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Genetic Differences in Behavioral Sensitivity to a Neuroactive Steroid

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ABSTRACT

Recent work found that lower endogenous levels of the γ-aminobutyric acid-agonist, neuroactive steroid 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP) may be correlated with increased ethanol withdrawal severity in the selectively bred Withdrawal Seizure-Prone and -Resistant mice. The present studies were conducted to determine whether decreased sensitivity to 3α,5α-THP was correlated with ethanol withdrawal hyperexcitability in another genetic mouse model, namely the C57BL/6 (B6) and DBA/2 (D2) inbred strains. These strains also differ in ethanol withdrawal severity (D2 ≫ B6). B6 and D2 male mice were injected with 3α,5α-THP (0–10 mg/kg i.p.) 15 min before the timed tail vein infusion of pentylenetetrazol. B6 mice were more sensitive than D2 animals to the anticonvulsant effect of 3α,5α-THP. Subsequent studies measured sensitivity to several of the pharmacological effects of 3α,5α-THP. B6 and D2 male mice were injected with 3α,5α-THP (0–32 mg/kg) before testing for locomotor activation (total number of entries) and anxiolysis (percent open arm entries) on the elevated plus maze, muscle relaxation (impairment of forelimb grip strength), ataxia (impairment of Rotarod performance) and seizure susceptibility to pentylenetetrazol. B6 mice were more sensitive than D2 animals to the anxiolytic, locomotor stimulant and anticonvulsant effects of 3α,5α-THP. In contrast, D2 mice were more sensitive than B6 mice to 3α,5α-THP-induced muscle relaxation and ataxia. Plasma 3α,5α-THP levels did not differ in the B6 and D2 mice injected with this steroid, suggesting that the strain differences were not pharmacokinetic. Collectively, the results in selectively bred Withdrawal Seizure-Prone and -Resistant mice and B6 and D2 inbred strains suggest that genetic differences in neuroactive steroid sensitivity and biosynthesis may contribute to ethanol withdrawal severity.

Sex steroids were reported to influence brain excitability as long ago as 1942 (Seyle, 1942). However, it was not until 1984 that the synthetic steroid alphaxalone (3α-hydroxy-5α-pregnan-11,20-dione) was electrophysiologically demonstrated to potentiate GABA-gated chloride conductance (Harrison and Simmonds, 1984). Subsequent studies determined that the progesterone metabolite 3α,5α-THP and the deoxycorticosterone metabolite 3α,5α-THDOC were potent GABA-agonist modulators of the GRC *via* a stereospecific interaction at a unique steroid recognition site associated with the GRC (for reviews, see Belelli *et al.*, 1990; Paul and Purdy, 1992; Lambert *et al.*, 1995). Both 3α,5α-THP and 3α,5α-THDOC enhance GABA-stimulated chloride uptake in rat brain synaptoneurosomes at nanomolar concentrations (Morrow *et al.*, 1987) and interact with the known sites on the GRC in a noncompetitive manner (for review, see Belelli *et al.*, 1990). These *in vitro* demonstrations provide evidence that some steroid metabolites have rapid membrane actions that are distinct from the genomic action of "classical" steroid hormones.

Consistent with their GABA-agonist pharmacological profiles, exogenous administration of 3α,5α-THP or 3α,5α-THDOC produces anesthetic (Mok *et al.*, 1991), hypnotic (Mendelson *et al.*, 1987), anticonvulsant (Belelli *et al.*, 1989; Finn and Gee, 1994) and anxiolytic (Crawley *et al.*, 1986; Bitran *et al.*, 1991; Weiland *et al.*, 1995) effects. Both 3α,5α-THP and 3α,5α-THDOC, as well as the synthetic steroid anesthetic alphaxalone, were potent anxiolytics in several animal models of anxiety, *i.e.*, the light/dark transition test (Crawley *et al.*, 1986; Weiland *et al.*, 1995), the open field test (Weiland *et al.*, 1991), the conflict test (Crawley *et al.*, 1986; Britton *et al.*, 1991; Weiland *et al.*, 1995) and the elevated plus maze (Bitran *et al.*, 1991; Britton *et al.*, 1991). In addition, 3α,5α-THP was anticonvulsant against PTZ-, (+)-bicuculline- and picrotoxin-induced seizures, with maximum po-

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ABBREVIATIONS: B6, C57BL/6; D2, DBA/2; FF clonus, face and forelimb clonus; GABA, γ-aminobutyric acid; GRC, γ-aminobutyric $acid_A$ receptor complex; MC twitch, myoclonic twitch; PTZ, pentylenetetrazol; RB clonus, running bouncing clonus; RIA, radioimmunoassay; 3α,5α-THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; THE, tonic hindlimb extension; 3α,5α-THP, 3α-hydroxy-5α-pregnan-20-one.

tency against (+)-bicuculline-induced convulsions (Belelli *et al.*, 1989). These behavioral responses closely follow the anticipated pharmacological patterns based on *in vitro* evidence.

Recent work has demonstrated that endogenous 3α,5α-THP can reach pharmacologically relevant concentrations (Paul and Purdy, 1992). After swim stress in male rats and during the estrus cycle in female rats, brain 3α,5α-THP levels increased to approximately 10 to 30 nM. Plasma 3α,5α-THP levels also reached 100 nM during the third trimester of pregnancy. These concentrations achieved *in vivo* have been shown to potentiate the action of GABA by *in vitro* studies. Therefore, the available evidence suggests, but does not prove, that fluctuations in endogenous GABAergic steroids can modify the functioning of central $GABA_A$ receptors *in vivo*.

