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Increased behavioral neurosteroid sensitivity in a rat line selectively bred for high alcohol sensitivity

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Abstract

Acute administration of a neurosteroid 5β-pregnan-3α-ol-20-one induced a greater impairment in motor performance of the selectively bred alcohol-sensitive (ANT) than alcohol-insensitive (AT) rats. This difference was not associated with the sensitivity of γ-aminobutyrate type A ($GABA_A$) receptors, as 5α-pregnan-3α-ol-20-one (allopregnanolone) decreased the autoradiographic signals of *t*-butylbicyclophosphoro[^{35}S]thionate binding to $GABA_A$ receptor-associated ionophores more in the brain sections of AT than ANT rats. Nor was the difference associated with baseline levels of neuroactive progesterone metabolites, as 5α-pregnan-3,20-dione (5α-DHP) and 5α-pregnan-3α-ol-20-one were lower in the ANT rats. After ethanol (2 g/kg, i.p.) administration and the subsequent motor performance test, the increased brain concentrations of these metabolites were still lower in the ANT than AT rats, although especially in the cerebellum the relative increases were greater in the ANT than AT rats. The present data suggest that the mechanisms mediating neurosteroid-induced motor impairment are susceptible to genetic variation in rat lines selected for differences in ethanol intoxication.

Keywords: Selected rat line; $GABA_A$ receptor; Progesterone; Pregnanolone; Motor impairment; Allopregnanolone

1. Introduction

Endogenous progesterone metabolites have marked actions on behavior, being especially interesting as potential endogenous positive allosteric modulators at the central nervous inhibitory synapses. These metabolites, termed here as neurosteroids, are formed locally in the brain, independent of their peripheral concentrations (Baulieu and Robel, 1990; Cheney et al., 1995; Paul and Purdy, 1992). 5α-Reductase transforms progesterone to 5α-pregnan-3,20-dione (5α-DHP), which can act on gene transcription via progesterone receptors of brain cells (Rupprecht et al., 1993). 5α-DHP is reduced by 3α-hydroxysteroid oxidoreductase to 5α-pregnan-3α-ol-20-one (allopregnanolone). Allopregnanolone is a potent and efficient agonist at γ-aminobutyric acid type A ($GABA_A$) receptors, being an anesthetic on its own (Mok et al., 1991) and apparently responsible for anxiolytic actions of progesterone in rats (Bitran et al., 1993; Lancel et al., 1996).

Previous work has implicated a role of neurosteroids in alcohol behaviors: first, acute alcohol actions, such as anxiolysis, ataxia, hypnosis and anticonvulsant action, are mimicked by neurosteroids (Finn et al., 1997). Ethanol-induced anticonvulsant action and inhibition of spontaneous neuronal activity of septal neurons are blocked by finasteride, a drug that inhibits the formation of 5-α reduced neurosteroids (VanDoren et al., 2000). These data suggest

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that endogenous neurosteroids mediate the actions of alcohol, which is in keeping with the fact that alcohol administration increases the concentration of neurosteroids in the brain (Barbaccia et al., 1999). Second, plasma levels of neurosteroids are markedly reduced in alcoholic patients during the early withdrawal phase, when anxiety and depression scores are higher (Romeo et al., 1996). Alcohol withdrawal symptoms in rats and mice are alleviated by neurosteroids (Devaud et al., 1995; Finn et al., 1995). Third, chronic alcohol administration enhances the anticonvulsant efficacy of neurosteroids (Devaud et al., 1996), indicating a unique mechanism of action and lack of cross-tolerance with alcohol. The actions of the neurosteroid allopregnanolone have been suggested to be mediated by the brain $GABA_A$ receptors, which are activated at a site not shared by any other known $GABA_A$ receptor ligand. Although neurosteroids have been considered as endogenous barbiturates, their binding sites on the $GABA_A$ receptor are not identical with those of barbiturates (Lambert et al., 1995; Peters et al., 1988; Puia et al., 1990; Turner et al., 1989).

