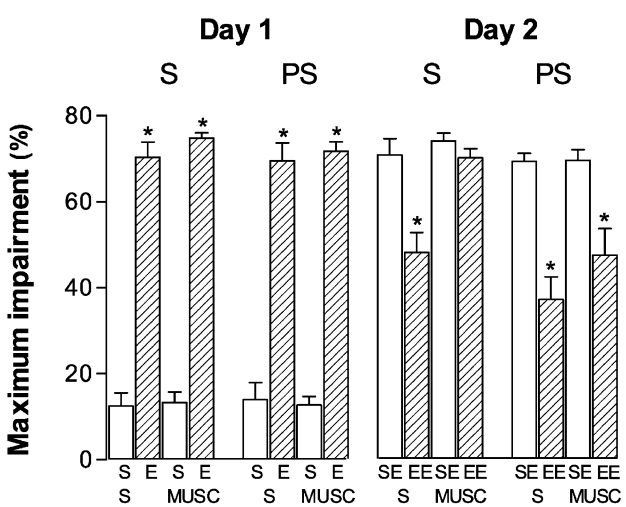

Fig. 7. Effect of pretreatment with pregnenolone sulfate on the impairment of rapid tolerance to ethanol produced by muscimol. On Day 1, four groups received saline (S) and another four groups received pregnenolone sulfate (PS; 0.08 mg/kg i.p.), and after 15 min, each group received muscimol (MUSC; 0.60 mg/kg i.p.) or saline, respectively. Thirty minutes later, each group was again divided in order to receive ethanol (E; 2.25 g/kg i.p.) or saline. After 24 h (Day 2), all mice were injected with ethanol (2.25 g/kg). Results shown are means ± S.E.M. for 10 animals per group, and * $P < 0.05$ compared to respective control (Tukey test).

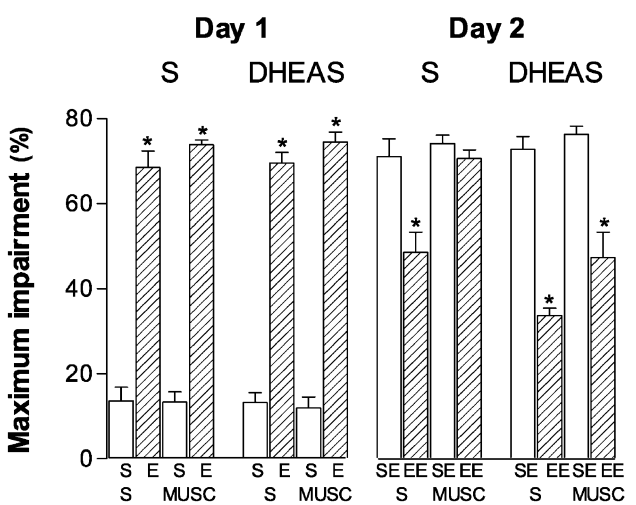

Fig. 8. Effect of pretreatment with dehydroepiandrosterone sulfate on the impairment of rapid tolerance to ethanol produced by muscimol. On Day 1, four groups received saline (S) and another four groups received dehydroepiandrosterone sulfate (DHEAS; 0.15 mg/kg i.p.), and after 15 min, each group received muscimol (MUSC; 0.60 mg/kg i.p.) or saline, respectively. Thirty minutes later, each group was again divided in order to receive ethanol (E; 2.25 g/kg i.p.) or saline. After 24 h (Day 2), all mice were injected with ethanol (2.25 g/kg). Results shown are means ± S.E.M. for 10 animals per group, and * $P < 0.05$ compared to respective control (Tukey test).

cated that muscimol interfered with the development of rapid tolerance to ethanol and that the pretreatment with dehydroepiandrosterone sulfate blocked the impairment produced by muscimol (Tukey test). Blood ethanol concentration was not significantly affected by the treatment with pregnenolone sulfate (135 ± 7 mg/dl), dehydroepiandrosterone sulfate (134 ± 10 mg/dl), (+)-MK-801 (138 ± 9 mg/dl) and muscimol (150 ± 9 mg/dl) as compared to the respective controls (156 ± 2 and 182 ± 11 mg/dl).

4. Discussion

The results of the present study show a clear development of rapid tolerance to ethanol in mice administered with doses of 2.0 and 2.25 g/kg of this drug on both days of the experiment. The doses of 1.9, 2.5 and 3.0 g/kg failed to produce an improvement of rota-rod performance, possibly because a certain degree of motor impairment on Day 1 is required for the development of rapid tolerance on Day 2 in this model (Barreto et al., 1998; Zaleski et al., 2001). Moreover, our study confirms and extends previous findings obtained with rats submitted to the tilt plane test and mice submitted to the rota-rod test, namely, that the extent of rapid tolerance to the motor impairment produced by a single administration of ethanol on Day 1 is similar to that produced by two divided doses of ethanol administered 2 h apart on Day 1 (Khanna et al., 1996; Barreto et al., 1998; Zaleski et al., 2001). In the present study, acute injection of pregnenolone sulfate, a positive allosteric modulator of NMDA receptors and a negative allosteric modulator of $GABA_A$ receptors (Wu et al., 1991; Bowlby, 1993; Majewska et al., 1988) and of dehydroepiandrosterone sulfate, a negative allosteric modulator of $GABA_A$ receptors (Demirgoren et al., 1991; Majewska et al., 1990), before administration of ethanol significantly stimulated the development of rapid tolerance to ethanol. These results agree with our previous study in which chronic tolerance to the effects of ethanol was affected by neurosteroids (Barbosa and Morato, 2000), thus strengthening the suggestion that rapid tolerance can be a predictor of chronic tolerance (Khanna et al., 1991, 1992).

