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Effects of Pentobarbital in Mice Selected for Differential Sensitivity to Ethanol

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O'CONNOR, M. F., T. C. HOWERTON AND A. C. COLLINS. *Effects of pentobarbital in mice selected for differential sensitivity to ethanol.* PHARMAC. BIOCHEM. BEHAV. 17(2) 245–248, 1982.—Two lines of mice have been genetically selected for differential sensitivity to ethanol. These lines have been designated long sleep (LS) and short sleep (SS) on the basis of their hypnotic response to the ethanol selection dose. Earlier studies of these mice suggested that this difference was limited to alcohols and did not extend to other classes of hypnotics. The present study examined hypnotic and hypothermic responses produced by pentobarbital in recent generations of these mice. Dose-dependent differences in sleep time and in hypothermia were found, with SS mice affected to a greater degree than LS mice. Pharmacokinetic studies showed that the half-life of pentobarbital disappearence from SS blood was twice that reported for SS mice of the 18th generation. The half-life in the LS line had not changed. The volumes of distribution and waking brain concentrations were identical in LS and SS mice. An altered rate of elimination (not differential CNS sensitivity) appeared to be the major factor responsible for the differences observed between these lines.

Pentobarbital Ethanol sensitivity Hypnosis Hypothermia Pharmacogenetics Pharmacokinetics

NUMEROUS studies have attempted to provide an explanation for the behavioral effects of depressant drugs such as barbiturates and ethanol. The observation that ethanol and barbiturates, such as pentobarbital, cause behavioral activation at low doses and depression at higher doses [6] suggests similar mechanisms or sites of action. Several investigators have attempted to ascertain whether ethanol and barbiturates have similar actions by studying the long-sleep (LS) and short-sleep (SS) lines of mice. These mouse lines were selectively bred for differential sensitivity to hypnotic doses of ethanol [6]. The foundation population for the breeding program was a heterogeneous stock (HS) that had been derived by intercrossing eight inbred mouse strains (A, AKR, BALB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII). Animals from the HS stock were injected with a hypnotic dose of ethanol, and duration of the loss of righting reflex (ethanol-induced "sleep time") was recorded. Mice with the shortest sleep times were mated to produce the progenitors of the SS line, while mice which slept the longest were used as parents of the initial LS generation. Selection pressure has been maintained (exception for generations 6, 7 and 19–24) with a resultant increase in the differential sensitivity to ethanol.

The LS and SS lines of mice differ in duration of ethanol-induced sleep time primarily because of a difference in CNS sensitivity to the depressant effects of ethanol. The difference in rate of ethanol metabolism between lines is not of sufficient magnitude to explain the difference in ethanol-induced sleep time [4].

Several studies [1, 8, 9] have examined the effects of non-alcohol hypnotics in these lines. Two of these studies [1,8] found no difference between the lines with respect to pentobarbital-induced sleep time, while the third [9] indicated that the SS mice slept longer than the LS when given this barbiturate. The latter study also found no differences between the lines in the rate of disappearance of pentobarbital from the blood, or in brain pentobarbital concentrations upon regaining the righting reflex. These three experiments were conducted on different generations earlier in the selective breeding program, and increased duration of pentobarbital-induced sleep was seen in both lines in later generations (Fig. 1). When the effects of low doses of ethanol and pentobarbital on open-field activity were examined [7], mice of the SS line were found to be activated to a greater degree by both ethanol and pentobarbital than were LS mice.

The possibility that the two lines may have developed a differential response to the hypnotic actions of other depressants during the selection for ethanol sensitivity has already been proposed and rejected [8]. However, this rejection was based on data from mice up to the 21st generation. Recently, using 29th generation animals, we tested LS and SS mice with pentobarbital in the course of studies on barbiturate dependence and found greater differences than previ-

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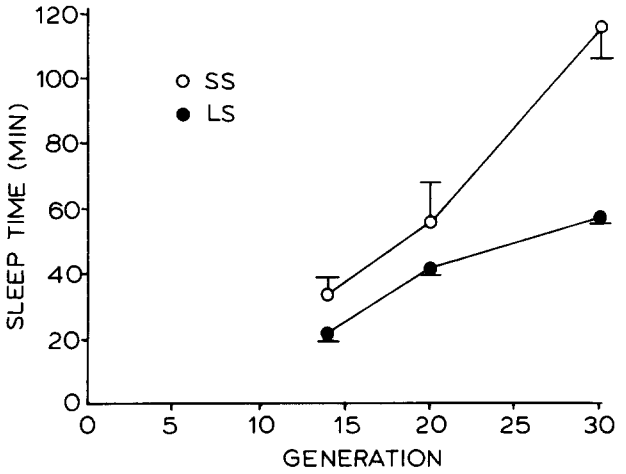