The present study was aimed at investigating the role of neurosteroids in alcohol sensitivity difference between alcohol-sensitive ANT (alcohol non-tolerant) and alcohol-insensitive AT (alcohol tolerant) rat lines. These rat lines have been produced by selective breeding for high and low motor impairment, respectively, by a moderate dose of ethanol (2 g/kg, i.p.) (see Korpi, 1994). The ANT rats possess a pharmacologically significant point mutation in a cerebellar granule cell-specific $GABA_A$ receptor subunit (Korpi et al., 1993). The receptors containing this subunit ($\alpha 6$) are normally insensitive to benzodiazepine agonists (Lüddens et al., 1990), but in the ANT rats are abnormally sensitive to benzodiazepines due to a glutamine residue replacing the wild-type arginine at the position 100 in the putative extracellular part of the subunit (Korpi et al., 1993). The ANT rats are thus highly sensitive to incoordinating actions of benzodiazepine agonists (Hellevuo et al., 1989; Wong et al., 1996). However, they also exhibit enhanced sensitivity to barbiturates (Hellevuo et al., 1989), but no pronounced abnormality in barbiturate actions on their $GABA_A$ receptors has been observed. The EC_{50} concentration of barbital for stimulation of GABA agonist [^{3}H]muscimol binding to brain membranes tends to be higher in the ANT than AT rats (Uusi-Oukari and Korpi, 1992). It is thus plausible that other $GABA_A$ receptor-related or unrelated alterations are present in the ANT rats.

Since the neurosteroid agonists have their unique mechanisms of action at the $GABA_A$ receptors, we have now compared the ANT and AT rats in their behavioral (motor performance) and neurochemical ($GABA_A$ receptor assay) sensitivities to neurosteroid agonists, and attempted to correlate the endogenous levels of allopregnanolone, 5α-DHP and progesterone with the innate sensitivity differences in conditions with or without the behavioral test used in the selection of these rat lines.

2. Materials and methods

2.1. Animals

Adult (4–5 months old) male ANT and AT rats (Eriksson and Rusi, 1981) of the generations F_{48} and F_{50} were bred and maintained in groups of four to six under a 12/12-h light/dark cycle (lights on at 06:00 a.m.) at an ambient temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. The animals had free access to tap water and RM1 (E) SQC pellet food (SDS Ltd., Witham, Essex, England). All animals were naive to drugs and other experimental procedure before experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committees.

2.2. Tilting plane test

To measure the effects of a neurosteroid agonist on motor performance, the tilting plane test was used (Hellevuo et al., 1989). The mean body weights ($\pm$S.E.M.) were 391 ± 7 g ($n = 18$) and 374 ± 5 g ($n = 18$) for the AT and ANT rats, respectively. In the tilting plane test, the animals are placed on a wire-cloth covered plane which is tilted at a constant speed from horizontal to vertical, which forces the animals to adjust their posture in order to retain their position on the plane. The tilting of the plane is stopped by the rat sliding against the lower edge of the plane, and the sliding angle is then recorded. Each rat was first given a three-trial pre-drug test, and thereafter, they were injected with vehicle [1 ml/kg, 20% Chremophor, Sigma, St. Louis, MO, in 0.25% (w/v) sodium chloride] or 5β-pregnan-3α-ol-20-one (pregnanolone, 0.5, 0.75, 1.0 or 1.5 mg/kg; Sigma) as a bolus injection into a tail vein in a volume of 1 ml/kg, followed with a flush with 0.5 ml of the vehicle solution. The change in the mean sliding angles (pre-drug–drug) at 5, 10 and 20 min after the injection was used as a measure of motor impairment.

2.3. Neurosteroid determinations after ethanol administration and tilting plane test

Tissues of AT and ANT rats in unstressed control conditions and after ethanol administration were collected for the analysis of progesterone, dihydroprogesterone and allopregnanolone to compare the rat lines and their neurosteroid responses. Control animals were sacrificed immediately after transfer from their home cages into the experimental room. Only one or two rats per cage were used for this purpose to avoid any general stress-related alterations. Other rats received the standard dose of ethanol (2 g/kg, i.p.), and 30 min later, they were subjected to three-trial tilting plane tests in order to use exactly the same behavioural manipulation as in the selection of the AT and ANT rat lines (the whole procedure comprising the "selec-

tion test"). Then the animals were immediately decapitated, trunk blood collected into heparinized test tubes for separation of plasma. Adrenal glands and several brain regions (cerebral cortex, hippocampus, cerebellum, olfactory bulb, striatum) were dissected out and frozen on dry ice.

