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Genetic Influences on the Central Nervous System Depressant and Membrane-Disordering Actions of Ethanol and Sodium Valproate

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SUMMARY

The effects of the two central nervous system (CNS) depressant drugs ethanol and sodium valproate were compared using two pairs of mouse lines that had been selected from a heterogeneous stock for differential sensitivity to ethanol. The LS/SS lines differ in sensitivity to ethanol-induced sedation, and the WSP/WSR lines differ in the severity of their withdrawal convulsions after chronic ethanol treatment. We used these lines to test the hypothesis that ethanol and valproate act by the same mechanism. CNS depressant action was assessed by determining the brain drug concentration at which the mice lost their ability to balance on a stationary wooden dowel. LS mice were about twice as sensitive as SS mice to valproate-induced ataxia, in agreement with their reported relative sensitivity to ethanol. The WSR and WSP mice did not differ significantly in sensitivity to ethanol or valproate in this test. The intrinsic order and sensitivity to disordering of synaptosomal plasma membranes prepared from the four lines were measured using fluorescence polarization with the probe 1,6-diphenyl-1,3,5-hexatriene and EPR spectroscopy with 5-doxylstearic acid. No differences in the intrinsic membrane order of the four lines were detected with either technique. The sensitivities of the membranes from the four lines to ethanol- or valproate-induced disordering were not significantly different when measured by fluorescence polarization, but EPR spectroscopy revealed line differences in disordering sensitivity that correlated with the relative sensitivity of the four lines to the CNS depressant action of these drugs. These studies show that genetic factors modulate sensitivity to ethanol and valproate in a similar manner both *in vivo* and *in vitro*, suggesting that these drugs act by the same membrane-disordering mechanism.

INTRODUCTION

The antiepileptic agent sodium valproate is a small, branched-chain fatty acid (di-n-propylacetate). Although it is known to affect the brain γ-aminobutyric acid system, valproate's true mechanism of anticonvulsant action has not yet been defined (1, 2). Because of its simple amphiphilic structure and relatively low potency, valproate might be expected to act by disordering neuronal membranes, a proposed mechanism of action of ethanol and the general anesthetics. These agents are thought to partition nonspecifically into the lipid bilayer of neuronal membranes and alter membrane structure and function (3). Many studies using a variety of physical chemical techniques have shown that anesthetic agents can increase the fluidity of biological membranes and liposomes (4). We recently demonstrated that the membrane-disordering potencies of short-chain fatty acids, including valproate, correlate with their CNS[1] depressant and anticonvulsant potencies (5), which suggests that the actions of valproate might be mediated by its membrane effects.

To further characterize the membrane-disordering and CNS depressant actions of valproate, we have utilized two pairs of mouse lines that were bred for differential sensitivity to ethanol: the LS/SS and the WSP/WSR lines. McClearn and Kakihana (6) selected two lines of mice that differ in their sensitivity to ethanol-induced hypnosis. After a standard dose of ethanol, LS mice lose their righting reflex for a longer period than do SS mice. As measured by "waking" blood ethanol levels (7, 8) or by ED_{50} values for loss of righting reflex (7, 9, 10), the LS mice are about twice as sensitive as SS mice to ethanol. *In vitro*, synaptosomal plasma membranes prepared from LS mice were found to be more sensitive than

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[1] The abbreviations used are: CNS, central nervous system; LS, long sleep; SS, short sleep; WSP, withdrawal seizure prone; WSR, withdrawal seizure resistant; SPM, synaptosomal plasma membrane; DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline.

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SS membranes to ethanol disordering (11), providing a strong link between the sedative/hypnotic and membrane-disordering actions of ethanol. The two lines of mice are not differentially sensitive to pentobarbital (12), indicating that this CNS depressant does not act through the site affected by the selection pressure. This illustrates the power of a genetic approach in identifying common mechanisms of actions for drugs with similar pharmacological effects.

Crabbe *et al.* (13) have recently carried out a selective breeding program based on intensity of ethanol withdrawal signs. These lines, WSP and WSR, were bred for differential severity of withdrawal convulsions after a standard regimen of chronic ethanol treatment.

