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Differential Response to Flurazepam in Long-Sleep and Short-Sleep Mice

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MARLEY, R. J., R. K. FREUND AND J. M. WEHNER. *Differential response to flurazepam in long-sleep and short-sleep mice.* PHARMACOL BIOCHEM BEHAV 31(2) 453–458, 1988.— In addition to differing in ethanol sensitivity, long-sleep (LS) and short-sleep (SS) mice also differ in response to GABAergic agents. In the present study the sensitivity of LS and SS mice to the anesthetic, hypothermic and anticonvulsant effects of benzodiazepine, flurazepam, was determined. Flurazepam (75–300 mg/kg) induced a dose-dependent loss of righting response in both lines. The LS line displayed a two-fold greater sensitivity to the anesthetic effects of flurazepam. A dose-dependent decrease in body temperature was also observed following administration of flurazepam (25–150 mg/kg), but the two lines did not differ on this measure. Determination of the anticonvulsant effects of flurazepam (1–6 mg/kg) against seizures induced by 3-mercaptopropionic acid revealed that the SS line was more sensitive to the anticonvulsant effects of this benzodiazepine. These studies demonstrate that LS and SS mice differ in response to flurazepam, but the nature of the difference depends on the type of response measured and the dose of flurazepam employed.

Benzodiazepine Long-sleep and short-sleep mice Anesthesia Hypothermia Anticonvulsant Seizure Flurazepam 3-Mercaptopropionic acid

IT has been fairly well established that ethanol affects various neurotransmitter systems (14). How ethanol alters receptor functioning and the relationship of these effects to behavioral and physiological effects of ethanol, however, still remains to be determined. Numerous studies have implicated the GABAergic system in some of the actions of ethanol in the CNS. The hypothesis that ethanol interacts with the GABAergic system is supported by behavioral data obtained from both human (38) and animal studies (4, 6, 16, 17, 28). Biochemical studies have demonstrated that ethanol potentiates muscimol-stimulated Cl^- flux in isolated rat cortical vesicles (35) and in primary cultures of spinal cord neurons (37). Although controversial (19), some behavioral and biochemical actions of ethanol can be blocked by the imidazobenzodiazepine, Ro 15-4513 (34,36), and another inverse agonist, FG 7142 (18), providing further support for at least partial mediation of ethanol's actions via modulation of the GABAergic receptor system. In general, the result of the enhancement of GABAergic function by ethanol is an increased inhibitory influence on neuronal functions (14), a mechanism consistent with the depressant nature of ethanol.

Selected lines of mice such as the long-sleep (LS) and short-sleep mice (SS) have provided a tool to investigate the relationship of ethanol sensitivity to the GABAergic system. These mouse lines were developed by selective breeding for differences in duration of ethanol-induced anesthesia following the IP injection of ethanol (22). The foundation population for selection was a heterogeneous stock of mice that resulted from an initial cross of eight inbred strains. Although modest differences in ethanol elimination exist between LS and SS mice (8,9), the major influence on the sleep-time differential appears to be central nervous system sensitivity to this agent (10).

It has been shown that LS and SS mice differ in sensitivity to certain GABA mimetics (28), seizure-producing agents (26), as well as to a number of anesthetic agents (12,13). Some of the differential response in LS and SS mice to anesthetics may be related to the intrinsic properties of the specific anesthetic since differences in sensitivity to alcohols and barbiturates in these mice are correlated to lipid solubility, with larger differences observed for more water-soluble anesthetics (27).

Interestingly, the diazepam-sensitive (DS) and diazepam-resistant (DR) mice show differential ethanol sensitivity (7). These mice were selected for sensitivity to the benzodiazepine, diazepam, from the same heterogenous stock of mice as LS and SS mice. Taking into account the responses of these mice and those of the LS and SS mice, it may be that

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some of the genes regulating sensitivity in the GABAergic system are common to those genes regulating sensitivity to ethanol's depressant properties.