Plasma ethanol concentrations determined with headspace gas chromatography (Eriksson et al., 1977) were similar ($P = 0.32$) in the above ANT and AT animals [48.9 ± 1.1 and 50.6 ± 1.2 mM (mean $\pm$ S.E.M., $n = 12$) for the ANT and AT rats, respectively], but the ANT rats slid down at lower angles ($P < 0.0001$) than the AT rats in the tilting plane test (45.5 ± 1.1 vs. $60.4 \pm 1.8°$, respectively), indicating enhanced motor impairment by ethanol in the ANT rats.

Determination of neurosteroids was performed using a combined high-performance liquid chromatography (HPLC) gas chromatography/mass spectrometry analysis as described elsewhere (Cheney et al., 1995; Romeo et al., 1996). Briefly, after extraction with ethyl acetate, separation by HPLC, the eluate containing allopregnanolone or progesterone was lyophilized and derivatized with heptafluorobutyric acid anhydride. 5α-DHP was derivatized with methoxyamine HCl (2%) in pyridine. The steroids were assayed in electron impact mode with the ions at m/z 510, 496 and 343 being selectively monitored. The detection limit of the assay for these steroids was 10 fmol, and their recoveries after extraction and purification were about 85%. The final concentrations were not corrected for recoveries.

Plasma corticosterone levels were quantified with a ^{125}I-corticosterone radioimmunoassay kit for rats (ICN Biomedicals, Costa Mesa, CA).

2.4. Ligand autoradiography for neurosteroid sensitivity of $GABA_A$ receptors

For ligand autoradiography, the animals were sacrificed and whole brains carefully dissected out and frozen on dry ice. Horizontal serial (14 μm) sections were cut from both rat lines using a Leitz 1720 cryostat, thaw-mounted onto gelatin-coated object glasses, and stored frozen under desiccant at −20°C. The autoradiographic procedures for regional localization the $GABA_A$ receptor convulsant sites labelled by *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) (DuPont de Nemours, NEN Division, Dreieich, Germany) were carried out as described in detail (Korpi et al., 1995). To remove the endogenous GABA, which could interfere with determination of cerebellar α6 subunit pharmacology (Korpi and Lüddens, 1993), the sections were preincubated in an ice-water bath for 3×10 min in 50 mM Tris–HCl supplemented with 1 mM Na_2–EDTA (pH 7.4). The final incubation in 50 mM Tris–HCl supplemented with 120 mM NaCl was performed with 6 nM [^{35}S]TBPS at room temperature (22°C) for 90 min, by using 750-μl liquid bubbles over sections on object glasses in a humid chamber. Effects of 0.1–3 μM 5α-pregnan-3α-ol-20-one (allopregnanolone, Research Biochemicals, Natick, MA, USA) in the presence and absence of 0.5 or 3 μM GABA (Sigma) were tested on [^{35}S]TBPS binding. Non-specific binding was determined in the presence of 20 μM picrotoxinin (Sigma). After the incubation, sections were washed 3×15 s in an ice-cold incubation buffer. Sections were then dipped into distilled water, air-dried under a fan at room temperature, and exposed with plastic [^{14}C] standards to Hyperfilm-β_{max} (Amersham), respectively, for 3–5 days.

Images on autoradiography films were quantitated using MCID M4 image analysis devices and programs (Imaging Research, St. Catharines, Canada) (Korpi et al., 1995). The