We measured the relative sensitivity of LS and SS mice to valproate-induced ataxia, and studied the SPM prepared from these mice using fluorescence polarization and EPR spectroscopy. We also compared the actions of ethanol and valproate in the WSP and WSR lines. Our findings indicate that these genetic selections did not discriminate between valproate and ethanol either *in vivo* or *in vitro*, suggesting that the two drugs might act by a similar membrane-disordering mechanism.

MATERIALS AND METHODS

LS and SS male mice, 5 to 6 weeks old, were obtained from the Institute for Behavioral Genetics, University of Colorado, Boulder, CO. The mice were from the 37th and 38th generations of the SS and LS lines. Selection pressure had been relaxed after the 33rd generation (the 25th selection). WSP and WSR male mice from the 9th and 10th selected generations (8 to 9 weeks old) were provided by John C. Crabbe, Oregon Health Sciences University, Portland, OR. Mice were housed with a 12-hr light/dark cycle for 1 to 3 weeks before testing. Water and Purina Rat Chow were available *ad libitum*.

Solutions of sodium valproate were made by dissolving the acid (gift of Abbott Laboratories, North Chicago, IL) in 95% ethanol and adding a slight excess of aqueous NaOH. After shaking for several hours, the solutions were lyophilized to remove the solvent. For administration to mice, the valproate was dissolved in glass-distilled water and adjusted to pH 7.4. For studies *in vitro*, the valproate was dissolved in a 10 mM phosphate buffer containing 2.7 mM KCl, and the solutions were brought to pH 7.4 and to a sodium concentration of 153 mM.

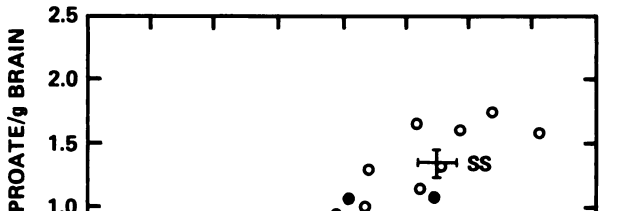

FIG. 1. *Thresholds for ataxia in LS and SS mice*

LS and SS mice received intraperitoneal injections of 8 mmol/kg valproate and then were placed on a stationary wooden dowel. When the mice fell, they were immediately killed, and brain drug concentrations were determined by gas chromatography. Fall times and valproate brain concentrations (micromoles of valproate/g brain wet weight) are plotted for each mouse. Error bars represent the standard error for the mean fall times and brain concentrations (n = 8–9; ●, LS; ○, SS).

CNS depressant action. The sensitivity of the mice to drug-induced ataxia was assessed by determining the brain concentration of drug at which the mice lost their ability to balance. Mice received an intraperitoneal injection of either 40 mmol/kg ethanol or 8 mmol/kg valproate. After 20 sec, the mice were placed on a stationary wooden dowel clamped in a horizontal position 45 cm above a bed of shavings. The mice fell within 3 min, presumably when the drugs had reached an effective concentration in the brain. They were immediately decapitated, and the brains were homogenized in 9 volumes of ice-cold 0.9% NaCl (for assay of valproate) or 3.4% perchloric acid (for assay of ethanol). One-ml aliquots were stored at −20° for later determination of drug concentrations.

Brain levels of ethanol were determined enzymatically (14). Valproate brain levels were determined by gas chromatography as previously described (5). The column was packed with 10% SP 216 PS (Supelco, Inc., Bellefonte, PA). Standard curves, prepared by adding