Based on recent results from our laboratory, we believed that one way to provide support for the hypothesis that 3α,5α-THP represented a physiologically significant endogenous neuromodulator would be to demonstrate that lower endogenous levels of, or decreased sensitivity to, 3α,5α-THP were correlated with genetic differences in basal or ethanol withdrawal hyperexcitability. The B6 and D2 inbred strains differ in a number of behaviors, including locomotor and exploratory activity (Lhotellier *et al.*, 1993), learning and memory (Fordyce and Wehner, 1993; Rossi-Arnaud and Ammassari-Teule, 1994), anxiety (Trullas and Skolnick, 1993) and seizure susceptibility (Kosobud and Crabbe, 1990; Ferraro *et al.*, 1995). When tested for susceptibility to a number of convulsants, B6 mice are generally very seizure resistant, in comparison with other inbred strains, whereas D2 animals are relatively seizure prone (Kosobud and Crabbe, 1990). B6 and D2 mice also differ markedly in many ethanol-related behaviors, of which ethanol preference, ethanol-induced locomotor activation and ethanol withdrawal severity are the most notable (for review, see Phillips and Crabbe, 1991). D2 mice exhibit more severe handling-induced convulsions than do B6 animals after withdrawal from both acute (Roberts *et al.*, 1992) and chronic (Crabbe *et al.*, 1983) ethanol administration. Therefore, B6 and D2 mice represent useful animal models to test the hypothesis that genetic differences in the modulatory effects of 3α,5α-THP on ethanol withdrawal severity might result from differences in 3α,5α-THP sensitivity or biosynthesis.

Preliminary results suggested that B6 and D2 mice do not differ in biosynthesis of 3α,5α-THP after 24-hr exposure to ethanol vapor (Finn *et al.*, 1995a). In both B6 and D2 mice, plasma 3α,5α-THP levels were unchanged during peak ethanol withdrawal and were increased in only the animals that were scored hourly for withdrawal. Therefore, the present studies were conducted to test the hypothesis that differences in 3α,5α-THP sensitivity may contribute to ethanol withdrawal hyperexcitability differences in B6 and D2 animals (*i.e.*, B6 and D2 mice would differ in sensitivity to 3α,5α-THP in a manner that was consistent with their genetic difference in ethanol withdrawal severity). Because ethanol withdrawal is characterized by multiple behavioral changes in addition to convulsions, a number of other behaviors were evaluated, *i.e.*, anxiolytic effects and locomotor activity (measured on an elevated plus maze), ataxic effects (measured by Rotarod performance), muscle relaxation (measured in a test of grip strength) and anticonvulsant effects (measured by tail vein infusion of PTZ), to determine whether a difference between B6 and D2 mice in sensitivity to 3α,5α-THP could be generalized to all pharmacological properties of 3α,5α-THP.

Methods

Subjects

Drug-naive male B6 and D2 mice were used in all experiments. The animals were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5 to 6 weeks of age, housed four per cage with *ad libitum* access to food and water and acclimated to a 12/12-hr light/dark cycle for a minimum of 1 week before experimentation. All procedures adhered to the United States Public Health Service/National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by two local institutional Animal Care and Use Committees.

Experiment 1: Anticonvulsant Sensitivity to 3α,5α-THP

The anticonvulsant effect of 3α,5α-THP was measured by administering 3α,5α-THP (5 or 10 mg/kg) or an equivalent volume of vehicle (20% w/v 2-hydroxypropyl-β-cyclodextrin; Research Biochemicals International, Natick, MA) by i.p. injection 15 min before timed tail vein infusion of PTZ (5 mg/ml in saline, 0.5 ml/min; Sigma Chemical Co., St. Louis, MO). The 3α,5α-THP was prepared as a 0.5 or 1.0 mg/ml solution in 20% 2-hydroxypropyl-β-cyclodextrin and injected in a volume of 0.01 ml/g body weight. The apparatus and procedure for tail vein infusion have been described in detail (Kosobud and Crabbe, 1990). The infusion was terminated when the animals exhibited THE. The animals were euthanized by decapitation, and trunk blood was collected for subsequent analysis of plasma 3α,5α-THP concentration by RIA.

The convulsion end-points are described in detail elsewhere (Kosobud and Crabbe, 1990). Briefly, clonus indicates rapid rhythmic movements due to alternating contraction and relaxation of muscles, whereas tonus indicates rigidity due to contraction of muscles. Four convulsion signs, which occur in progression, characterize PTZ-induced convulsions, *i.e.*, MC twitch (sudden involuntary muscle jerks), FF clonus (rapid writhing movements of the head and neck and forelimb clonus), RB clonus (violent whole-body clonus, including running and explosive jumps) and THE (extreme rigidity, with forelimbs and hindlimbs extended caudally). Latency to each sign was recorded in seconds and subsequently converted to threshold convulsant dosage (*i.e.*, milligrams of drug per kilogram of body weight).

Experiment 2: Behavioral Sensitivity to 3α,5α-THP

We recently established a procedure for measuring a series of behaviors, taking advantage of our experience with the individual behavioral protocols and validating the accuracy of repeated testing of individual animals (E. J. Gallaher, unpublished observations). The anticonvulsant effect of 3α,5α-THP was reexamined in this study for two reasons; 1) an expanded dose range was evaluated and 2) the timed tail vein infusion of PTZ occurred at the end of this series of behavioral tests, so that comparisons between experiments would yield a measure of the reliability of repeated behavioral testing in a single animal.

Each mouse was pretested for forelimb grip strength (three trials; time −20 min) and Rotarod baseline performance (three trials; time −10 min) and then weighed and injected with 3α,5α-THP (0, 1, 3.2, 10, 17 or 32 mg/kg in 20% 2-hydroxypropyl-β-cyclodextrin i.p.) at time 0. At time +20 min the mouse was placed on the plus maze for a 5-min trial. Total arm entries (locomotor activation) and percent open arm entries (anxiolysis) were recorded. At time +30 min the mouse was tested for forelimb grip strength (compared with baseline). At time +40 min the mouse was tested for impairment on the accelerating Rotarod (compared with baseline). At time +50 min the mouse was infused, *via* a lateral tail vein, with the convulsant PTZ

and was observed for latency to MC twitch, FF clonus, RB clonus and THE. The threshold dose of PTZ for each sign was calculated from the infusion rate, body weight and latency. The infusion was terminated when the animals exhibited THE or at 4 min (in cases where the mice were protected by 3α,5α-THP). The animal was then euthanized by decapitation, and trunk blood was collected for subsequent analysis of plasma 3α,5α-THP concentration by RIA.

Behavioral Assessments

Muscle relaxation. In this test, mice are placed on a tray, allowed to grasp a horizontal bar connected to a strain gauge and then pulled gently until they lose their grip. For comparative purposes, grip strength decreases in a dose-dependent manner after graded doses of chlordiazepoxide (3, 9 and 27 mg/kg) and phenobarbital (20, 40 and 80 mg/kg). The lowest doses produce a 10 to 20% decrease in grip strength and the higher doses a 50 to 60% decrease (Meyer *et al.*, 1979).