In the present study, we have explored further behavioral responses mediated by the GABAergic system in LS and SS mice by assessing the actions of the benzodiazepine, flurazepam. Benzodiazepines are thought to enhance GABAergic function in the CNS by binding at a separate benzodiazepine receptor protein (2). These drugs have multiple behavioral effects, but are capable of inducing anesthesia when administered at high doses. In the present study, this action was measured by conducting sleep-time experiments.

LS and SS mice also differ in their hypothermic response to ethanol, with the LS mice being more sensitive (13). For this reason, the induction of hypothermia by flurazepam was examined.

Lastly, the anticonvulsant properties of benzodiazepines were investigated. Numerous studies have implicated GABA as having an important role in both human epilepsy and the control of various types of experimentally-induced seizure activity (5,20). Compounds which inhibit GABA synthesis or block GABAergic transmission usually have convulsant or proconvulsant activity (11,32). In contrast, benzodiazepines and barbiturates which augment GABAergic transmission reduce or inhibit both spontaneous and exogenously-induced seizure activity (21,29). Therefore, the anticonvulsant actions of benzodiazepines were monitored by assessing the ability of flurazepam to antagonize seizures induced by 3-mercaptopropionic acid (MP), an inhibitor of GABA synthesis (21).

METHOD

Animals

Female LS and SS production mice were obtained from the Institute for Behavioral Genetics. All mice were weaned at 25 days of age and housed with two to five like-sex litter mates. Animals were maintained on a 12-hr light/12-hr dark cycle (lights on 7 a.m. until 7 p.m.) and were permitted free access to food (Wayne Lab Blox) and water. All testing was done when the animals were between 60 and 90 days of age and no animal was tested more than once.

Flurazepam-Induced Loss of Righting Response

The anesthetic potency of flurazepam (Hoffmann-La Roche, Nutley, NJ) was measured as the duration of the loss of righting response (sleep time) following administration of doses of flurazepam ranging from 75 mg/kg to 225 mg/kg for the LS mice and from 225 mg/kg to 300 mg/kg for the SS mice. Flurazepam was prepared fresh daily in physiological saline and administered intraperitoneally (IP) in an injection volume of 0.01 ml/g. All sleep time experiments were conducted in an isolated room in which the temperature was maintained at 26°C. Following injection of the drug, each animal was placed on its back in a V-shaped trough. Sleep time was recorded as the time period between loss and regaining of the righting response. The animals were considered to have regained the righting response after they righted themselves three times in 30 seconds. Observation periods were limited to 3 hr for each animal and any animal not regaining the righting response within this period was given a score of 180 min.

Dose-response curves for flurazepam-induced sleep times were analyzed by least squares linear regression techniques to determine the slope ±the standard error of the estimate (S.E.E.) and the ED_{60} ±S.E.E. for each dose-response curve. The ED_{60} value represents the dose of flurazepam predicted to produce an average sleep time of 60 min. These values were compared between the two lines by use of Student's *t*-tests modified for analysis of the parameters of regression lines.

Flurazepam-Induced Hypothermia

Body temperature was measured with a rectal probe (Bailey Instruments, Saddle Brook, NJ). The probe was lubricated with peanut oil and inserted 2.5 cm into the rectal cavity. Prior to the administration of flurazepam, each animal's baseline body temperature was determined. Mice were then injected with flurazepam (25–150 mg/kg IP) in an injection volume of 0.01 ml/g and placed singly in holding cages. Control animals were administered physiological saline in the same manner. Body temperatures for each animal were measured 30 min after injection of flurazepam or saline and recorded as the decrease in °C from the animal's baseline body temperature. This time point was chosen based on preliminary time-course experiments in which it was observed that by 30 min following flurazepam injection the decrease in body temperature had stabilized at a maximal level.

Analysis of the flurazepam-induced decrease in body temperature was accomplished using analysis of variance (ANOVA) techniques to evaluate the effects of dose and population. Least squares regression analysis was also used to determine the slopes of the dose-response curves and the dose of flurazepam expected to produce a decrease in body temperature of 3°C ($ED_{3°}$). Three degrees was chosen to reflect approximately 50% of the maximal decrease in body temperature from which the animals could recover.