FIG. 1. Mean sleep time±S.E.M. of LS and SS mice produced by sodium pentobarbital (60 mg/kg, IP) at different generations. Values for the 14th and the 19th–21st generations were taken from [1] and [8], respectively. The value for the 28th–31st generations was calculated from sleep times of 15 mice per line.

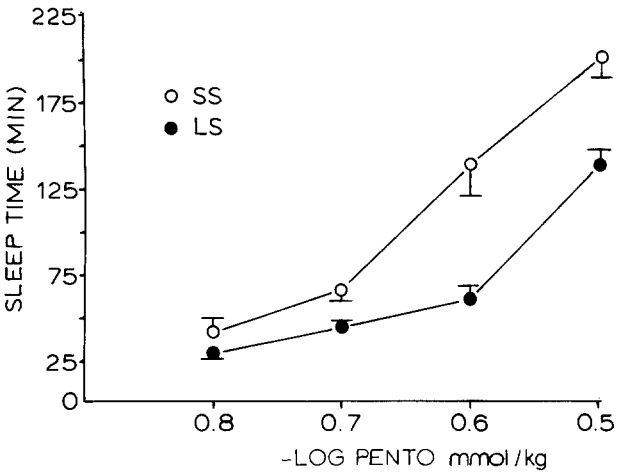

FIG. 2. Mean sleep time±S.E.M. of LS and SS mice following IP injection of sodium pentobarbital. Negative logs of the doses correspond to 0.158, 0.198, 0.249, and 0.314 mmol sodium pentobarbital/kg (N=8).

ously reported. Since selection pressure has been reapplied on the LS and SS lines, it seemed reasonable to examine again the possibility that mice selected for differences in sensitivity to ethanol may have also developed a differential response to pentobarbital. This paper presents the results of those studies.

METHOD

Male and female LS and SS mice of generations 28–31 from the Institute for Behavioral Genetics were used in all experiments. All mice were 60–90 days old at time of testing. Sodium pentobarbital (PENTO) dissolved in saline was injected IP in a volume of 0.01 ml/g. Doses were calculated as mmol/kg and are presented in some figures as negative logs, e.g., −log 0.249 mmol/kg=0.6. Sleep time was measured as described previously [8]. Rectal temperatures were taken every 30 min with a 2.5-cm probe attached to a Digitec 5810 thermometer. Sleep time and temperature were assessed in the same animals.

For the pharmacokinetic experiments, groups of six mice per line were injected with 1 μCi ^{14}C-pentobarbital (New England Nuclear) and 60 mg/kg sodium pentobarbital and were sacrificed at specific times. Blood (75 μl), brain, and fat (50–100 mg, removed from between the stomach and spleen) samples were obtained from each mouse and homogenized in 1.0 or 1.5 ml (brain) of 0.1 M acetate buffer, pH 5, which contained 10% w/v NaCl. Unchanged ^{14}C-pentobarbital was extracted according to the procedure of Siemens and Chan [9]. Following evaporation, radioactivity in the samples was determined in a Beckman LS 7000 liquid scintillation counter. Recovery of radioactive pentobarbital using this procedure was essentially complete for blood, 96–98% for fat, and 89–92% for brain.

RESULTS

Two studies [1,8] which examined the hypnotic effect of pentobarbital in earlier generations of LS and SS mice used a dose of 60 mg/kg (0.242 mmol/kg). No difference was found between the lines in these studies. The data presented in Fig. 1 for mice of the 28th–31st generations tested with the same dose indicate a significant difference in sleep times between the LS and SS mice, $t=4.92$, $p<0.001$. This result is in agreement with a previous finding [9] that SS mice were more affected than LS mice by a 50 mg/kg (0.201 mmol/kg) dose of sodium pentobarbital. The ratio of average sleep times (SS:LS) in that study was 2 to 1. The value for this ratio in our study was comparable (1.95 to 1).