Ataxia. The Rotarod test for ataxia was first described by Dunham and Miya (1957). The Rotarod is a horizontal rotating dowel (5 cm in diameter) suspended 60 cm above a bed of sawdust. It is divided into six segments (10.2-cm wide) by means of opaque white disks 28 cm in diameter. The surface of the dowel is covered with 320-grit, wet-dry sandpaper to ensure a uniform surface. Typically, mice are placed on the Rotarod and observed for ability to remain on the dowel as it turns. In the present experiment, each mouse was placed on a stationary Rotarod, which began to accelerate linearly (20 rpm/min) until the mouse fell off. The latency to fall was then used to calculate the speed (rpm) at which the mouse could no longer remain on the Rotarod. This procedure allowed each mouse to be tested for baseline ability. The drug effect was then expressed as a change from baseline ability.

Anxiolysis. The animal model of anxiety that was used was the elevated plus maze method (Pellow *et al.*, 1985; Lister, 1987; Trullas and Skolnick, 1993; Cole and Rodgers, 1994), which is based on a rodent's natural avoidance of open elevated alleys (Montgomery, 1958). The elevated plus maze consists of two open and two enclosed horizontal perpendicular arms extending from a central platform (5 × 5 cm), 50 cm above the floor. Each mouse was placed on the central platform and allowed to explore freely for 5 min. During the 5-min test period, the number of entries into the open and closed arms and the amount of time spent in the open and closed arms were measured. For an arm entry to be measured, all four paws had to be within the arm. Mice normally prefer the closed arms of the plus maze. Anxiolytic drugs typically increase the proportion of open arm entries and the time spent on the open arm (anxiolysis). The elevated plus maze is able to detect both anxiolytic and anxiogenic agents in mice (Lister, 1987).

Low-dose locomotor activation. Sedative/hypnotic drugs commonly cause locomotor activation after low doses. This has been ascribed to an anxiolytic effect, because locomotion may be an expression of increased exploration in the absence of anxiety. The information obtained from elevated plus maze testing (*i.e.*, total number of arm entries) was used as the estimate of locomotor activation.

Seizure protection. Mice were administered the convulsant PTZ (5 mg/ml in saline) *via* timed tail vein infusion into a lateral vein (0.5 ml/min infusion rate). Latencies to each convulsion measure were recorded in seconds and subsequently converted to threshold convulsant dosage (*i.e.*, milligram of drug per kilogram of body weight). This method allowed for observation and qualitative analysis of several different endpoints (*i.e.*, MC twitch, FF clonus, RB clonus and THE). Drugs that alter seizure threshold can be tested for pro- or anticonvulsant activity by pretreating mice and observing the effect on PTZ seizure threshold. A decreased PTZ seizure threshold indicates proconvulsant activity, whereas an increased PTZ threshold suggests anticonvulsant activity. This simple method has been used to demonstrate the anticonvulsant efficacy of 3α,5α-THP (Finn and Gee, 1994; Finn *et al.*, 1995a,b).

RIA

The RIA for 3α,5α-THP was adapted from the method of Purdy *et al.* (1990) and is described in detail elsewhere (Finn and Gee, 1994). The RIA used a polyclonal antiserum, which was kindly provided by CoCensys, Inc. (Irvine, CA), and [^{3}H]3α,5α-THP (54 Ci/mmol; New England Nuclear, Boston, MA). Counts per minute were normalized and fit to a least-squares-fit regression equation produced by log-logit transformation of the standards. The mass of samples was calculated by interpolation of the standards and correction for recovery. The minimum detectable limit in the present assay was 25 pg. The intraassay coefficient of variation averaged 14%, and the interassay coefficient of variation in seven assays averaged 15%.

Data Analysis

The data are expressed as the mean ± S.E. Analysis of variance was used to assess strain and dose effects on the dependent variables muscle relaxation (change in baseline forelimb grip strength), ataxia (change in baseline Rotarod performance), locomotor activity (total arm entries on the elevated plus maze), anxiolysis (percent open arm entries on the plus maze), seizure protection (threshold dose for onset to MC twitch, FF clonus, RB clonus and THE) and plasma 3α,5α-THP concentration. When appropriate, simple main-effects analyses followed by *post hoc* comparisons were used to examine significant dose effects within each strain. Because the results of experiment 1 indicated that B6 and D2 mice differed in sensitivity to 3α,5α-THP, statistical analyses for experiment 2 were conducted on each strain separately.

Results

Experiment 1: anticonvulsant sensitivity to 3α,5α-THP. To evaluate whether differences in sensitivity to an endogenous anticonvulsant steroid (*i.e.*, 3α,5α-THP) contribute to the genetic differences in ethanol withdrawal severity found in B6 and D2 mice, it was important to first investigate anticonvulsant sensitivity to 3α,5α-THP in ethanol-naive animals. The results indicated that vehicle-treated D2 mice were more sensitive to PTZ than B6 animals, as illustrated in figure 1 for all convulsion measures (compare with vehicle-treated animals in fig. 1). More importantly, B6 mice were more sensitive than D2 mice to the anticonvulsant effect of 3α,5α-THP, as measured by the increase in PTZ seizure threshold for onset to MC twitch (fig. 1A) and FF clonus (fig. 1B). There were significant main effects of strain [$F(1,54) > 94.3$, $P < .0001$] and dose [$F(2,54) > 28.7$, $P < .0001$], with significant interactions between strain and dose [$F(2,54) > 6.5$, $P < .005$]. *Post hoc* tests indicated that both doses of 3α,5α-THP significantly increased PTZ seizure threshold in B6 mice, whereas only the 10 mg/kg dose significantly increased seizure threshold in D2 mice. Analysis of the RB clonus (fig. 1C) and THE (fig. 1D) results indicated that there were significant main effects of strain [$F(1,52) > 9.5$, $P < .005$] and dose [$F(2,52) > 21.7$, $P < .0001$]. The interaction between main effects was a nonsignificant trend for RB clonus [$F(2,52) = 2.73$, $P = .07$] and was not significant for THE [$F(2,52) = 1.18$], suggesting that the anticonvulsant effects of 3α,5α-THP against PTZ-induced RB clonus and THE were similar in B6 and D2 mice.