Determination of the Anticonvulsant Properties of Flurazepam

To assess the anticonvulsant effects of flurazepam in LS and SS mice, experiments were conducted at doses of MP that were equal to or greater than those doses which we have previously found to induce seizure activity in 100% of the animals from both lines of mice (26). Prior to measurement of seizure susceptibility, all animals were placed in an isolated, air conditioned, testing room with 34-watt overhead fluorescent lighting for at least 1 hr. The mice were injected with various doses of flurazepam (1–6 mg/kg IP) or saline, in an injection volume of 0.01 ml/g, 30 min prior to the administration of MP. The protocol for the determination of susceptibility to MP-induced seizures was identical to that previously reported (26). MP was dissolved in 0.9% saline and administered IP in a volume of 0.02 ml/g. All solutions were prepared fresh daily.

Mice were placed individually in a 1.5-liter Pyrex jar for observation of seizure activity. Latencies from injection of MP to clonus or wild running were recorded to the nearest 5 sec. These stages of seizure activity were identified as follows: 1) clonus was defined as the loss of body posture, with convulsive movements in all extremities and 2) wild running consisted of a bout of uninhibited running and jumping, distinct from the running and jumping associated with a fear response. For purposes of analysis, the onset of seizure activity was defined as the first occurrence of either clonus or wild running. Observation periods were limited to 15 min and any animal not displaying seizure activity within this

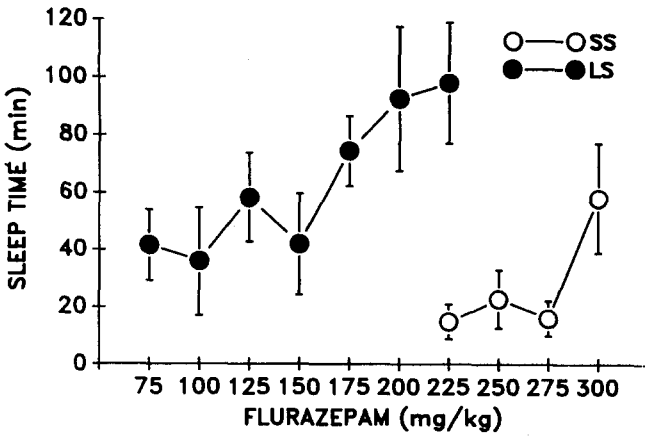

FIG. 1. Flurazepam-induced loss of righting response in female LS and SS mice. Each point represents the mean±S.E.M. duration of loss of righting response for 7–9 mice.

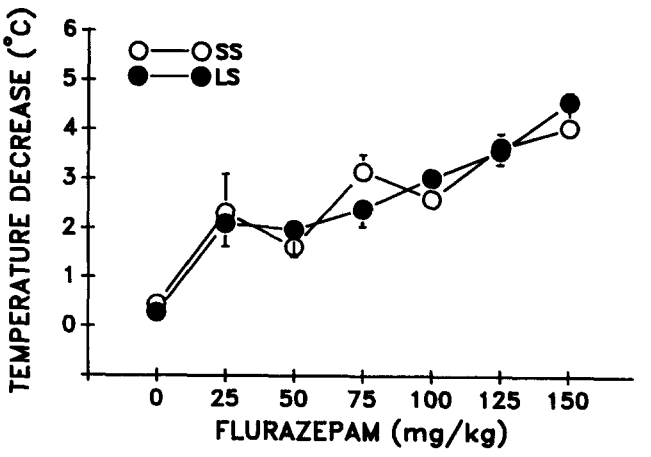

FIG. 2. Flurazepam-induced hypothermia in female LS and SS mice. Each point reflects the mean±S.E.M. decrease in body temperature 30 min after the administration of flurazepam for 6 mice.