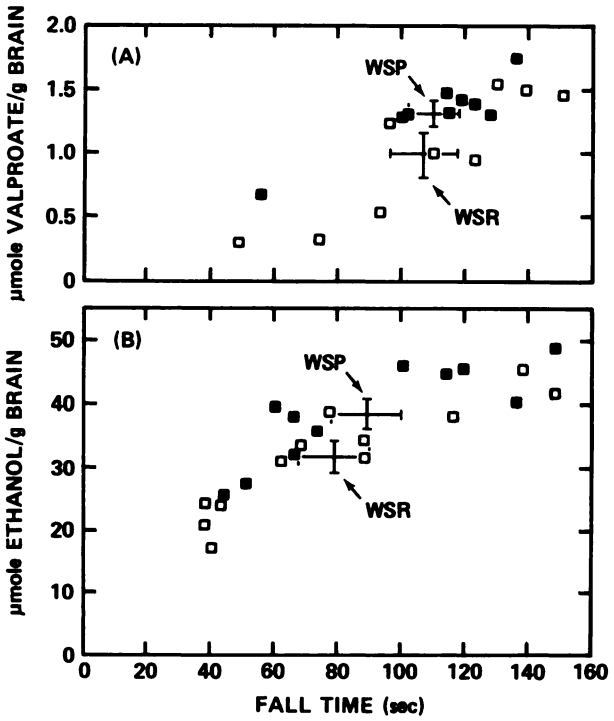

FIG. 2. *Thresholds for ataxia in WSP and WSR mice*

Fall times and brain concentrations are shown as in Fig. 1 (■, WSP; □, WSR). A, mice tested with 8 mmol/kg valproate (n = 8–9). B, mice tested with 40 mmol/kg ethanol (n = 11–12).

TABLE 1

Intrinsic membrane order

The baseline order of the SPM prepared from the mice was determined at 25° using fluorescence polarization with the probe DPH and EPR spectroscopy with the probe 5-doxylstearic acid. The data are averages ± standard error of three to eight samples from each line (each prepared from the pooled brains of two to four mice). No significant line differences were seen with either technique.

Line	Fluorescence anisotropy	EPR order parameter
LS	0.2505 ± 0.0003	0.6682 ± 0.0002
SS	0.2507 ± 0.0002	0.6678 ± 0.0003
WSP	0.2503 ± 0.0002	0.6683 ± 0.0005
WSR	0.2504 ± 0.0003	0.6685 ± 0.0004

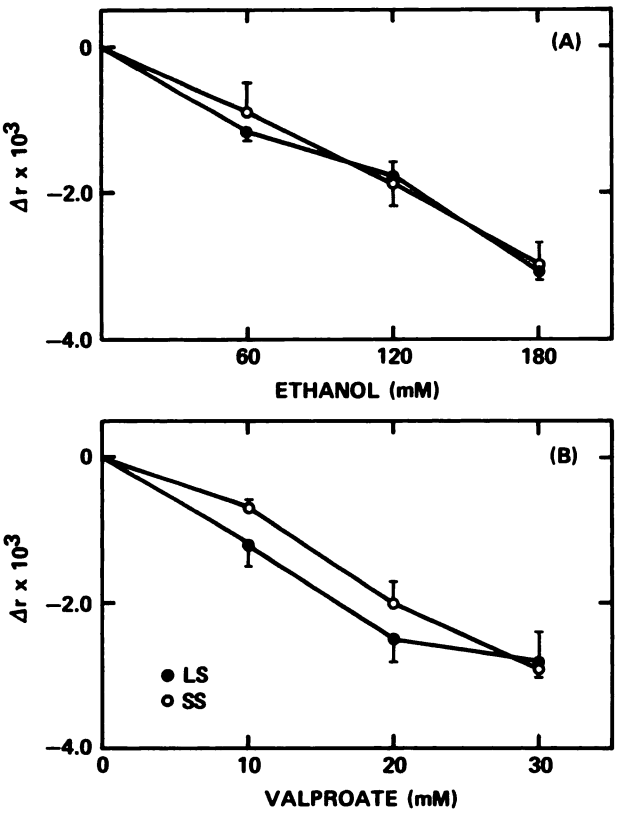

FIG. 3. *Fluorescence anisotropy of DPH in membranes from LS and SS mice*

Anisotropy was measured in the presence of various concentrations of (A) ethanol or (B) valproate. Points (●, LS; ○, SS) represent the means of four membrane preparations and vertical bars represent the standard error. Membranes were prepared from the pooled brains of two to four mice. Fluorescence anisotropy in the absence of added drug (intrinsic order) is shown in Table 1.