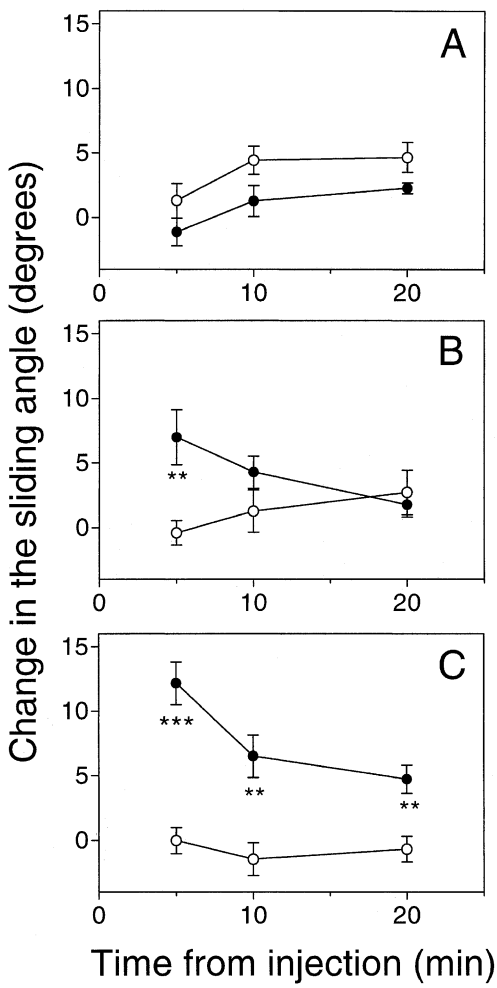

Fig. 1. Motor impairment of ANT (●) and AT (○) rats in the tilting plane test induced by acute intravenous injection of pregnanolone. After determining the pre-test sliding angles, the animals were injected with pregnanolone (0.75 mg/kg, panel (B); 1.0 mg/kg, panel (C)) or with vehicle solution [20% (w/v) Chremophor, 1 ml/kg, panel (A)], and tested for their tilting plane test performance 5, 10 and 20 min later. Drug-naive animals were used in each group ($n = 6$). The data points are mean ± S.E.M. values. Statistical significance of the difference from the corresponding AT value (ANOVA followed by Student's *t*-test): $^{**}P < 0.01$, $^{***}P < 0.001$.

standards exposed simultaneously with brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray matter areas (nCi/g).

2.5. Statistical procedures

Results are analysed using two-way analysis of variance (ANOVA) with the Prism 2.0 program (GraphPad Software, San Diego, CA, USA), followed by Student's *t*-test comparisons of the individual differences, when ANOVA was significant, or with Student's *t*-test if only two groups were compared.

3. Results

3.1. Pregnanolone-induced motor impairment

Acute intravenous injection of pregnanolone (5β-pregnan-3α-ol-20-one), which is equipotent to allopregnanolone as positive modulator at $GABA_A$ receptors (Carboni et al., 1996; Lambert et al., 1995), produced dose-dependent impairment in the tilting plane test performance. The usable dose range was very tight, as 0.5 mg/kg induced no impairment in either rat lines and 2.0 mg/kg caused loss of righting reflex shortly after injection in both lines (data not shown). Fig. 1 illustrates that at the doses of 0.75 and 1.0 mg/kg the ANT animals were significantly more impaired than the AT animals. The action of pregnanolone was short-lasting, the maximal impairment being already measured at 5 min. ANOVA revealed significant rat line effects [$F(1,30) = 6.56$, $P < 0.02$] and [$F(1,30) = 63.82$, $P < 0.0001$] for the 0.75 and 1.0 mg/kg doses, respectively.

3.2. Responses of neurosteroid concentrations to selection test

Since the ANT rats were more sensitive to a neurosteroid agonist (Fig. 1), we wanted to extend the study into the endogenous neurosteroid agonist, allopregnanolone, and its immediate precursors, progesterone and 5α-DHP, by comparing their concentrations in various brain regions, adrenal glands and blood plasma between the naive ANT and AT rats, either unstressed, or stressed by the selection test (ethanol administration followed by tilting plane test). We found that the neurosteroids are clearly lower in the ANT rats in most tissues in both conditions (Tables 1–3). The baseline plasma corticosterone levels were 23.5 ± 5.9 and 15.3 ± 3.1 ng/ml (mean ± S.E.M., $n = 12$) for the AT and ANT rats, respectively. The selection test procedures elevated the corticosterone level to a higher level in the AT than ANT rats (619 ± 20 vs. 491 ± 17 ng/ml, respectively, $P < 0.001$). These corticosterone results confirm our previous findings of lower corticosterone levels in the ANT than AT rats after a number of manipulations (Tuominen and Korpi, 1991).

Progesterone concentrations were similar in the brain but differed between the ANT and AT rat lines in the adrenal glands, the ANTs having lower levels (Table 1). The selection test failed to alter progesterone levels in the brain, but slightly increased them in the adrenals and plasma.

In contrast, 5α-DHP and allopregnanolone concentrations clearly differed between the rat lines in most tissues studied (Tables 2 and 3). In the non-stressed animals, the levels of both compounds were about 40% lower in the ANT than in the AT rats. After the selection test, 5α-DHP and allopregnanolone levels were increased in both rat lines. There were clear brain regional differences in the

Table 1
Effects of the selection test on progesterone concentrations in brain and peripheral tissues of AT and ANT rats
Progesterone concentrations were determined in samples derived from rats sacrificed immediately after the selection test used in the generation of the ANT and AT rat lines [acute administration of ethanol (EtOH, 2 g/kg, i.p.) followed by a tilting plane test 30 min later]. Control rats were sacrificed without any pretreatment. Values (expressed in pmol/g tissue or ml plasma) are means of four animals per group with S.E.M. values. Two-way ANOVA results are for rat line (R) and treatment (T) effects, no significant rat line × treatment interactions. ns: Non-significant.