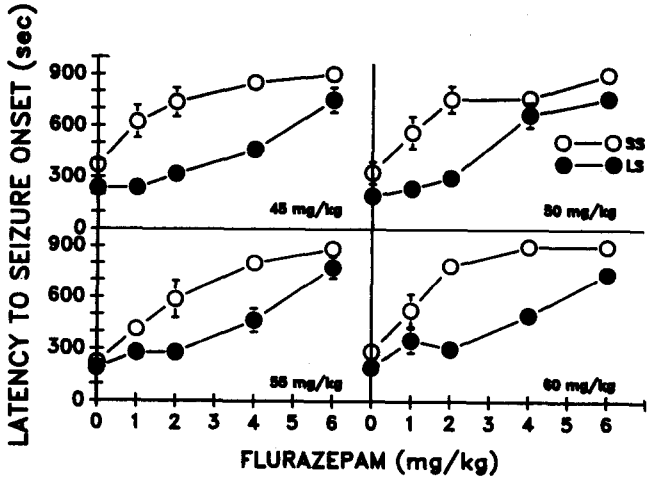

FIG. 3. Anticonvulsant actions of flurazepam against seizures induced by various doses of 3-mercaptopropionic acid in female LS and SS mice. Each point represents the mean±S.E.M. latency to the onset of seizure for 6–11 mice.

period was given a score of 900 sec. Separate dose-response analyses of the anticonvulsant properties of flurazepam in LS and SS mice were conducted at 45, 50, 55 and 60 mg/kg MP. Latencies from the injection of MP to the onset of seizure activity were analyzed using ANOVA techniques to assess the effects of line and dose of flurazepam at each dose of MP. Slopes and ED_{600} values (the dose of flurazepam predicted to produce an average latency to the onset of seizure activity of 600 sec) were calculated by least squares linear regression on each dose-response curve.

RESULTS

Flurazepam induced a loss of righting response in both LS and SS mice, but at different dose ranges (Fig. 1). The LS mice were more sensitive to the anesthetic effects of flurazepam than were the SS mice. Only at a dose of 225 mg/kg did animals from both lines lose the righting response. The similarity of slopes of the dose-response curves for the LS (0.44±0.04) and for the SS mice (0.52±0.02) indicated that the curves were parallel. However, the anesthetic potency of flurazepam, as reflected by the ED_{60} values (the dose required to produce an average sleep time of 60 min), was significantly greater in LS mice, $t(81)=5.1$, $p<0.001$. The ED_{60} values were 143.9±16.5 for the LS mice and 321.2±15.1 for the SS mice.

Administration of flurazepam resulted in a dose-dependent decrease in body temperature for both lines of mice (Fig. 2). The two lines, however, did not differ in their hypothermic response to flurazepam. The dose-response curves for the two lines were characterized by slopes of 0.024±0.001 and 0.021±0.001 and by ED_{3° values of 96.9±8.9 and 93.3±5.8 for LS and SS mice, respectively.

The antagonism of the onset of MP-induced seizures, by flurazepam, is illustrated in Fig. 3 for the four doses of MP tested. Flurazepam delayed the onset of MP-induced seizures in a dose-dependent manner in both lines of mice [$F(4,86)=25.2$, $p<0.001$; $F(4,84)=34.6$, $p<0.001$; $F(4,71)=43.7$, $p<0.001$; and $F(4,74)=35.2$, $p<0.001$, at 45, 50, 55, and 60 mg/kg MP, respectively]. We have previously observed (26) that SS mice were more resistant to MP-induced seizures than LS mice. This difference in seizure susceptibility is reflected in the present study by a greater resistance to MP-induced seizures for the SS line in the absence of flurazepam (0 mg/kg) at all doses of MP except 55 mg/kg. The dose-response curves for the effects of flurazepam on seizure latency were significantly different between LS and SS mice at all doses of MP tested [$F(1,86)=68.9$, $p<0.001$; $F(1,84)=46.3$, $p<0.001$; $F(1,71)=28.9$, $p<0.001$; and $F(1,74)=45.8$, $p<0.001$, at 45, 50, 55, and 60 mg/kg MP, respectively]. However, as some of the differences between the two lines reflect baseline differences in seizures susceptibility, it was important to examine the interaction between the effects due to line differences and the effects due to the dose of MP. At all doses of MP significant dose-by-line interactions were observed [$F(4,86)=2.9$, $p<0.05$; $F(4,84)=3.9$, $p<0.01$; $F(4,71)=3.0$, $p<0.05$; and $F(4,74)=5.2$, $p<0.001$, at 45, 50, 55, and 60 mg/kg, respectively]. Slopes and ED_{600} values for these dose-response curves are presented in Table 1. Inspection of the tabled values indicates that the anticonvulsant potency of