Plasma samples were taken approximately 30 min after injection of 3α,5α-THP. Analysis of the RIA data (fig. 2) showed that the dose of 3α,5α-THP [$F(2,47) = 165.62$, $P < .0001$], but not the strain [$F(1,47) = 1.41$], significantly altered plasma 3α,5α-THP levels. The interaction between strain and dose was also not significant [$F(2,47) = .05$],

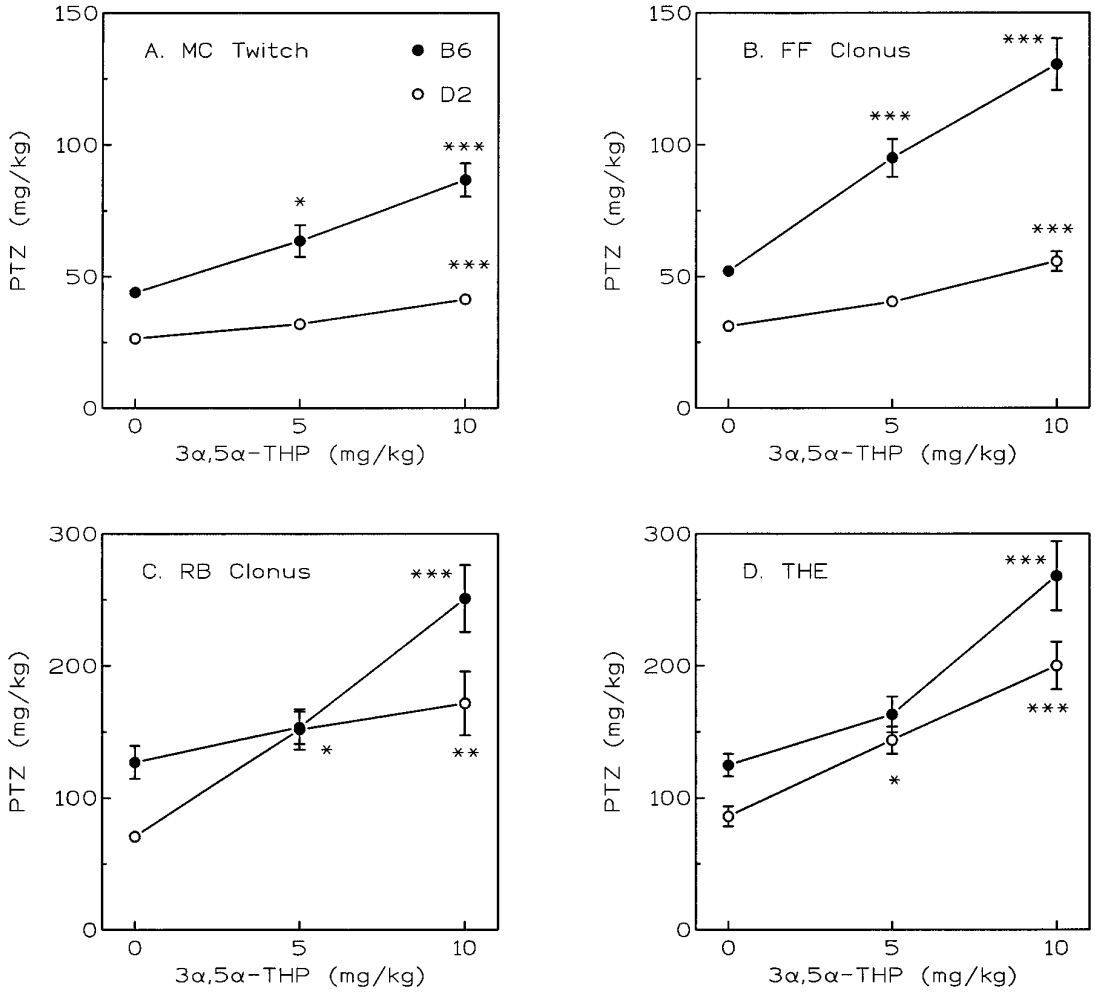

Fig. 1. Anticonvulsant effects of 3α,5α-THP against PTZ-induced MC twitch (A), FF clonus (B), RB clonus (C) and THE (D) in B6 and D2 mice. Administration of 3α,5α-THP 15 min before tail vein infusion of PTZ significantly increased the threshold dose of PTZ for onset for all four convulsion measures. Values represent the mean $\pm$ S.E. for 8 to 10 animals per dose and strain. In cases where the S.E. is not visible, it is contained within the symbol. *P $<$ .05, **P $<$ .01, ***P $<$ .001 *vs.* respective vehicle-treated group.

suggesting that injection of 3α,5α-THP produced similar increases in plasma 3α,5α-THP concentrations in both B6 and D2 mice.

Experiment 2: behavioral sensitivity to 3α,5α-THP. The results of experiment 1 indicated that B6 mice were more sensitive than D2 animals to the anticonvulsant effect of 3α,5α-THP and that this difference in sensitivity was not pharmacokinetic. Subsequent studies evaluated whether this strain difference in sensitivity to the anticonvulsant effect of 3α,5α-THP was generalized across several of the pharmacological effects of 3α,5α-THP.

The results of these series of tests are shown in figures 3 to 5. Administration of 3α,5α-THP produced locomotor stimulation and anxiolysis (measured on the elevated plus maze) (fig. 3), varying amounts of seizure protection (fig. 4) and muscle relaxation and ataxia (fig. 5). The differences between the two strains in the doses of 3α,5α-THP producing pharmacological effects suggested that B6 mice were more sensitive than D2 mice to the anxiolytic, locomotor stimulant and anticonvulsant effects of 3α,5α-THP. In contrast, D2 mice appeared to be more sensitive than B6 mice to the muscle relaxation and ataxia produced by 3α,5α-THP.