Tissue	AT rats		ANT rats		ANOVA results
	Control	Selection test	Control	Selection test	
Cerebral cortex	27 ± 4	27 ± 4	32 ± 4	33 ± 4	ns
Hippocampus	35 ± 5	37 ± 4	33 ± 3	38 ± 5	ns
Cerebellum	32 ± 4	33 ± 3	34 ± 5	29 ± 3	ns
Olfactory bulb	93 ± 11	94 ± 8	93 ± 7	88 ± 6	ns
Striatum	48 ± 6	51 ± 5	51 ± 3	55 ± 4	ns
Adrenal glands	3.2 ± 0.3	3.8 ± 0.4	2.0 ± 0.1[a]	2.7 ± 0.2[b]	R[c],T[b]
Blood plasma	2.8 ± 0.3	3.4 ± 0.1	3.1 ± 0.2	3.7 ± 0.3	T[b]

[a] Significance of the difference from the corresponding AT value (Student's *t*-test): $P < 0.05$.
[b] Significance of the difference from the corresponding control value (Student's *t*-test): $P < 0.05$.
[c] Significance of the difference from the corresponding control value (Student's *t*-test): $P < 0.01$.

Table 2
Effects of the selection test on 5α-pregnan-3,20-dione (5α-DHP) concentrations in brain and peripheral tissues of AT and ANT rats
5α-Pregnan-3,20-dione concentrations were determined in samples derived from rats sacrificed immediately after the selection test used in the generation of the ANT and AT rat lines [acute administration of ethanol (EtOH, 2 g/kg, i.p.) followed by a tilting plane test 30 min later]. Control rats were sacrificed without any pretreatment. Values (expressed in pmol/g tissue or ml plasma) are means of four animals per group with S.E.M. values. Two-way ANOVA results are for rat line effect (R), treatment effect (T) and for rat line × treatment interaction ($R \times T$).

Tissue	AT rats		ANT rats		ANOVA results
	Control	Selection test	Control	Selection test	
Cerebral cortex	2.3 ± 0.3	4.4 ± 0.4^{a}	1.2 ± 0.1^{b}	3.6 ± 0.2^{c}	R^{a}, T^{c}
Hippocampus	3.1 ± 0.6	4.4 ± 0.2	1.8 ± 0.1	3.5 ± 0.3^{a}	R^{d}, T^{a}
Cerebellum	2.7 ± 0.4	4.0 ± 0.2^{d}	1.5 ± 0.1^{b}	$3.1 \pm 0.2^{b,c}$	R^{c}, T^{c}
Olfactory bulb	7.1 ± 0.3	32.0 ± 2.0^{c}	4.6 ± 0.4^{e}	$17.5 \pm 1.3^{c,f}$	$R^{c}, T^{c}, R \times T^{c}$
Striatum	4.3 ± 0.5	6.4 ± 0.4^{d}	3.3 ± 0.2	$4.4 \pm 0.2^{a,e}$	R^{a}, T^{c}
Adrenal glands	0.40 ± 0.04	0.85 ± 0.10^{a}	0.28 ± 0.03^{b}	$0.50 \pm 0.04^{a,b}$	R^{a}, T^{c}
Blood plasma	0.60 ± 0.07	1.38 ± 0.10^{c}	0.40 ± 0.04^{b}	$0.88 \pm 0.06^{c,e}$	R^{c}, T^{c}

[a] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.01$.
[b] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.05$.
[c] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.001$.
[d] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.05$.
[e] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.01$.
[f] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.001$.

response of these compounds, the response in olfactory bulbs being the greatest and those in the hippocampus, cerebellum and striatum the lowest. The brain regional concentration profiles of 5α-DHP and allopregnanolone agree with the findings of Cheney et al. (1995) on non-selected rodents. ANOVA indicated significant rat line × treatment interactions for 5α-DHP in the olfactory bulb and for allopregnanolone in the olfactory bulb, adrenals and plasma. In all these cases, the neurosteroid concentrations still remained lower in the ANT rats. On the other hand, the relative increases from the control level tended to be greater in various brain regions of the ANT than AT rats, especially for allopregnanolone in the cerebellum [$F(1,12) = 3.96,\ P = 0.07$].

