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Differential Modulation of Benzodiazepine Receptor Binding by Ethanol in LS and SS Mice[1]

LAWRENCE G. MILLER,[2] DAVID J. GREENBLATT, JAMIE G BARNHILL AND RICHARD I. SHADER

Division of Clinical Pharmacology, Departments of Psychiatry and Medicine Tufts University School of Medicine and New England Medical Center, Boston, MA

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MILLER, L G , D J GREENBLATT, J G BARNHILL AND R I SHADER *Differential modulation of benzodiazepine receptor binding by ethanol in LS and SS mice* PHARMACOL BIOCHEM BEHAV 29(3) 471–477, 1988 — The LS and SS lines of mice were initially selected based on sedative responses to ethanol, but have been found to differ in response to a variety of hypnotics and anesthetics These differences do not appear to be due to pharmacokinetic factors and several lines of evidence suggest involvement of the GABAergic system To examine an important component of this system, the benzodiazepine receptor, we analyzed benzodiazepine receptor binding *in vivo* in LS and SS mice, and modulation of receptor binding by three interventions known to increase binding in other strains pentobarbital, defeat stress, and ethanol Receptor binding was determined by specific uptake of [^{3}H]-Ro15-1788 Receptor binding was increased in cortex and hippocampus of LS mice compared to SS mice, with the increase in cortex most likely due to increased receptor number rather than a change in apparent affinity Pentobarbital (30 mg/kg IP) induced similar increases in binding in both lines in several brain regions Defeat stress caused increased binding in several brain regions of both SS and LS mice, with greater binding in cortex of LS mice In contrast, ethanol at 3 doses (0 5, 1, and 2 g/kg) led to greater increases in binding in SS mice compared to LS mice in most brain regions None of the interventions altered nonspecific binding Ethanol concentrations were slightly greater in plasma and brain of LS mice These results indicate differences in benzodiazepine receptor binding in LS and SS mice, with differential modulation of binding by ethanol but not by pentobarbital or stress These differences may contribute to differential pharmacodynamic responses in the two lines of mice

LS/SS mice Ethanol Benzodiazepine receptor

THE LS and SS lines of mice were initially selected based on differential loss of the righting reflex ("sleep time") after ethanol administration [21] Subsequent studies reported a small difference in ethanol elimination between the two lines, but this was not sufficient to account for effects on sleep time [11,12] The LS line also appears to be more sensitive to hypnotic effects of paraldehyde, trichloroethanol, thiopental, pentobarbital, phenobarbital, barbital, chlordiazepoxide, nitrous oxide, isoflurane, and enflurane [6, 7, 19, 24, 33] The differential in sensitivity to a broad variety of hypnotic agents suggests that selection for the initial ethanol-responsive phenotype in fact resulted in selection for a more general process Several lines of evidence implicate the GABA system as a locus for selection (1) Most of the hypnotic agents listed above exert at least part of their effects via the GABAergic system [32,35], (2) LS and SS lines also differ in response to convulsive agents which appear to act via GABA receptors [23], and (3) LS mice are more affected by the GABA agonists THIP and baclofen than SS mice [20] Since whole brain GABA levels and GABA uptake kinetics do not appear to differ in the two lines [22], it is more likely that differences exist in postsynaptic actions of GABA

The postsynaptic $GABA_A$ receptor is a complex structure, including a GABA binding site, an allosterically-coupled benzodiazepine binding site, and a chloride ionophore [10] A recent report based on binding of [^{3}H]-flunitrazepam to brain membrane preparations *in vitro* indicated no difference in benzodiazepine receptor number or affinity between the LS and SS lines, except in midbrain. However, GABA-enhanced benzodiazepine binding was greater in cortex and cerebellum of SS mice [18] Another report described no difference in high affinity [^{3}H]-muscimol binding, but increased sensitivity of LS mice to mus-