To verify whether NMDA-mediated neurotransmission participated in this effect, we studied the influence of (+)-MK-801, a competitive NMDA receptor antagonist, on the effect of the neurosteroids pregnenolone sulfate and dehydroepiandrosterone sulfate. The administration of (+)-MK-801 reversed the pregnenolone sulfate-induced facilitation of tolerance, suggesting that this facilitation by the neurosteroid is at least partially mediated by NMDA receptors. Nevertheless, (+)-MK-801 did not reverse the stimulatory action of dehydroepiandrosterone sulfate on tolerance development. This effect does not support an NMDA-receptor mechanism in the stimulatory action of dehydroepiandrosterone sulfate on the development of rapid tolerance to ethanol. In fact, our result was expected since dehydroepiandrosterone sulfate is considered to be mainly a negative allosteric modulator of the $GABA_A$ receptor system (Majewska, 1992). However, studies have shown that this neurosteroid can act as a positive allosteric modulator of the NMDA receptor complex at the σ site (Begeron et al., 1996) and that memory impairments induced by dizocilpine [(+)-MK-801] may be reduced by dehydroepiandrosterone sulfate through an interaction with the σ_1 site (Maurice et al., 1997). Moreover, other studies have shown that neurosteroids, which are modulators at the NMDA receptor, act at distinct sites on this receptor complex (Park-Chung et al., 1997; Yaghoubi et al., 1998). Although (+)-MK-801 produced a significant increase in the motor-impairing effect of ethanol on Day 1 in some experiments of the present study, there was no residual effect on the motor coordination of animals or cross-tolerance to the effects of ethanol on Day 2. These results are consistent with the results of Khanna et al. (1993).

An interesting finding of this study was the blockade of rapid tolerance by muscimol, a $GABA_A$ receptor agonist. Pretreatment with pregnenolone sulfate and dehydroepiandrosterone sulfate prevented the inhibitory action of muscimol on the development of rapid tolerance to ethanol. These effects suggest that a stimulatory action on tolerance development by these neurosteroids could be mediated via an antagonistic interaction with the $GABA_A$ receptor. These results are consistent with previous studies demonstrating that negative allosteric modulators of the $GABA_A$ receptor (pregnenolone sulfate and dehydroepiandrosterone sulfate) act non-competitively to reduce the activity of $GABA_A$ receptors (Majewska et al., 1988; Majewska, 1992; Mienville and Vicini, 1989).

It could be argued that the results obtained by using the combination of pregnenolone sulfate plus (+)-MK-801 or muscimol in rats given with 1.9 mg/kg of ethanol simply reflect their algebraically additive effects on rapid tolerance development. However, this seems unlikely as this was clearly not the case for the dehydroepiandrosterone sulfate plus (+)-MK-801 combination. Furthermore, when the pregnenolone sulfate plus (+)-MK-801 combination was tested in rats given with 2.25 g/kg of ethanol, the neurosteroid clearly overrode the effect of (+)-MK-801. Finally, even though neither pregnenolone nor dehydroepiandrosterone sulfate potentiated the development of rapid tolerance induced by 2.25 g/kg of ethanol, per se, each of these neurosteroids clearly overrode the blocking effect of muscimol on the development of rapid tolerance.

All of the results of the present study were obtained with doses of neurosteroids that did not interfere with the motor coordination of the animals on Day 1 and did not produce any residual effects on Day 2. Furthermore, the effects of these neurosteroids seem to be pharmacodynamic rather than pharmacokinetic since our results and data in the literature show that these drugs do not interfere with alcohol metabolism (Mechior and Allen, 1992). However, a recent study showed that the administration of

ethanol to rats (at 2 g/kg) increased plasma levels of allopregnanolone, progesterone and corticosterone (Van Doren et al., 2000). If these effects can be extended to mice, a more complex interaction among neurosteroids can be expected.

Studies suggest that the NMDA and $GABA_A$ receptor systems are involved in processes underlying memory and learning (Collingridge et al., 1992; Morrisett and Swartzwelder, 1993) and that learning may be involved in rapid tolerance acquisition (Bitrán and Kalant, 1991). Furthermore, other studies have shown that neurosteroids can facilitate or impair learning (Mathis et al., 1994, 1996; Flood et al., 1992; Flood and Roberts, 1988; Mechior and Ritzmann, 1996) and that dehydroepiandrosterone sulfate seems to be involved in the modulation of synaptic transmission and long-term potentiation (Meyer et al., 1999). Thus, a possible explanation for our results could be the influence of these neurosteroids in processes related to synaptic plasticity. Taken together, our results suggest that neurosteroids may stimulate the development of rapid tolerance to ethanol, possibly through mechanisms related to the NMDA or $GABA_A$ receptor systems.

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