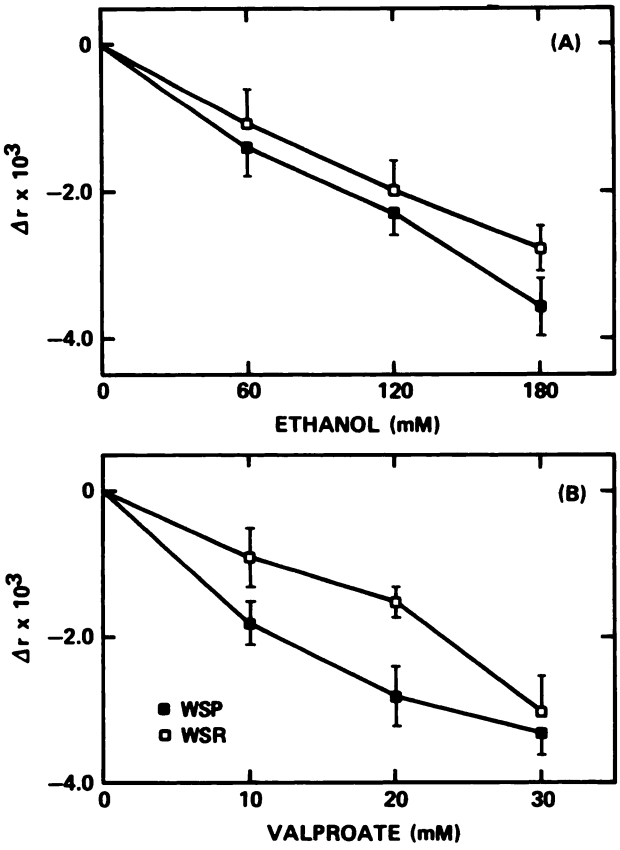

FIG. 4. *Fluorescence anisotropy of DPH in membranes from WSP and WSR mice*

See legend for Fig. 3 (■, WSP; □, WSR).

known amounts of drug to brain homogenates, were linear in the range of 0.06 to 2.0 μmol valproate per g brain (wet weight).

Preparation of membranes. Synaptosomal plasma membranes were prepared from whole brains (2 to 4 brains/tube) by the sucrose gradient centrifugation technique of Jones and Matus (15). The SPM fraction was removed from the gradient, pelleted by centrifugation at 27,000 × *g*, and resuspended in PBS without Ca^{2+} or Mg^{2+} at pH 7.4 at a concentration of about 20 mg of protein per ml. The suspensions were stored in 60–100-μl aliquots under nitrogen at −80° before use. Protein concentrations were determined by the method of Lowry *et al.* (16).

Fluorescence polarization. SPM aliquots were thawed and diluted with PBS. Then 250 μM DPH (Sigma Chemical Co., St. Louis, MO) in tetrahydrofuran (Aldrich Chemical Co., Milwaukee, WI) was added to a concentration of about 1 mol of dye per 500 mol of membrane phospholipid. The labeled membrane suspension was dispensed into silanized tubes, and PBS or drug solution was added. The final protein concentration was 30 μg/ml. The samples were incubated in the dark at 25° in a shaking water bath for at least 30 min before measurements were taken.

Fluorescence intensity and steady state anisotropy values were determined at 25.0 ± 0.1° using an SLM 4048 polarization spectrofluorometer (SLM Instruments Inc., Urbana, IL) as previously described (5). The excitation wavelength was 360 nm. Emission above 420 nm was isolated using a combination of bandpass and cutoff filters; with this arrangement, the effects of light scattering on the measured anisotropy values were negligible. Four different membrane preparations from each line were measured in duplicate at each drug concentration. Previous studies have shown that these concentrations of ethanol or valproate do not significantly affect the fluorescence lifetime of DPH in SPM (5).

EPR studies. Membrane samples were spin-labeled with 5-doxylstearic acid and incubated with or without drugs as previously described (17). Each sample was scanned three times at 25° to produce an average order parameter (18). Three or four membrane preparations from each line were tested in duplicate at each drug concentration.