TABLE 1

SLOPE AND ED_{600} VALUES FOR THE INHIBITION OF MP-INDUCED SEIZURES BY FLURAZEPAM

	ED_{600}*		Slope†	
MP (mg/kg)	SS	LS	SS	LS
45	1.34 ± 0.36	4.84 ± 0.24	110.7 ± 4.11	87.2 ± 1.34
50	1.72 ± 0.41	4.20 ± 0.20	102.3 ± 4.60	106.0 ± 1.35
55	2.42 ± 0.24	4.72 ± 0.23	142.6 ± 3.98	94.9 ± 1.47
60	1.28 ± 0.16	4.79 ± 0.26	250.7 ± 9.01	84.6 ± 1.48

*ED_{600} values represent the dose of flurazepam (mg/kg ± S.E.E.) expected to produce a latency of 600 sec to seizure onset.

†Slope values represent the slope (±S.E.E.) of the seizure latencies regressed on the dose of flurazepam.

flurazepam, as reflected by the ED_{600} values, is from two to four times greater in SS mice than in LS mice.

DISCUSSION

The results presented here illustrate that LS mice are much more sensitive than SS mice to the anesthetic effects of flurazepam. LS mice have also been shown to be more sensitive to the anesthetic effects of the benzodiazepine, chlordiazepoxide (23). Thus, it appears that the LS mice which are more sensitive to anesthetic effects of ethanol are also more sensitive to the anesthetic properties of benzodiazepines than are SS mice. There is some evidence that the loss of righting response is mediated by the cerebellum (33). Previous studies have indicated that ethanol-induced inhibition of spontaneous firing of cerebellar Purkinje cells is different in LS and SS mice (33). However, it is unknown whether benzodiazepines produce similar differential actions in electrophysiological preparations from these mice. Evaluations of various components of GABA receptor complex in cerebellar tissue have been performed. There is no significant difference in the number or affinity of benzodiazepine receptors in LS and SS cerebellar tissue measured by ^{3}H-flunitrazepam binding or ^{3}H-Ro 15-1788 binding (24,25), or in heat-denaturation patterns for cerebellar benzodiazepine receptors (24). However, GABA protection from heat denaturation of cerebellar benzodiazepine receptors is greater in LS mice (24). The functional status of receptor as measured using $^{36}Cl^-$ flux also has been revealing. In LS mice muscimol is a more potent stimulator of $^{36}Cl^-$-flux in microsacs prepared from LS cerebellar tissue (1). Moreover, ethanol potentiates muscimol-stimulated $^{36}Cl^-$-flux in this preparation from LS mice, but not from SS mice (1). Because these differences are observed in the absence of a difference for benzodiazepine binding (25), or high affinity muscimol binding (1), it may be that subtle differences in coupling of receptors to the ionophore exist between GABAergic receptor systems of LS and SS cerebellum.