Administration of 3α,5α-THP before testing on the elevated plus maze produced locomotor stimulation, as measured by the total number of entries, in B6 [$F(5,39) = 3.28$, $P < .02$] and D2 [$F(5,39) = 2.28$, $P < .07$] mice, although the effect was marginally significant in D2 animals (fig. 3A). *Post hoc* analyses indicated that the 10 mg/kg dose of 3α,5α-THP significantly increased the total number of entries for B6 animals *vs.* vehicle. In addition, the number of entries for B6 mice injected with 3.2 and 10 mg/kg was significantly greater than for animals injected with the 1 mg/kg dose. Exogenous administration of 3α,5α-THP also produced anxiolysis, as measured by the percent open arm entries on the plus maze, in both B6 mice [$F(5,36) = 2.55$, $P < .05$] and D2 mice [$F(5,39) = 2.80$, $P < .05$] (fig. 3B). *Post hoc* tests found that there was a significant increase in the percent open arm entries in B6 mice administered 3.2 and 10 mg/kg 3α,5α-THP and a nonsignificant trend for an increase in percent open arm entries in D2 animals injected with 17 and 32 mg/kg 3α,5α-THP *vs.*

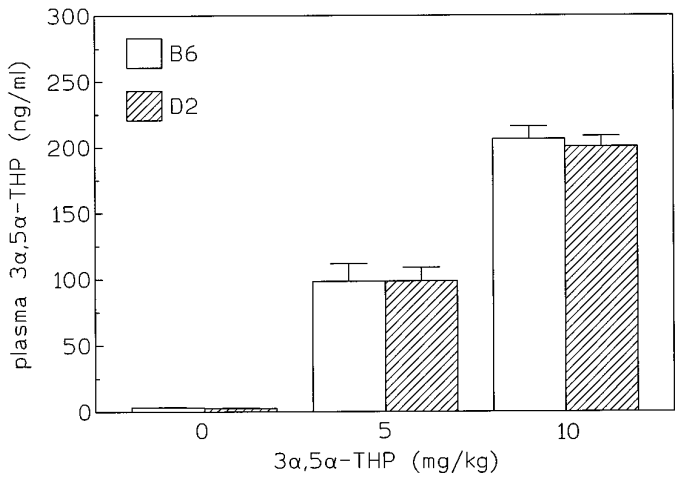

Fig. 2. Plasma 3α,5α-THP levels in B6 and D2 mice. Plasma samples were taken upon completion of the behavioral testing (*i.e.*, approximately 30 min after injection of 3α,5α-THP). Administration of 3α,5α-THP significantly increased plasma 3α,5α-THP concentrations in a dose-dependent manner. Values represent the mean ± S.E. for the animals depicted in figure 1.

the respective vehicle-treated animals. Thus, the dose-response curve for B6 mice appeared to be shifted to the left of that for D2 mice (*i.e.*, greater sensitivity to 3α,5α-THP in B6 *vs.* D2 mice).

Consistent with the results of experiment 1, B6 mice were more sensitive than D2 animals to the anticonvulsant effect of 3α,5α-THP against PTZ-induced convulsions (fig. 4). 3α,5α-THP significantly increased the PTZ-induced threshold dose for onset to MC twitch (fig. 4A) and FF clonus (fig. 4B) in both B6 mice [$F(5,33) > 4.97$, $P \leq .002$] and D2 mice [$F(5,37) > 3.70$, $P < .01$]. *Post hoc* tests for the B6 mice found a dose-dependent increase in PTZ seizure threshold for onset to MC twitch and FF clonus in the animals injected with doses ranging from 3.2 to 32 mg/kg 3α,5α-THP. In the D2 animals, administration of 32 mg/kg 3α,5α-THP significantly increased the threshold dose for onset to MC twitch, whereas 17 and 32 mg/kg 3α,5α-THP significantly increased the threshold dose for FF clonus. Analysis of the RB clonus (fig. 4C) and THE (fig. 4D) data indicated that 3α,5α-THP produced similar significant increases in PTZ seizure threshold in B6 mice [$F(5,32 > 8.84$, $P < .0001$] and D2 mice [$F(3,33) > 15.75$, $P < .0001$]. In both strains, the threshold dose for onset to RB clonus and THE was significantly higher in animals administered the 17 and 32 mg/kg doses of 3α,5α-THP, compared with the respective vehicle-treated animals as well as with the animals administered the 1, 3.2 and 10 mg/kg doses.

In contrast to the anticonvulsant and anxiolytic results, D2 animals appeared to be more sensitive than B6 mice to the muscle relaxant (fig. 5A) and ataxic (fig. 5B) effects of 3α,5α-THP. Administration of 3α,5α-THP produced significant muscle relaxation, measured by the percent change in baseline grip strength (fig. 5A), in both B6 mice [$F(5,39) = 3.92$, $P < .01$] and D2 mice [$F(5,39) = 36.05$, $P < .0001$]. Both the 17 and 32 mg/kg doses of 3α,5α-THP significantly decreased baseline grip strength in D2 mice, whereas only the 32 mg/kg dose significantly decreased grip strength in B6 animals. Injection of 3α,5α-THP also significantly affected Rotarod performance (fig. 5B) for both B6 mice [$F(5,37) = 2.86$, $P < .05$] and D2 mice [$F(5,39) = 3.27$, $P < .02$]. Whereas the 32 mg/kg dose significantly decreased Rotarod performance in D2 mice, the 10 mg/kg dose significantly increased Rotarod performance in B6 mice. The apparent enhancement of baseline Rotarod performance in B6 mice after 10 mg/kg 3α,5α-THP may be related to the locomotor activation also found in B6 mice after this dose of 3α,5α-THP (fig. 3A).

Analysis of the plasma samples (fig. 6), which were taken upon completion of the behavioral testing (*i.e.*, at approximately 60 min after injection of 3α,5α-THP), indicated that exogenous administration of 3α,5α-THP produced a dose-dependent increase in plasma 3α,5α-THP concentrations in both B6 mice [$F(5,30) = 26.36$, $P < .0001$] and D2 mice [$F(5,30) = 14.71$, $P < .0001$]. In B6 mice, plasma 3α,5α-THP levels were significantly higher in the animals injected with 10, 17 and 32 mg/kg *vs.* vehicle. The plasma 3α,5α-THP concentration in the 32 mg/kg-treated mice was also significantly higher than in all other 3α,5α-THP-injected animals, whereas the plasma 3α,5α-THP level in the 17 mg/kg-treated animals was significantly higher than in the animals injected with the 1, 3.2 and 10 mg/kg doses. In D2 mice, plasma 3α,5α-THP levels were significantly higher in the 17 and 32 mg/kg-treated animals, compared with vehicle-treated animals and animals injected with the 1, 3.2 and 10 mg/kg doses.