3.3. Brain regional $GABA_A$ receptor sensitivity to allopregnanolone in vitro

We used [^{35}S]TBPS autoradiography with brain sections to get an idea of the functional sensitivity of the native $GABA_A$ receptors in various brain regions to allopregnanolone, as a decrease in binding in the presence of

Table 3
Effects of the selection test on allopregnanolone concentrations in brain and peripheral tissues of AT and ANT rats
Allopregnanolone concentrations were determined in samples derived from rats sacrificed immediately after the selection test used in the generation of the ANT and AT rat lines [acute administration of ethanol (EtOH, 2 g/kg, i.p.) followed by a tilting plane test 30 min later]. Control rats were sacrificed without any pretreatment. Values (expressed in pmol/g tissue or ml plasma) are means of four animals per group with S.E.M. values. Two-way ANOVA results are for rat line effect (R), treatment effect (T) and for rat line × treatment interaction ($R \times T$).

Tissue	AT rats		ANT rats		ANOVA results
	Control	Selection test	Control	Selection test	
Cerebral cortex	2.6 ± 0.3	5.3 ± 0.4^{a}	1.3 ± 0.2^{b}	4.1 ± 0.6^{a}	R^{b}, T^{c}
Hippocampus	3.3 ± 0.2	4.6 ± 0.3^{b}	1.6 ± 0.1^{d}	$3.4 \pm 0.3^{a,e}$	R^{c}, T^{c}
Cerebellum	2.9 ± 0.3	4.7 ± 0.3^{a}	1.8 ± 0.1^{f}	4.6 ± 0.2^{c}	R^{b}, T^{c}
Olfactory bulb	7.8 ± 0.5	45.5 ± 5.4^{c}	5.4 ± 0.4^{f}	$14.3 \pm 1.9^{a,f}$	$R^{c}, T^{c}, R \times T^{c}$
Striatum	4.3 ± 0.3	6.1 ± 0.6^{b}	2.1 ± 0.1^{d}	$4.5 \pm 0.2^{c,e}$	R^{c}, T^{c}
Adrenal glands	0.18 ± 0.03	0.45 ± 0.03^{c}	0.10 ± 0.01^{e}	$0.23 \pm 0.03^{a,f}$	$R^{c}, T^{c}, R \times T^{c}$
Blood plasma	0.18 ± 0.03	0.50 ± 0.04^{c}	0.13 ± 0.03	0.18 ± 0.03^{d}	$R^{c}, T^{c}, R \times T^{c}$

[a] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.01$.
[b] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.05$.
[c] Significance of the difference from the corresponding control value (Student's t-test): $P < 0.001$.
[d] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.001$.
[e] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.05$.
[f] Significance of the difference from the corresponding AT value (Student's t-test): $P < 0.01$.

Table 4
Effects of allopregnanolone (Allo) on [^{35}S]TBPS binding in the presence of exogenous GABA in various brain regions of AT and ANT rats as revealed by quantitative autoradiography
Basal [^{35}S]TBPS binding values at 6 nM are given as nCi/g (mean ± S.D., $n = 3$), and the values in the presence of GABA (0.5 or 3 μM) are expressed as % of the basal binding. Values for GABA (0.5 μM or 3 μM) + allopregnanolone (0.1, 1 or 3 μM) are expressed as % of the corresponding binding in the presence of GABA. In the presence of 3 μM GABA, 3 μM allopregnanolone reduced binding to the background level (not shown). The concentrations stated in the columns for GABA and allopregnanolone (Allo) are in μM.