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[2]Requests for reprints should be addressed to David J Greenblatt, M D , Division of Clinical Pharmacology, Tufts-New England Medical Center, Box 1007, 171 Harrison Avenue, Boston, MA 02111

cimol displacement of [^{35}S]-t-butylbicyclophosphorothionate (TBPS), a putative chloride channel ligand [1] In addition, chloride uptake in response to ethanol was greater in membranes from LS than from SS mice [1] These results support the presence of functional, although not structural, differences in the GABA receptor complex between the LS and SS lines

Studies of *in vitro* binding to brain membrane preparations are commonly used for benzodiazepine receptor analyses, but are potentially limited by membrane preparative techniques and temperature and buffer conditions during binding assays [29] Techniques employed to remove GABA may also substantially affect binding results [8] The recent development of methods to assess benzodiazepine receptor binding in vivo by several groups circumvents many of these limitations [9,27] Results obtained from *in vivo* studies may not parallel those obtained *in vitro*, as has been reported in studies of stress and benzodiazepine or barbiturate administration [5, 27, 28, 30] We have used *in vivo* binding techniques to reassess benzodiazepine receptor binding in LS and SS lines, including effects of three interventions reported to modulate benzodiazepine receptor binding barbiturates, ethanol, and stress

METHOD

Materials

Male LS and SS mice were obtained from the Institute for Behavioral Genetics, University of Colorado, Boulder, CO Experiments were performed when mice were 6 to 8 weeks old CFW mice were obtained from Charles River Laboratories (Wilmington, MA) Mice were housed under a 12-hour light-dark cycle and fed water and laboratory chow ad lib [^{3}H]-Ro15-1788 (Spec Act 82 8 Ci/mmol) was obtained from New England Nuclear (Boston, MA) Clonazepam, desmethyl-flunitrazepam, methylclonazepam, and Ro15-1788 were kindly provided by Hoffmann-La Roche, Inc (Nutley, NJ) All other reagents were obtained from standard commercial sources

Method

LS and SS mice were compared for the following parameters

(1) Rotarod ataxia five mice of each line received clonazepam (0 02–2 mg/kg) one hour prior to testing,

(2) Clonazepam concentrations in cortex three mice of each line received clonazepam (0 2–2 mg/kg) one hour prior to sacrifice,

(3) Benzodiazepine receptor binding seven mice of each strain were evaluated for benzodiazepine binding *in vivo*,

(4) Pentobarbital three mice of each line received pentobarbital (30 mg/kg) one hour prior to determination of binding,

(5) Stress three mice of each line underwent defeat stress immediately prior to binding determination,

(6) Ethanol three mice of each line received 0 5 g/kg and six mice of each line received 1 and 2 g/kg received ethanol one hour prior to binding determination

Drug administration Benzodiazepines and pentobarbital were dissolved in propylene glycol or polyethylene glycol 400 and diluted to the appropriate concentration with saline Drugs were administered IP in a volume of 0 15 ml Clonazepam was administered in doses ranging from 0 02 to 2 mg/kg Ro15-1788 was administered at a dose of 6 mg/kg Ethanol was diluted to a 20% (v/v) solution with water and the appropriate amounts administered IP

Rotarod ataxia Rotarod ataxia was performed according to the method of Kalir *et al* [13] Mice were injected with varying doses of clonazepam IP (0 02–2 mg/kg) and rotarod performance was evaluated one hour after dosage Results are expressed as seconds off the rotarod from a 2 minute period

Benzodiazepine and ethanol concentrations in plasma and brain At the appropriate time point, mice were sacrificed by cervical dislocation and decapitation, and trunk blood was collected into heparinized tubes Plasma was separated by centrifugation and stored at −20°C until analysis Brains were rapidly dissected on ice and cortices were removed In some experiments, cortices were divided into approximately equal parts and one half was used for receptor binding (see below) Cortical tissue was weighed and homogenized in 1 ml 0 025 M borate buffer (pH 8 3) with a Polytron (Brinkmann, Lucerne, setting 7, 15 seconds) Clonazepam was determined by gas-liquid chromatography by the method of Lister *et al* [15] Ethanol concentrations in plasma or brain homogenate were determined by enzymatic methods [16]