Discussion

The results of the present studies suggest that B6 mice were more sensitive than D2 animals to the anxiolytic, locomotor stimulant and anticonvulsant effects of 3α,5α-THP. In contrast, D2 mice appeared to be more sensitive than B6 mice to the muscle relaxation and ataxia produced by 3α,5α-THP. Although these results do not indicate that increased sensitivity to endogenous 3α,5α-THP in B6 mice is the factor responsible for their decreased seizure susceptibility, relative to D2 animals, the enhanced sensitivity to the anticonvulsant effect of exogenous 3α,5α-THP in B6 *vs.* D2 mice is consistent with their genetic differences in seizure susceptibility and ethanol withdrawal severity (*i.e.*, decreased seizure susceptibility and ethanol withdrawal severity in B6 *vs.* D2 mice). More important, the genetic differences in sensitivity to the pharmacological effects of 3α,5α-THP were not pharmacokinetic, because plasma 3α,5α-THP levels did not differ in B6 and D2 mice after injection of this neuroactive steroid.

The conclusion that B6 mice were more sensitive than D2 mice to the anxiolytic and locomotor stimulant effects of 3α,5α-THP was based on the results obtained from testing on the elevated plus maze (*i.e.*, percent open arm entries and total entries). Recent work has indicated that additional behavioral measures, collectively referred to as "risk assessment," may enhance the sensitivity of the elevated plus maze as an animal model of anxiety (Cole and Rodgers, 1994). Although these behavioral assessments were not evaluated in the present study, the 3α,5α-THP-induced anxiolysis in B6 and D2 mice is consistent with published results, which have used several animal models of anxiety (Bitran *et al.*, 1991; Weiland *et al.*, 1991, 1995). Nonetheless, future studies that incorporate these risk assessment behaviors may yield more information about differences between genotypes in 3α,5α-THP-induced anxiolysis.

Administration of 3α,5α-THP significantly increased plasma 3α,5α-THP concentrations in both inbred strains.

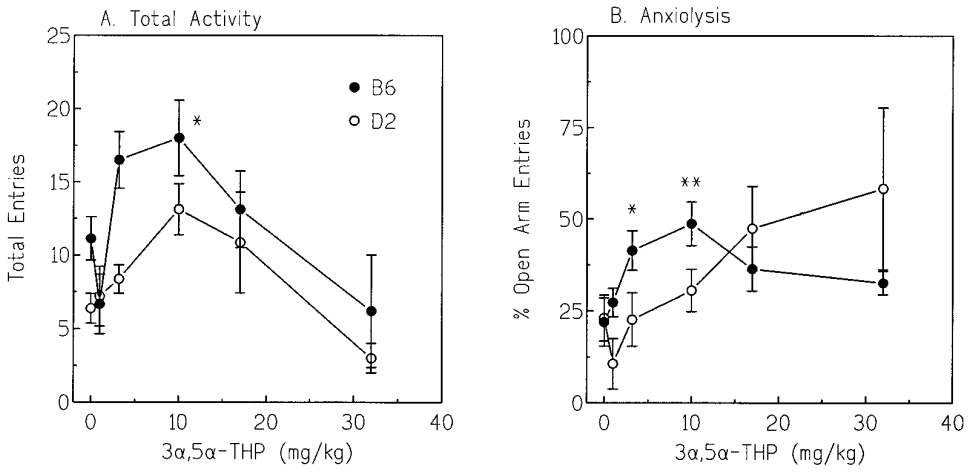

Fig. 3. Locomotor stimulant (A) and anxiolytic (B) effects of 3α,5α-THP in B6 and D2 mice. Mice were administered 3α,5α-THP 20 min before testing on the elevated plus maze. The total number of entries was used as an estimate of locomotor activity, and the percent open arm entries was used as a measure of anxiolysis. Values represent the mean ± S.E. for five to eight animals per strain and dose, except for D2 mice injected with 32 mg/kg 3α,5α-THP (n = 3). *P < .05, **P < .01 *vs.* respective vehicle-treated mice.