Brain region	Basal	GABA (0.5)	+ Allo (0.1)	+ Allo (1)	+ Allo (3)	GABA (3)	+ Allo (0.1)	+ Allo (1)
Frontoparietal cortex								
AT	242 ± 7	124 ± 4	92 ± 1	35 ± 8	14 ± 1	57 ± 6	58 ± 7	17 ± 5
ANT	216 ± 38	144 ± 14	86 ± 6	32 ± 4	16 ± 2	65 ± 12	78 ± 7[a]	20 ± 5
Hippocampus								
AT	203 ± 10	100 ± 7	93 ± 7	42 ± 9	20 ± 3	48 ± 1	64 ± 2	26 ± 9
ANT	209 ± 37	110 ± 17	86 ± 1	40 ± 4	21 ± 1	52 ± 9	80 ± 4[b]	26 ± 8
Caudate / putamen								
AT	137 ± 7	108 ± 12	96 ± 4	52 ± 12	25 ± 3	67 ± 2	69 ± 6	28 ± 9
ANT	123 ± 21	127 ± 9	92 ± 3	52 ± 5	30 ± 1	77 ± 9	93 ± 13[a]	33 ± 6
Thalamus								
AT	175 ± 11	107 ± 8	98 ± 7	53 ± 8	24 ± 1	68 ± 4	70 ± 4	25 ± 8
ANT	155 ± 31	123 ± 13	90 ± 3	51 ± 7	26 ± 1	74 ± 11	87 ± 4[b]	29 ± 7
Inferior colliculus								
AT	272 ± 19	121 ± 1	96 ± 9	36 ± 7	13 ± 1	57 ± 5	68 ± 3	17 ± 8
ANT	239 ± 42	128 ± 10	98 ± 9	42 ± 12	17 ± 4	70 ± 6[a]	76 ± 12	19 ± 5
Superior colliculus								
AT	182 ± 18	133 ± 17	100 ± 4	54 ± 14	20 ± 4	86 ± 11	72 ± 8	19 ± 8
ANT	169 ± 21	164 ± 39	90 ± 13	46 ± 5	21 ± 1	95 ± 12	80 ± 9	22 ± 6
Cerebellar granule cell layer								
AT	266 ± 25	57 ± 4	82 ± 2	32 ± 4	16 ± 4	31 ± 1	57 ± 6	25 ± 11
ANT	235 ± 9	72 ± 3[b]	76 ± 3[a]	28 ± 2	19 ± 1	32 ± 1	76 ± 7[a]	30 ± 2
Cerebellar molecular layer								
AT	193 ± 20	76 ± 12	107 ± 6	26 ± 1	13 ± 2	27 ± 1	61 ± 10	32 ± 17
ANT	186 ± 24	91 ± 6	86 ± 12	23 ± 9	15 ± 3	27 ± 4	79 ± 20	36 ± 3

[a] Statistical significance of the difference between rat lines (Student's t-test): $P < 0.05$.
[b] Statistical significance of the difference between rat lines (Student's t-test): $P < 0.01$.

GABA correlates with agonistic activity of the allosteric modulators including neurosteroid agonists (Hawkinson et al., 1994; Sapp et al., 1992). The basal binding was not significantly different in any brain region between the AT and ANT rats (Table 4). A low GABA concentration (0.5 μM) decreased the binding slightly more in the cerebellar granule cell layer of the AT than ANT rats, but the allopregnanolone modulation was similar in both rat lines in this condition. At a higher GABA concentration (3 μM), the binding was decreased in all regions, the cerebellum being the most sensitive. Allopregnanolone, at 100-nM concentration, was more potent in decreasing the binding in the AT rats in most regions analysed, but at higher allopregnanolone concentrations this difference disappeared.

4. Discussion

The primary finding of the present study was the observation that the alcohol-sensitive ANT rats have a higher sensitivity to acute motor-impairing actions of a neurosteroid agonist, pregnanolone, than the alcohol-insensitive AT rats. This extends the list of drugs producing greater motor coordination deficits in the ANT than AT rats [ethanol, sodium barbital, diazepam, lorazepam, propofol, halothane, desflurane and dizocilpine (Firestone et al., 2000; Hellevuo et al., 1989; Toropainen et al., 1997; Wong et al., 1996; Yildirim et al., 1997)] to a new class of compounds. It is likely that the sensitivity difference to pregnanolone is due to a pharmacodynamic difference, since intravenous administration can be considered to have largely avoided pharmacokinetic differences, which was supported by similar emergence of hypnotic action (loss of righting reflex) in both ANT and AT rats already at pregnanolone doses of 2 mg/kg (data not shown). High neurosteroid sensitivity of the ANT rats was not unexpected, since they are strongly facilitatory at the $GABA_A$ receptors, as most of the drug already demonstrated to produce a greater motor impairment in the ANT than AT rats (see above). Thus, collectively, the pharmacological

results on the ANT and AT rat lines strongly support the role of $GABA_A$ receptor-mediated inhibition as one of the genetic factors explaining the behavioural sensitivity difference caused between the AT and ANT rat lines obtained by selective breeding for high and low motor impairment by ethanol.