Benzodiazepine receptor binding Benzodiazepine receptor binding was determined by the method of Goeders and Kuhar [9] as modified by Miller *et al* [27] Briefly, after appropriate pre-treatment, mice were injected via the tail vein with 3 μCi [^{3}H]-Ro15-1788 After 20 minutes, mice were sacrificed and brains rapidly removed and dissected on ice Tissue was weighed and placed in vials containing 2 ml Protosol for 24 hours at 40°C Scintillation fluid (10 ml) was added and vials were allowed to stand at room temperature for 24 hours prior to counting by conventional scintillation spectrometry To determine nonspecific binding, mice were pre-treated with a saturating dose of clonazepam (5 mg/kg IP) 30 minutes prior to radioligand injection, and tissue was processed as above Results are expressed as specific binding total binding minus nonspecific binding Specific binding in cortex was greater than 80% regardless of pretreatment Administration of vehicle did not alter receptor binding in any brain region evaluated In some experiments, cortices were divided and segments were used for receptor binding and clonazepam or ethanol determination In clonazepam experiments, results were expressed as receptor occupancy in percent

$$\left[1 - \left(\frac{\text{Clonazepam binding/g} - \text{Nonspecific binding/g}}{\text{Total binding/g} - \text{Nonspecific binding/g}}\right)\right] \times 100$$

These data in combination with clonazepam concentrations were fitted to the modified Hill equation $y = x^{\gamma}/(B + x^{\gamma})$ yielding a sigmoidal function from which IC_{50} can be calculated [27]

Defeat stress Defeat stress was performed according to the method of Miczek *et al* [25] Briefly, "intruder" LS and SS mice were introduced into the home cages of resident CFW mice As previously described, residents attacked the intruders in a stylized fashion with bites on the rump Intruders were transferred to the cage of a new resident after 10 to 20 bites, until 100 bites had been sustained Intruder mice were then removed and receptor binding was performed as described above

Statistical analysis Comparisons between two groups were made using two-tailed *t*-tests or the Wilcoxon test for

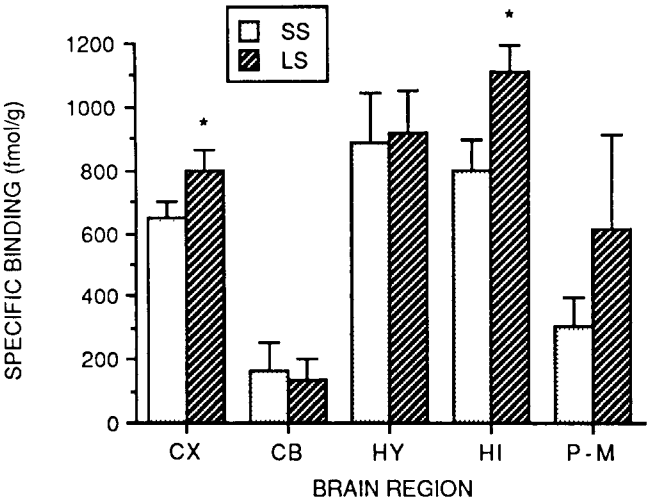

FIG 1 Benzodiazepine receptor binding in LS and SS mice Receptor binding was determined by specific uptake of [^{3}H]-Ro15-1788 *in vivo* CX=cortex, CB=cerebellum, HY=hypothalamus, P-M=pons-medulla Results are mean±SEM, n=7 in each group *$p<0$ 05 compared to SS mice