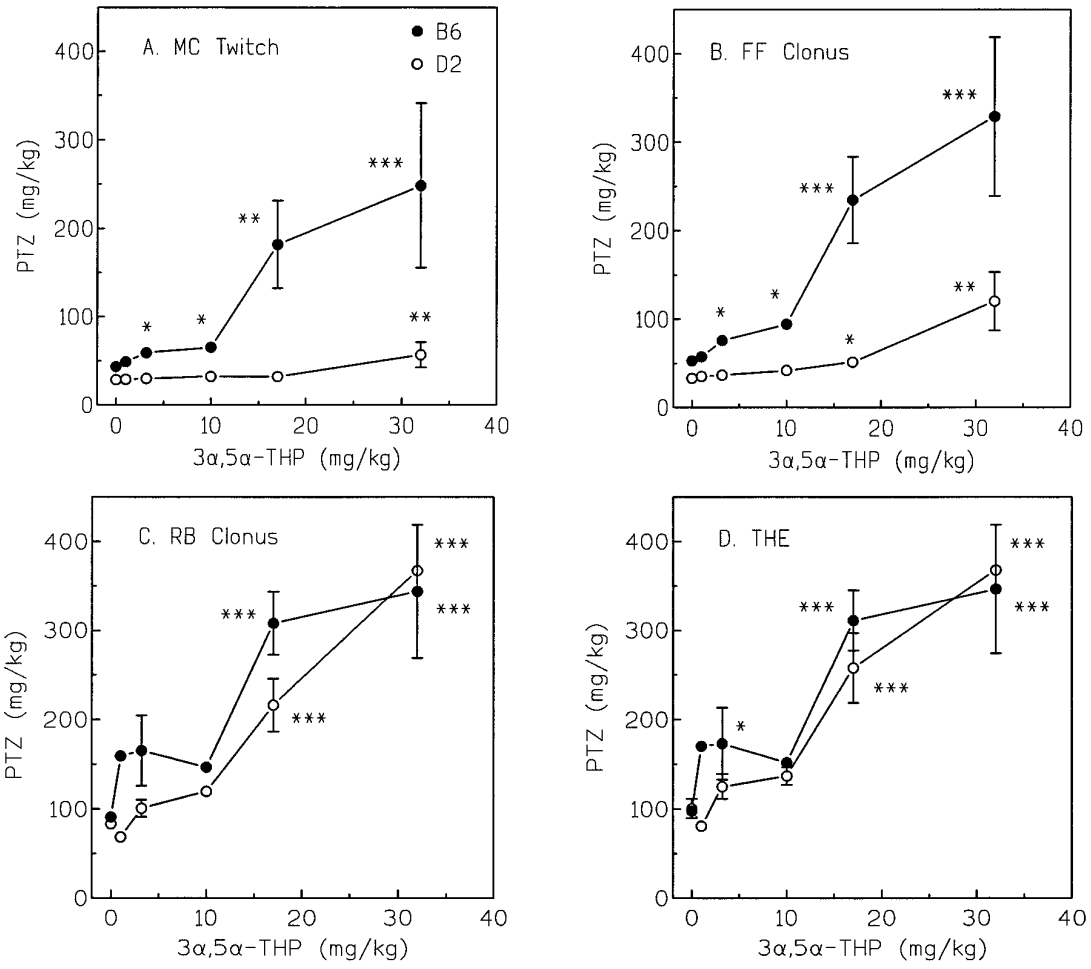

Fig. 4. Anticonvulsant effects of 3α,5α-THP against PTZ-induced MC twitch (A), FF clonus (B), RB clonus (C) and THE (D) in B6 and D2 mice. 3α,5α-THP was administered 50 min before the timed tail vein infusion of PTZ and significantly increased the threshold dose of PTZ required for onset for all four convulsion measures. Values represent the mean ± S.E. for the animals depicted in figure 3. *P < .05, **P < .01, ***P < .001 *vs.* respective vehicle-treated mice.

Depending on the experiment, doses of 3α,5α-THP of ≥5 mg/kg produced a significant increase in plasma 3α,5α-THP levels, compared with the respective vehicle-treated animals. Even though the highest dose of 3α,5α-THP administered (*i.e.*, 32 mg/kg) produced significant ataxia and plasma concentrations ranging from 450 to 480 ng/ml, these levels are considerably lower than the anesthetic levels recently reported for mice (Mok *et al.*, 1991). Brain 3α,5α-THP levels were not measured in the present study. However, the data reported by Mok *et al.* (1991) indicated that i.v. administration of 3α,5α-THP produced peak brain levels within 1 min of injection. Because the animals in the present studies were euthanized for determination of plasma 3α,5α-THP concentrations at approximately 30 or 60 min after injection of

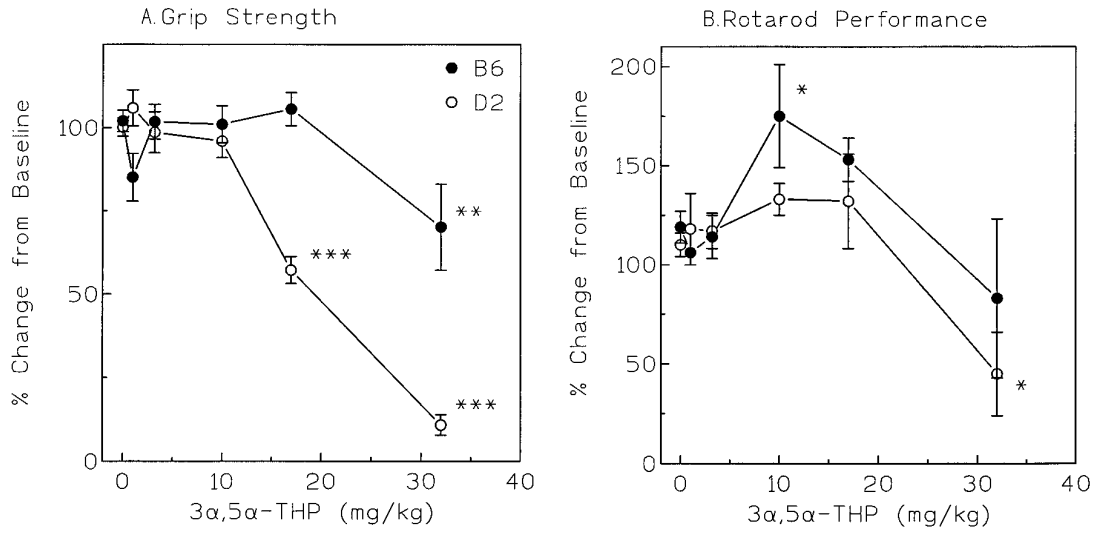

Fig. 5. Effect of 3α,5α-THP on forelimb grip strength (A) and Rotarod performance (B) in B6 and D2 mice. Animals were injected with 3α,5α-THP and then tested for forelimb grip strength (compared with baseline) at 30 min after injection and tested for performance on the accelerating Rotarod (compared with baseline) at 40 min after injection. Values represent the mean ± S.E. (percent change from baseline) for the animals depicted in figures 3 and 4. *P < .05, **P < .01, ***P < .001 *vs.* respective vehicle-treated group.

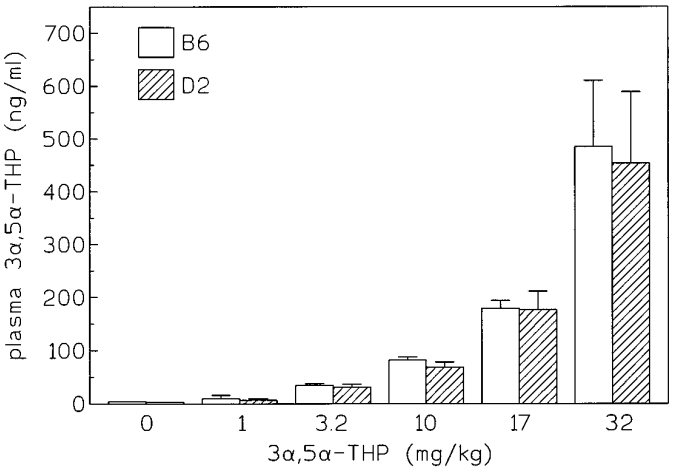

Fig. 6. Plasma 3α,5α-THP levels in B6 and D2 mice. Plasma samples were taken upon completion of the behavioral testing (*i.e.*, approximately 60 min after injection of 3α,5α-THP). Values represent the mean ± S.E. for the animals depicted in figures 3 to 5. See "Results" for details regarding group differences.