Because the neurosteroid agonists are also present endogenously, we were able to search for a correlation between the endogenous neurosteroid levels and the behavioral responses to ethanol. We found significantly lower levels of allopregnanolone and 5α-DHP in many brain regions of the ANT than AT rats at control, non-handled condition. The brains of the ANT rats are adapted to lower neurosteroid levels, which might enhance their behavioural sensitivity to increases of both endogenous and exogenous neurosteroids. However, the concentrations of endogenous neurosteroids clearly fail to explain the differential action of ethanol on motor performance in these rats, even if the tendency for greater relative increases of cerebellar neurosteroid levels after the selection test in the ANT rats might make it possible to induce a stronger acute facilitation of the $GABA_A$ receptors in the ANT than AT rats in the presence of ethanol (Criswell et al., 1999).

The lower concentrations of 5α-DHP and allopregnanolone, with practically unaltered progesterone levels, in the ANT rats suggest that these animals might have a down-regulation of the synthesizing enzymes, e.g. 5α-reductase, and/or an up-regulation of allopregnanolone catabolism. The increased levels of the progesterone metabolites, again with unaltered progesterone levels, after the selection test procedure, suggest that progesterone synthesis is accelerated to balance its enhanced metabolism.

The ANT rats have a unique point mutation in a pharmacologically critical region of the cerebellar granule cell-specific $GABA_A$ receptor α6 subunit (Korpi and Seeburg, 1993; Korpi et al., 1993), which makes the normally benzodiazepine-agonist insensitive $GABA_A$ receptor sensitive to this class of compounds, such as lorazepam and diazepam in the ANT rats (Hellevuo et al., 1989; Wong et al., 1996). This difference can be clearly visualized by autoradiography (Korpi et al., 1993), which technique we now applied to see whether the $GABA_A$ receptors would be directly differentially sensitive to allopregnanolone. However, allopregnanolone was slightly more effective in the brain sections of the AT than the ANT rats to enhance the GABA inhibition of convulsant binding, a measure of agonistic effect at the $GABA_A$ receptors (Hawkinson et al., 1994). A similar situation has emerged from experiments with propofol, which is slightly more potent on the AT than ANT receptors in vitro, but produces stronger motor impairment in the ANT rats in vivo (Yildirim et al., 1997). Therefore, it is unlikely that the high behavioral sensitivity of the ANT rats to pregnanolone would be a direct consequence of high sensitivity of their $GABA_A$ receptors or that the point mutation of the α6 subunits in the ANT rats would be responsible for the enhanced behavioral sensitivity of these rats to neurosteroid agonists. It has been demonstrated that chronic neurosteroid agonist administration leads to down-regulation of $GABA_A$ receptor function (Friedman et al., 1993; Yu and Ticku, 1995), perhaps due to selective alterations in receptor subunit expression, which might then be different between the ANT and AT rats. This possibility seems unlikely, since the concentrations of allopregnanolone needed to modulate $GABA_A$ receptor function in vitro were much greater (100 nM) than their endogenous levels (low nmol concentrations, Tables 2 and 3).

Our conclusion that neurosteroid action on the $GABA_A$ receptors does not explain the differential motor impairment between the rat lines is at variance with the data indicating generally that the potency of the neurosteroids at $GABA_A$ receptors shows a positive correlation with their behavioral potencies (Kokate et al., 1994). It should be kept in mind that neurosteroids may facilitate $GABA_A$ receptor function via indirect actions on intracellular protein kinases (Brussaard et al., 2000; Fancsik et al., 2000), which effect might not be detectable at all by the ligand binding assay used in the present study. Thus, the sensitivity differences in the indirect mechanisms cannot be excluded by the present experiments.

In conclusion, we have demonstrated that alcohol-sensitive ANT rats show high sensitivity to motor-impairing action of a neurosteroid agonist. However, no direct evidence was obtained for enhanced direct sensitivity of the brain $GABA_A$ receptors in the ANT rats to neurosteroid agonists, nor for the role of baseline and selection test-induced increases in the endogenous levels of these potent compounds. The data indicate that mechanisms important for neurosteroid sensitivity may be at least partly similar to those involved in genetically determined alcohol sensitivity.

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