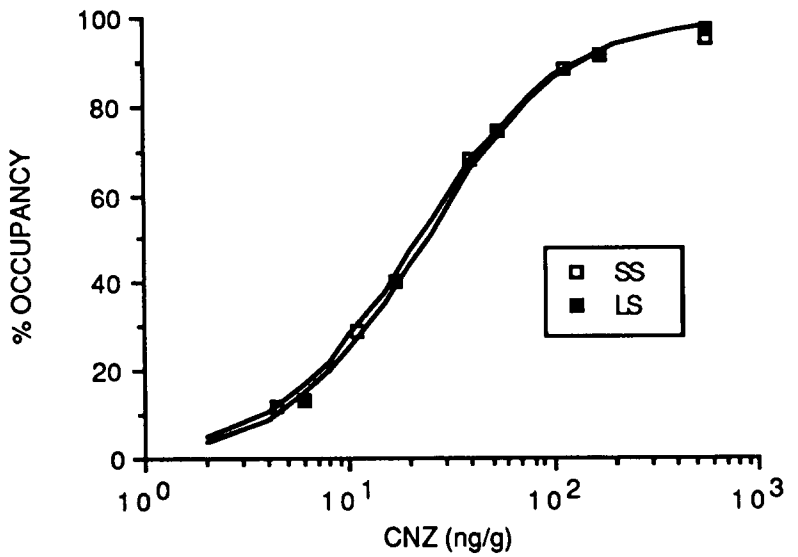

FIG 2 Receptor occupancy versus cortex clonazepam concentration in LS and SS mice The IC_{50} values for LS and SS mice were similar (22 vs 24 ng/g)

non-Gaussian distributions Comparisons between more than two groups were made using analysis of variance with correction for multiple comparisons

RESULTS

Benzodiazepine receptor binding *in vivo* was increased in cortex and hippocampus of LS as compared to SS mice ($p<0.05$ in each region, Fig 1) In the other brain regions evaluated, cerebellum, hypothalamus, and pons-medulla, no differences in binding were observed between the two strains There were no differences in nonspecific binding between LS and SS mice in any of the 5 brain regions

Comparison of apparent affinities for clonazepam in cortex *in vivo* indicated little difference between LS and SS mice (Fig 2) The IC_{50} value for clonazepam in LS mice was 22 ng/g compared to 24 ng/g for SS mice Thus, the increase in receptor binding in cortex in LS mice is most likely due to increases in receptor number rather than apparent affinity

Pentobarbital, 30 mg/kg IP, caused increases in receptor binding in cortex, hippocampus, and pons-medulla of SS mice, and cortex, hypothalamus and pons-medulla of LS mice at one hour after administration ($p<0$ 05, Table 1) Specific binding was similar after pentobarbital in LS and SS mice except for an increase in hypothalamus in LS mice ($p<0$ 05) The degree of increase in binding above control levels due to pentobarbital was similar in LS and SS mice in all brain regions examined

TABLE 1

RECEPTOR BINDING IN LS AND SS MICE AFTER STRESS AND PENTOBARBITAL

Region	Receptor Binding (fmol/g) Control	Stress	Pentobarbital (30 mg/kg)
LS Cortex	849 9 ± 59 1*	1781 6 ± 106 9†	1009 0 ± 173 8
Cerebellum	122 7 ± 50 0	331 8 ± 45 4	168 2 ± 72 2
Hypothalamus	913 5 ± 95 4	3049 7 ± 109 6	2145 0 ± 112 3‡
Hippocampus	1109 0 ± 54 5*	1477 1 ± 120 3	1568 0 ± 358 3
Pons-Medulla	613 6 ± 186 3	1563 5 ± 125 7	1536 2 ± 259 3
SS Cortex	649 9 ± 36 4	1254 4 ± 69 5	1136 3 ± 40 1
Cerebellum	163 6 ± 63 6	313 6 ± 136 4	304 5 ± 139 0
Hypothalamus	840 0 ± 109 1	2608 8 ± 411 7	1290 8 ± 136 4
Hippocampus	709 0 ± 100 0	977 2 ± 125 7	1545 3 ± 344 9
Pons-Medulla	545 4 ± 104 5	1350 0 ± 125 7	1286 2 ± 556 1