3α,5α-THP, the plasma levels should reflect brain 3α,5α-THP levels. Therefore, it is unlikely that differences in brain 3α,5α-THP levels between B6 and D2 mice administered 3α,5α-THP contributed to the differences in sensitivity to the pharmacological effects of 3α,5α-THP.

Comparisons between endogenous 3α,5α-THP levels and plasma 3α,5α-THP concentrations after injection suggest that exogenous administration of low doses of 3α,5α-THP may lead to physiologically relevant concentrations. Doses of 3α,5α-THP ranging from 3.2 to 32 mg/kg were significantly anticonvulsant in B6 mice, with doses of 3.2 and 10 mg/kg producing significant anxiolysis in these animals. Although data are limited, endogenous plasma 3α,5α-THP levels of $\geq$30 ng/ml or $\geq$100 nM have been reported to occur under some conditions (Paul and Purdy, 1992). Therefore, the 3.2 mg/kg dose of 3α,5α-THP, which produced a 34 ng/ml plasma level in B6 mice and was anxiolytic and anticonvulsant in these animals, might be physiologically relevant. Administration of the 5 or 10 mg/kg doses produced plasma 3α,5α-THP concentrations that were at least 2- to 3-fold higher than published endogenous levels measured after swim stress or during pregnancy (Paul and Purdy, 1992). Because recent work demonstrated that endogenous 3α,5α-THP levels were higher in brain than in plasma (Corpechot *et al.*, 1993), the possibility exists that the plasma 3α,5α-THP concentrations achieved after exogenous administration of 5 or 10 mg/kg 3α,5α-THP in the present studies may more closely reflect values attainable in the brain under some circumstances.

The strain differences in sensitivity to the anticonvulsant effect of 3α,5α-THP were similar in the two experiments, in which animals were injected 15 min (experiment 1) *vs.* 50 min (experiment 2) before infusion of PTZ. Although there was a slight decrease in the protection provided by 3α,5α-THP in the animals tested at 50 min after injection, the dose-dependent increase in PTZ threshold was still evident in B6 mice and lacking in D2 mice. This time-dependent decrease in sensitivity to the anticonvulsant effect of the same dose of 3α,5α-THP in experiment 2 *vs.* experiment 1 is exemplified by the differences between the two experiments in plasma 3α,5α-THP concentrations after the same dose of 3α,5α-THP (*e.g.*, 10 mg/kg). These results also are consistent with recent work, which determined that protection by 3α,5α-THP against PTZ-induced convulsions peaked at 15 min after injection of 3α,5α-THP, had decreased to approximately 60% by 60 min after injection and was no longer apparent at 180 min after injection (Kokate *et al.*, 1994). More importantly, there was no difference in the PTZ thresholds of vehicle-treated animals tested at 15 or 50 min after injection. This emphasizes that the series of behavioral tests did not alter basal seizure susceptibility to PTZ.

The strain difference in sensitivity to the anticonvulsant effect of 3α,5α-THP was not generalized across all seizure measures. B6 mice were more sensitive than D2 mice to the 3α,5α-THP-induced increase in the threshold dose of PTZ for onset to MC twitch and FF clonus. The two strains had similar increases in the threshold dose for onset to PTZ-

induced RB clonus and THE after administration of 3α,5α-THP. These strain differences in anticonvulsant sensitivity to 3α,5α-THP, which vary with the seizure measure, may be related to the different anatomical systems that are believed to underlie the two major types of convulsions. Specifically, forebrain substrates (predominantly limbic) appear to be important in mediating MC twitch and FF clonus, whereas brainstem circuitry (*e.g.*, caudal midbrain and pontine reticular formation) appears to be important for RB clonus and THE (for review, see Gale, 1988). Because there are brain regional variations in the distribution of $GABA_A$ receptor subunit mRNAs (Vicini, 1991; Laurie *et al.*, 1992; Wisden *et al.*, 1992) and in the potency of 3α,5α-THP as a modulator of the GRC (Gee and Lan, 1991; Sapp *et al.*, 1992), it is possible that the potency of 3α,5α-THP at its site on the GRC in these two strains may differ within specific brain regions. In other words, increased sensitivity of $GABA_A$ receptors in the hippocampus, amygdala and other limbic structures to 3α,5α-THP in B6 *vs.* D2 mice would be consistent with the increased sensitivity to 3α,5α-THP-induced anxiolysis and anticonvulsant effects, as measured by the threshold dose for onset to MC twitch and FF clonus, in B6 *vs.* D2 mice. Likewise, the similar increase in threshold dose for onset to RB clonus and THE in B6 and D2 mice suggests that the sensitivities of $GABA_A$ receptors to 3α,5α-THP in brainstem circuitry are similar in these animals.

It is noteworthy that administration of the 10 mg/kg dose of 3α,5α-THP to B6 mice was anxiolytic and anticonvulsant and stimulated activity but did not produce muscle relaxation or ataxia. This result is consistent with recent findings in both genetically heterogeneous and inbred mouse strains (Weiland *et al.*, 1995) and suggests that the pharmacological effects of 3α,5α-THP can be dissociated in some genotypes. However, in the present studies D2 animals were sensitive to the anxiolytic and anticonvulsant effects of 3α,5α-THP only at doses that also produced muscle relaxation and ataxia.

Overall, the present results suggest that there are genetic differences in sensitivity to 3α,5α-THP, which vary, depending on the pharmacological effect. Coupled with recent results obtained in the selectively bred Withdrawal Seizure-Prone and -Resistant mice, indicating that chronic ethanol treatment significantly decreased endogenous 3α,5α-THP levels only in Withdrawl Seizure-Prone mice (Finn *et al.*, 1994), these results are consistent with the hypothesis that genetic differences in neuroactive steroid sensitivity and biosynthesis may contribute to ethanol withdrawal severity. In addition, the differences in sensitivity to 3α,5α-THP found in B6 and D2 mice can be further evaluated, because these animals are the progenitor strains from which 26 recombinant inbred strains have been derived by F2 crosses (*i.e.*, BXD Recombinant Inbred strains). Therefore, future studies can use this genetic animal model to determine genetic correlations between measures of 3α,5α-THP sensitivity and ethanol-related traits, including withdrawal severity.

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