RESULTS

Effects of valproate and ethanol in vivo. The 8 mmol/kg dose of valproate caused the mice from all four lines to fall from the dowel within 2.5 min, during the time that brain concentrations of valproate were rising. The LS mice fell off the dowel earlier and with lower brain valproate levels than the SS mice (Fig. 1), indicating that LS mice are more sensitive than SS mice to valproate-induced ataxia. There was little overlap in brain levels or fall times between the two lines ($p < 0.005$ in both cases). The average brain concentrations of valproate at the onset of ataxia, 0.68 ± 0.13 μmol/g brain for LS mice and 1.35 ± 0.11 μmol/g brain for SS mice (± standard error, $n = 8$ and 9, respectively), demonstrate that LS mice are about twice as sensitive as SS mice to valproate. A similar line difference in sensitivity to ethanol has been reported (7).

In contrast, the WSP and WSR mice lines did not differ significantly in sensitivity to either ethanol or valproate. With each drug the fall times of the two lines were virtually the same (Fig. 2), and the brain levels of

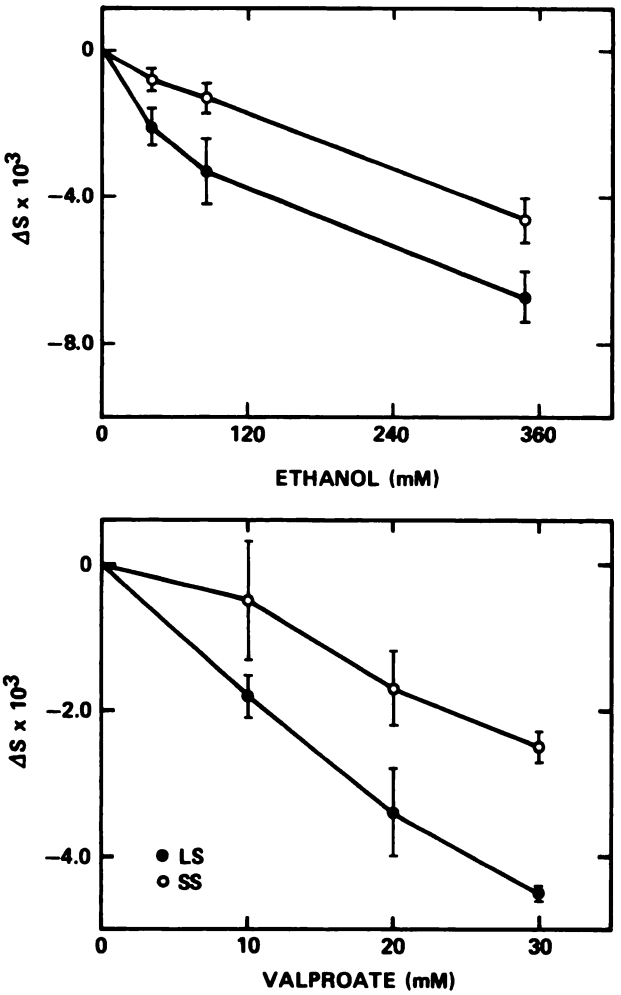

FIG. 5. *EPR spectroscopy of membranes from LS and SS mice*
A, SPM prepared from LS and SS mice were labeled with 5-doxylstearic acid, and order parameters were measured at 37° in the presence of various concentrations of ethanol (reproduced from ref. 11 with permission). B, order parameters were measured at 25° in the presence of various concentrations of valproate. Points (●, LS; ○, SS) represent the means of three membrane preparations and vertical bars represent the standard deviation.

drug were not significantly different. Both the fall times and the valproate brain levels of the WSR and WSP mice were close to the corresponding values for SS mice.

Membrane-disordering actions of valproate and ethanol. With DPH fluorescence polarization no significant differences in the membranes prepared from the four lines were seen. The intrinsic order of the membranes from the four lines (the fluorescence anisotropy in the absence of added drug) were virtually identical (Table 1). Both valproate (10–30 mM) and ethanol (60–180 mM) caused a concentration-dependent decrease in the order of the membranes (Figs. 3 and 4). The change in fluorescence anisotropy per millimolar concentration of drug reflects the sensitivity of the membranes to drug-induced disordering. Comparison of the slopes of the linear regression lines using Student's *t* test demonstrated that the disordering sensitivities of the membranes from the four lines were not significantly different.