Results are mean ± SEM, n=7 for controls, n=3 for stress and pentobarbital
*$p<0.05$ vs SS controls
†$p<0.05$ vs SS stress
‡$p<0.05$ vs SS pentobarbital

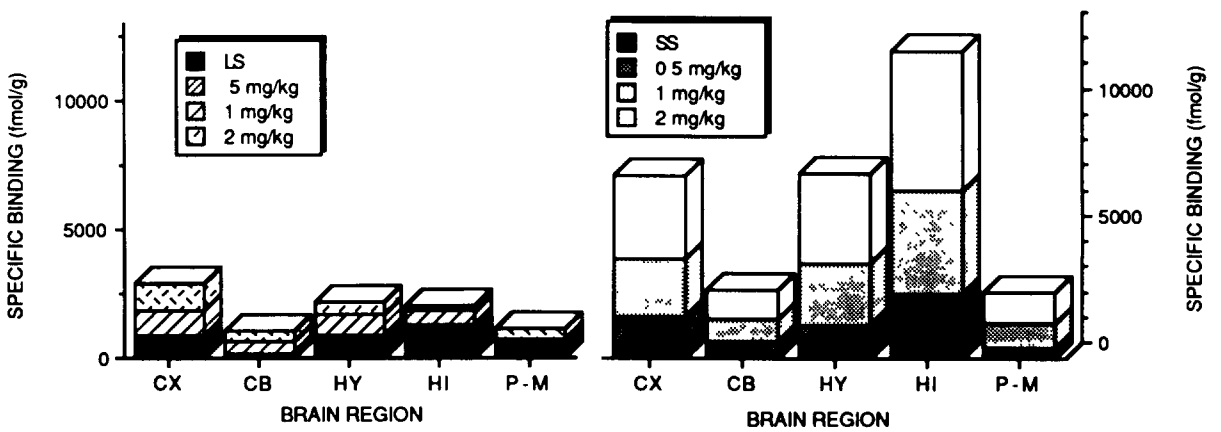

FIG 3 Effects of ethanol administration on benzodiazepine receptor binding in LS and SS mice CX=cortex, CB=cerebellum, HY=hypothalamus, HI=hippocampus, P-M=pons-medulla Results are mean±SEM, n=3 at 0 5 g/kg, n=6 at 1 and 2 g/kg SEM are omitted for clarity For comparisons see text

Defeat stress, a model of social stress involving a resident-intruder interaction, led to increases in benzodiazepine receptor binding in cortex, hypothalamus, and pons-medulla of both LS and SS mice ($p<0.05$ in each region, Table 1) Specific binding after stress was greater in cortex of LS mice compared to SS mice ($p<0.05$) The degree of increase in binding relative to control binding was similar in SS and LS mice

Binding was also examined one hour after three doses of ethanol (0 5, 1, 2, g/kg IP) in LS and SS mice (Fig 3). At the lowest dose, 0 5 g/kg, there were no significant changes in binding in LS mice in any brain region examined In contrast, significant increases in binding were observed in cortex, cerebellum, and hippocampus in SS mice ($p<0.05$), with a trend toward increased binding in hypothalamus ($p=0.14$) Specific binding in cortex cerebellum, and hippocampus was greater in SS than LS mice at this dose ($p<0.05$ in each region) After ethanol 1 g/kg, increases in binding compared to controls were observed in cortex, cerebellum, hypothalamus, and hippocampus in LS mice ($p<0.05$) and further increases in binding compared to the 0 5 g/kg dose were observed in all brain regions in SS mice ($p<0.05$) At this dose, binding was increased in SS mice compared to LS mice in cerebellum, hypothalamus, and hippocampus ($p<0.05$) At the highest dose of ethanol evaluated, 2 g/kg, there were nonsignificant trends toward increased binding compared to the 1 g/kg dose in cortex, cerebellum, and hypothalamus of LS mice ($p<0.15$) At this dose in SS mice, binding was further increased compared to 1 g/kg in cortex ($p<0.15$), with nonsignificant trends toward increase in cerebellum and hypothalamus ($p<0.15$) Specific binding at 2 g/kg was increased in SS versus LS mice in hypothalamus and hippocampus ($p<0.05$) These doses of ethanol did not alter nonspecific binding in either strain In sum, at 3 ethanol doses, binding was increased in several brain regions in SS compared to LS mice, despite lower baseline binding in SS mice