Mechanistic differences between the actions of ethanol and flurazepam are supported by the results of our hypothermia experiments. LS and SS mice do not differ in their hypothermic response to the benzodiazepine, flurazepam, whereas they do differ in their hypothermic response to ethanol (13). These results indicate that GABA/benzodiazepine influences on thermoregulation are probably the same in LS and SS mice. The lack of similar patterns of differences between the two lines for benzodiazepine-induced anesthesia and benzodiazepine-induced hypothermia implies that benzodiazepines induce these two responses via different mechanisms and/or through actions in different brain regions. Thermoregulation is believed to be mediated primarily via components of the hypothalamus (15), while the evidence regarding the loss of the righting response in LS and SS mice suggest a cerebellar involvement (33). Given the multimeric nature of the GABA receptor complex, it is also conceivable that the components of the receptor complex could be differentially coupled in the different regions of the brain responsible for thermoregulation and anesthesia. At the benzodiazepine receptor level, no differences in the number of hypothalamic receptors have been observed (26). Functional studies of hypothalamic GABA-stimulated Cl^- flux have not, however, been performed.

SS mice are substantially more sensitive than LS mice to the anticonvulsant effects of flurazepam. The greater sensitivity of SS mice to the anticonvulsant properties of benzodiazepines, however, is in contrast to their lesser sensitivity to the anesthetic effects and their equal sensitivity to the hypothermic effects of benzodiazepines. This again indicates that the various behavior correlates of GABA and benzodiazepine actions probably are the result of different mechanisms of action and/or differential control by different brain regions.

The cortex, hippocampus and midbrain areas have been implicated in the origination and propogation of seizures (3, 30, 31). Cortex and hippocampus do not differ in the number of benzodiazepine receptors as measured by ^{3}H-flunitrazepam or ^{3}H-Ro 15-1788 binding [(24), and unpublished data]. However, we have previously reported that LS and SS mice differ in the degree of GABA enhancement of flunitrazepam binding in forebrain tissue (cortex containing striatum and hippocampus) with enhancement being greater in SS mice (25). A correlational study in several genetic stocks of mice showed that the degree of GABA enhancement was correlated to the resistance to seizure induction by MP (26). If forebrain areas are involved in the control of seizure activity, it may be that SS mice are more sensitive to the anticonvulsant effects of flurazepam because they have a more tightly coupled interaction between the benzodiazepine and GABA receptor. LS and SS mice differ in specific ^{3}H-flunitrazepam binding in the midbrain (25). This difference may also account for their differences in sensitivity to the anticonvulsant actions of flurazepam.

It is interesting to note that the concentrations of

flurazepam required for the three measures reported here are quite different. Benzodiazepines exert their anticonvulsant properties at relatively low doses (less than 5 mg/kg), whereas their anesthetic actions are apparent at doses 10 to 20 times greater. The hypothermic effects occur in a range that is intermediate to the anticonvulsant and anesthetic effects. These differences could relate to the percent of receptors that are occupied at a given concentration of flurazepam as well as to the benzodiazepine concentration present in various brain regions after an IP injection of flurazepam. Braestrup and Nielsen (2) have estimated the percent of benzodiazepine receptors that must be occupied to elicit different responses to benzodiazepines. A higher percentage occupancy was required for anxiolytic effects than for anticonvulsant effects. Conceivably, LS and SS mice could differ in the percent of receptors required to elicit the anticonvulsant and/or anesthetic responses.

A comparison of the results of previous work using both lipid- and water-soluble alcohols and barbiturates (27) with the results from the flurazepam-induced sleep-time experiments might lead to the prediction that LS and SS mice would not differ in their anesthetic response to benzodiazepine because these molecules are very lipid-soluble. While this prediction is not supported in the present work, it should be noted that flurazepam is one of the more water-soluble benzodiazepines. In order to perform a correlational analysis, it will be necessary to perform more extensive studies with a series of benzodiazepines.

In summary, ethanol-sensitive LS mice are also more sensitive to the anesthetic properties of benzodiazepines, while the ethanol- resistant SS mice are more sensitive to the anticonvulsant actions of the benzodiazepine, flurazepam. The two lines do not differ in their hypothermic responsiveness to flurazepam. These studies demonstrate that LS and SS mice differ in response to benzodiazepines, but the nature of these differences is dependent on the type of response measured and/or the dose of the drug employed.

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