EPR spectroscopy with 5-doxylstearic acid also did not

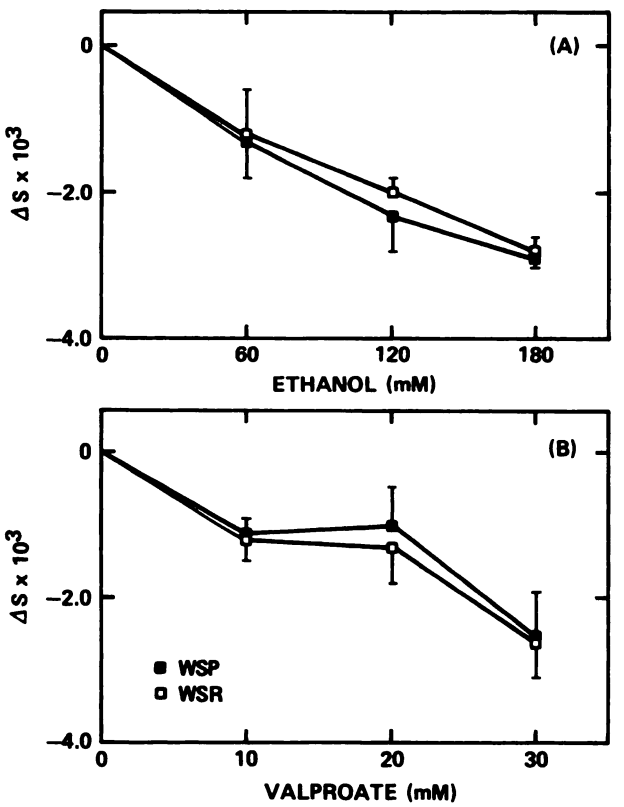

FIG. 6. *EPR spectroscopy of membranes from WSP and WSR mice*
5-Doxyl order parameters of the SPM prepared from WSP and WSR mice were measured in the presence of (A) ethanol and (B) valproate. Points (■, WSP; □, WSR) represent the means of four membrane preparations and vertical bars represent the standard error.

TABLE 2
Summary of line differences in sensitivity to ethanol and valproate.

	LS/SS		WSP/WSR	
	Ethanol	Valproate	Ethanol	Valproate
CNS depression	LS>SS[a]	LS>SS[b]	NS[c]	NS
EPR disordering	LS>SS[d]	LS>SS[b]	NS	NS
Fluorescence polarization disordering	NS	NS	NS	NS

[a] From refs. 7–10.
[b] $p < 0.005$.
[c] NS, the within-pair difference was not significant.
[d] From ref. 11.

detect any significant differences in the baseline order of the membranes from the four lines (Table 1). However, there were clear differences in sensitivity to disordering, which agreed with the differences seen *in vivo*. SPM prepared from LS mice were almost twice as sensitive as SS membranes to disordering by valproate (Fig. 5B). This is similar to the results obtained in a previous study of LS/SS sensitivity to ethanol-induced disordering (Fig. 5A). In contrast, the membranes from the WSR and WSP mice did not differ in their response to either ethanol or valproate (Fig. 6).

Table 2 summarizes the results for all four lines.

DISCUSSION

The relationship between lipid solubility and anesthetic potency has been known for some time (3), and

clear correlations between membrane-disordering potency and the actions of CNS depressants *in vivo* have recently been demonstrated (5, 19, 20). Although the disordering effects of these agents are significant, clinically relevant concentrations usually produce quite small changes in membrane order. Possibly a similar but more intense effect occurs at certain regions of the membrane such as the boundary lipids of membrane proteins, an effect that is averaged out when the fluidity of the entire lipid bilayer is determined. However, an actual link between a membrane perturbation and any behavioral effects has not yet been established, and a mechanism of anesthetic action involving a disordering of membrane lipids is not universally accepted (21).