To ensure that these differences in response to ethanol

were not due to ethanol absorption or delivery to brain, ethanol concentrations were evaluated in plasma and brain of treated mice There were small but non-significant increases in ethanol in plasma and brain of LS compared to SS mice at each dose evaluated (data not shown) To assess brain uptake of benzodiazepines in LS and SS mice, we determined clonazepam concentrations in cortex of these strains one hour after varying doses of clonazepam (0 02–2 mg/kg IP) Clonazepam concentrations were slightly greater in LS compared to SS mice at each dose, but these differences were not significant (data not shown) We also compared effects of varying clonazepam doses on rotarod performance, a parameter known to be affected by benzodiazepines At each dose, rotarod ataxia was greater in LS compared to SS mice, but these differences did not achieve significance (data not shown)

Differences in receptor binding as determined by specific uptake of [^{3}H]-Ro15-1788 might be related to delivery of the radioligand to brain rather than actual differences in binding To address this possibility, we administered saturating doses of unlabeled Ro15-1788 (6 mg/kg) to LS and SS mice and determined cortex concentrations of this compound at 20 minutes [15] Concentrations of Ro15-1788 were similar in the two strains (LS 96 9±6 5 ng/g, SS 86 6±4 4 ng/g, mean±SEM, n=3, p=0 4)

DISCUSSION

LS and SS mice differ both in pharmacokinetic and pharmacodynamic responses to ethanol and a variety of other sedatives [7, 11, 21] In accordance with prior reports, we found a trend toward increased pharmacodynamic effects of clonazepam in LS compared to SS mice We also demonstrated a trend toward increased clonazepam uptake in brain after acute administration in LS and SS mice, analogous to the small increases in ethanol concentrations observed in the LS line

With regard to benzodiazepine receptor binding, our results are in contrast to prior studies based on *in vitro* binding techniques, which reported alterations in [^{3}H]-flunitrazepam binding in membrane preparations only in midbrain of LS mice [10] We observed increases in benzodiazepine receptor binding as assessed by [^{3}H]-Ro15-1788 uptake *in vivo* in cortex and hippocampus of LS mice compared to SS mice The discrepancy between the *in vivo* binding data reported here and prior *in vitro* binding studies may be in part due to limitations of *in vitro* binding techniques, which may be altered by tissue preparation, temperature and buffer conditions, and techniques to remove GABA [8, 22, 29] *In vivo* methods appear to bypass these limitations, [9,27] and we and others have previously reported that increases in receptor binding *in vivo* due to stress or barbiturate administration may not be reflected in *in vitro* binding analyses [5,28] In addition, the *in vitro* studies in LS and SS lines were conducted in female mice, in contrast to male mice used in the present study Since there is some evidence that steroid metabolites may alter benzodiazepine receptor binding [4, 17, 31], it is possible that different phases of the estrus cycle might confound *in vitro* results

The observed differences in receptor binding might be due to alterations in receptor number or apparent affinity Our studies in clonazepam-treated mice indicate that apparent affinity for clonazepam in cortex is similar in LS and SS mice, suggesting that increases in binding are most likely due to increased receptor number An additional explanation for increased uptake of [^{3}H]-Ro15-1788 in LS mice is enhanced delivery of radioligand to brain rather than increased binding However, concentrations of unlabeled Ro15-1788 were similar in cortex of LS and SS mice While the dose of unlabeled Ro15-1788 was substantially greater than the tracer doses used in radioligand studies, uptake of this compound into brain is dose-dependent [15], making it unlikely that changes in delivery of radioligand can account for the increase in receptor binding in LS mice Further, the lack of difference in nonspecific binding in any brain region mitigates against an effect based on delivery of radioligand