The hypothesis that sedative/hypnotic agents act by disordering membranes is strengthened by the evidence that an animal's sensitivity to ethanol is influenced by the physical properties of its brain cell membranes (11, 22–25). Mice and rats with different degrees of sensitivity to the depressant actions of ethanol have been obtained by choosing animals with different innate sensitivities (11) and by using chronic ethanol treatment to produce functional tolerance (22–25). In both cases, the sensitivity of the brain membranes to disordering correlates with the sensitivity of the animals *in vivo*. The lipids extracted from the membranes of ethanol-tolerant and control animals are also differentially sensitive (23, 26), suggesting that the lipid bilayer is an important mediator of ethanol's effects.

The LS/SS lines, which would be expected to show differential sensitivity to all drugs that have the same mechanism of action as ethanol, can be used to study the mechanism of action of other anesthetic agents. Differential sensitivity of LS/SS mice has been reported in response to alcohols (7, 9), trichloroethanol and paraldehyde (27), and some inhalation anesthetics (28). However, the lines do not differ in sensitivity to pentobarbital (7, 27, 29), or halothane (8). Thus, although all these CNS depressants produce similar pharmacological effects, it appears that they do not all act at the primary site affected by the selective breeding. The fact that the two lines are not differentially sensitive to all the inhalation anesthetics is not yet understood.

LS mice are almost twice as sensitive as SS mice to the hypnotic (7–10) and membrane-disordering actions (11) of ethanol. In our present study, the LS mice were also about twice as sensitive as SS mice to valproate *in vivo*, whereas WSR and WSP mice were not differentially sensitive to valproate- or ethanol-induced ataxia. Similarly, in our EPR studies, the SPM from LS mice were almost twice as sensitive as SS membrane to disordering, whereas the disordering sensitivities of the membranes from WSP and WSR mice were not significantly different. Thus, the two different genetic selections provide concordant data demonstrating a correlation between the disordering sensitivity of neuronal membranes and the sensitivity of the whole animal to the CNS depressant action of these drugs.

In contrast to the EPR studies, fluorescence polarization was not able to distinguish among the four lines. Thus, a genetic link between membrane-disordering and CNS depressant action is not seen with this technique. This is surprising since we have previously seen strong correlations between membrane disordering and the anticonvulsant effects of CNS depressants using DPH fluorescence polarization (5). 5-Doxylstearic acid monitors membrane order near the polar head group region, while DPH is probably dispersed throughout the interior hydrophobic region of the membrane. Thus, the primary site affected by these genetic selections might be localized near the polar head group region of the membrane.

In conclusion, these data suggest that sodium valproate and ethanol might produce their CNS depressant effects via the same mechanism: by disordering neuronal membranes.