To assess possible differential modulation of receptor binding in the LS and SS lines, we evaluated effects on receptor binding of three interventions reported to increase *in vivo* binding in other strains barbiturates, stress, and ethanol [5, 28, 30] Increases in benzodiazepine receptor binding have been described both *in vitro* and *in vivo* after acute barbiturate administration in several mouse strains [14,30] We observed similar increases in several brain regions after a single dose of pentobarbital known to increase receptor binding in other strains We found differences in pentobarbital effects between the LS and SS lines only in hypothalamus, and we found no differences in the degree of increase in binding in the two lines Behavioral evidence indicates that this dose of pentobarbital has little effect on "sleep-time" in SS mice, but significantly increases "sleep-time" in LS mice [2] It is thus unlikely that differences in benzodiazepine binding account for the differential response to pentobarbital in these lines

Defeat stress has been shown to produce increases in receptor binding in several brain regions in CFW and B6AF1 strains [28] Defeat stress led to increases in binding in cortex, hypothalamus, and pons-medulla in both LS and SS lines Binding in cortex was greater after stress in LS versus SS mice, although the degree of increase in binding compared to control levels was similar in all brain regions evaluated Thus, the responses of SS and LS mice to pentobarbital and stress are similar to other mouse strains and similar between the two lines, with increases in specific binding in response to both stimuli only in single regions in LS mice

Ethanol increases benzodiazepine receptor binding *in vivo* in several strains of mice [5] The effects of ethanol on the LS and SS lines are of particular interest, given the differences in pharmacodynamic responses to ethanol in these lines and evidence from several studies indicating that ethanol exerts at least some of its effects via the benzodiazepine-GABA complex [34,35] Prior studies indicate that the ED_{50} for ethanol on loss of the righting reflex in LS mice is 1 65 g/kg, and in SS mice, 3 64 g/kg [11] Little effect was seen in either line at doses less than 1 g/kg [2], while a substantial increase in sleep-time was observed at a dose of 2 g/kg We observed no change in benzodiazepine receptor binding in LS mice at 0 5 g/kg, but significant increases were found at 1 and 2 g/kg corresponding to doses which increased sleep-time In contrast, binding was increased in SS mice at 0 5 g/kg and markedly increased at doses of 1 and 2 g/kg, despite little effect on sleep-time at these doses These data do not support a simple correlation of benzodiazepine receptor binding and increasing sleep-time in the two lines It is more likely that differences exist in the benzodiazepine receptor complex between LS and SS mice such that ethanol effects both receptor binding and receptor function differentially

As discussed above, prior studies reported *in vitro* differences in benzodiazepine receptor coupling to GABA, in

GABA coupling to the chloride ionophore, and in chloride flux in response to ethanol between the LS and SS lines [1, 10, 18] However, GABA concentrations were not differentially altered by ethanol in these lines [3] Our data indicating increases in benzodiazepine receptor binding *in vivo* in LS mice suggest structural as well as functional differences in benzodiazepine receptors in these lines We have also demonstrated modulation of benzodiazepine receptor binding *in vivo* in LS and SS mice by pentobarbital, stress and ethanol These data suggest that both receptor number and affinity can be increased in LS and SS lines, since pentobarbital appears to increase receptor affinity [30] and stress increases receptor number [28] The differential modulatioh of receptor binding by ethanol but not by pentobarbital or stress may indicate that these lines differ not only in general characteristics of the benzodiazepine-GABA complex, but also in specific responses of this complex to ethanol Differential modulation of receptor binding may contribute to the differing sedative responses to ethanol in LS and SS mice Further studies in selectively-bred lines may shed light on the effects of benzodiazepine receptor modulation, and perhaps on the structural determinants of receptor function

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