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REFERENCES

1. Kupferberg, H. J. Sodium valproate, in *Antiepileptic Drugs: Mechanisms of Action* (G. H. Glaser, J. K. Penry, and D. M. Woodbury, eds.). Raven Press, New York, 643–654 (1980).
2. Chapman, A., P. E. Keane, B. S. Meldrum, J. Simiand, and J. C. Vernieres. Mechanism of anticonvulsant action of valproate. *Prog. Neurobiol.* **19**:315–359 (1982).
3. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
4. Goldstein, D. B. The effects of drugs on membrane fluidity. *Annu. Rev. Pharmacol. Toxicol.* **24**:43–64 (1984).
5. Perlman, B. J., and D. B. Goldstein. Membrane disordering potency and anticonvulsant action of valproic acid and other short chain fatty acids. *Mol. Pharmacol.* **26**:83–89 (1984).
6. McClearn, G. E., and R. Kakihana. Selective breeding for ethanol sensitivity: short-sleep and long-sleep mice, in *Development of Animal Models as Pharmacogenetic Tools* (G. E. McClearn, R. A. Deitrich, and V. G. Erwin, eds.). United States Department of Health and Human Services, Washington, DC, 147–159 (1981).
7. Erwin, V. G., W. D. W. Heston, G. E. McClearn, and R. A. Deitrich. Effect of hypnotics on mice genetically selected for sensitivity to ethanol. *Pharmacol. Biochem. Behav.* **4**:679–683 (1976).
8. Baker, R., C. Melchior, and R. Deitrich. The effect of halothane on mice selectively bred for differential sensitivity to alcohol. *Pharmacol. Biochem. Behav.* **12**:691–695 (1980).
9. Howerton, T. C., M. F. O'Connor, and A. C. Collins. Differential effects of long-chain alcohols in long- and short-sleep mice. *Psychopharmacology* **79**:313–317 (1983).
10. Heston, W. D. W., V. G. Erwin, S. M. Anderson, and H. Robbins. A comparison of the effects of alcohol on mice selectively bred for differences in ethanol sleep-time. *Life Sci.* **14**:365–370 (1974).
11. Goldstein, D. B., J. H. Chin, and R. C. Lyon. Ethanol disordering of spin-labeled mouse brain membranes: correlation with genetically determined ethanol sensitivity of mice. *Proc. Natl. Acad. Sci. USA* **79**:4231–4233 (1982).
12. Collins, A. C. A review of research using short-sleep and long-sleep mice, in *Development of Animal Models as Pharmacogenetic Tools* (G. E. McClearn, R. A. Deitrich, and V. G. Erwin, eds.). United States Department of Health and Human Services, Washington, DC, 161–170 (1981).
13. Crabbe, J. C., A. Kosobud, and E. R. Young. Genetic selection for ethanol withdrawal severity: differences in replicate mouse lines. *Life Sci.* **33**:955–962 (1983).
14. Lundquist, F. The determination of ethyl alcohol in blood and tissues. *Methods Biochem. Anal.* **7**:217–251 (1959).
15. Jones, D. H., and A. I. Matus. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta* **356**:276–287 (1974).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. Chin, J. H., and D. B. Goldstein. Membrane-disordering action of ethanol: variation with membrane cholesterol content and depth of the spin label probe. *Mol. Pharmacol.* **19**:425–431 (1981).
18. Hubbell, W. L., and H. M. McConnell. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93**:314–326 (1971).
19. Lyon, R. C., J. A. McComb, J. Schreurs, and D. B. Goldstein. A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. *J. Pharmacol. Exp. Ther.* **218**:669–675 (1981).

20. Pang, K.-Y. Y., L. M. Braswell, L. Chang, T. J. Sommer, and K. W. Miller. The perturbation of lipid bilayers by general anesthetics: a quantitative test of the disordered lipid hypothesis. *Mol. Pharmacol.* **18**:84–90 (1980).
21. Richards, C. D., C. A. Keightley, T. R. Hesketh, and J. C. Metcalfe. A critical evaluation of the lipid hypotheses of anesthetic action, in *Molecular Mechanisms of Anesthesia* (*Progress in Anesthesiology, Vol 2*) (B. R. Fink, ed.). Raven Press, New York, 337–350 (1980).
22. Chin, J. H., and D. B. Goldstein. Drug tolerance in biomembranes: a spin label study of the effects of ethanol. *Science* **196**:684–685 (1977).
23. Rottenberg, H., A. Waring, and E. Rubin. Tolerance and cross-tolerance in chronic alcoholics: reduced membrane binding of ethanol and other drugs. *Science* **213**:583–585 (1981).
24. Lyon, R. C., and D. B. Goldstein. Changes in synaptic membrane order associated with chronic ethanol treatment in mice. *Mol. Pharmacol.* **23**:86–91 (1983).
25. Perlman, B. J., and D. B. Goldstein. Ethanol and sodium valproate disordering of membranes from chronic ethanol treated mice. *Fed. Proc.* **42**:2123 (1983).
26. Johnson, D. A., N. M. Lee, R. Cooke, and H. H. Loh. Ethanol-induced fluidization of brain lipid bilayers: required presence of cholesterol in membranes for the expression of tolerance. *Mol. Pharmacol.* **15**:739–746 (1979).
27. Sanders, B., S. K. Sharpless, A. C. Collins, G. E. McClearn, and C. Flanagan. Activating and anesthetic effects of general depressants. *Psychopharmacology* **56**:185–189 (1978).
28. Koblin, D. D., and J. E. Deady. Anaesthetic requirement in mice selectively bred for differences in ethanol sensitivity. *Br. J. Anaesth.* **53**:5–10 (1981).
29. Siemens, A. J., and A. W. K. Chan. Differential effects of pentobarbital and ethanol in mice. *Life Sci.* **19**:581–590 (1976).

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