In order to determine if this difference would be evident over a range of doses, dose-response curves were constructed. Again, the results revealed SS mice to be more responsive to the depressant action of pentobarbital (Fig. 2; ANOVA $F(1,56)=34.7$, $p<0.001$). A significant line × dose interaction, $F(3,56)=4.2$, $p<0.009$, indicated that this difference was dose-dependent.

The divergence between lines shown in Fig. 1 suggests that differential responsiveness to pentobarbital has increased during generations 14–31. In the SS line, pentobarbital sleep time increased 65.7% from generation 14 to generations 19–21, while it increased 91.7% in the LS line. From the 14th to the 28th–31st generations, the increase was 150.4% in the LS line and 234.6% in the SS.

Hypothermia produced by pentobarbital, as measured by area under the temperature curves, confirmed that SS mice of the 28th–31st generations were more affected than LS mice by this agent (Fig. 3; ANOVA $F(1,56)=28.6$, $p<0.001$). As was seen with the sleep-time results, this line difference was also dose-dependent, $F(3,56)=5.1$, $p<0.003$.

The pharmacokinetic data from 20–120 min after injection (Figs. 4 and 5) were calculated according to the equation used by Siemens and Chan [9]: $\log y = \log y_0 - kx/2.3026$, where y is the pentobarbital concentration at time x, y_0 is the concentration at zero time, and k is the rate constant. Using this equation we did not find any major line differences in the apparent potential concentrations of pentobarbital at zero time in blood (Fig. 4; SS: 43.0 μg/ml vs LS: 45.6 μg/ml), brain (Fig. 5; SS: 53.4 μg/g vs LS: 63.0 μg/g), or fat tissue

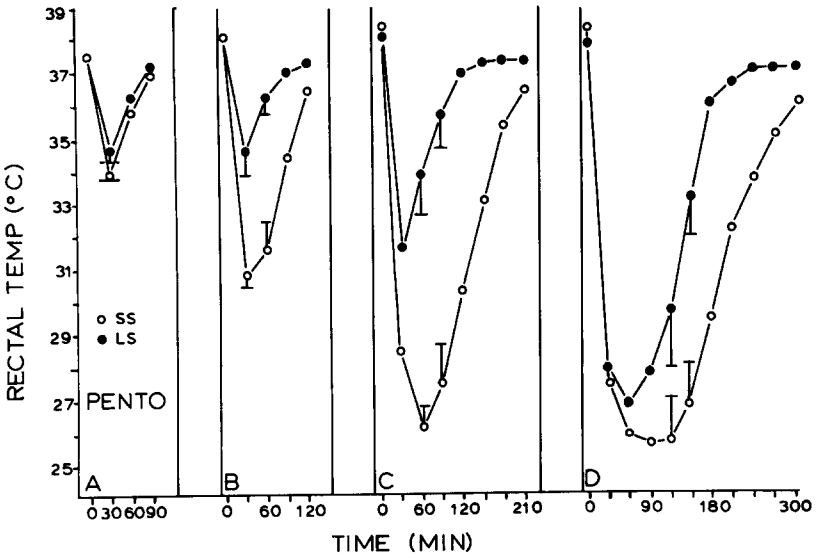

FIG. 3. Time course of sodium pentobarbital-induced hypothermia in LS and SS mice. Eight mice per line were injected IP with sodium pentobarbital at doses of 0.158 mmol/kg (A), 0.198 mmol/kg (B), 0.249 mmol/kg (C), or 0.314 mmol/kg (D). Symbols represent temperature means±S.E.M. Areas under the curves in units of degree × min were calculated for each line at each dose. The mean±S.E.M. areas at each dose were: LS—136.9±26.6, SS—151.9±18.7 (A); LS—214.3±26.1, SS—558.6±75.5 (B); LS—510.9±120.2, SS—1549.5±201.2 (C); LS—1523.8±183.7, SS—2559.2±322.9 (D).

(SS: 98.7 μg/g vs. LS; 110.5 μg/g). Consequently, there was no difference between lines in apparent volume of distribution. This finding is in contrast to an observation that LS mice have a greater volume of distribution than SS mice [9]. A large line difference did exist with respect to the half-life of pentobarbital. Substituting the calculated rate constant, k, into the equation for half-life, $t_{1/2}=0.693/k$, the half-lives of pentobarbital in blood, brain and fat of LS mice (34.7 min, 25.2 min, and 34.0 min, respectively) were much shorter than in the SS line (70.0 min, 62.4 min, and 89.5 min, respec-

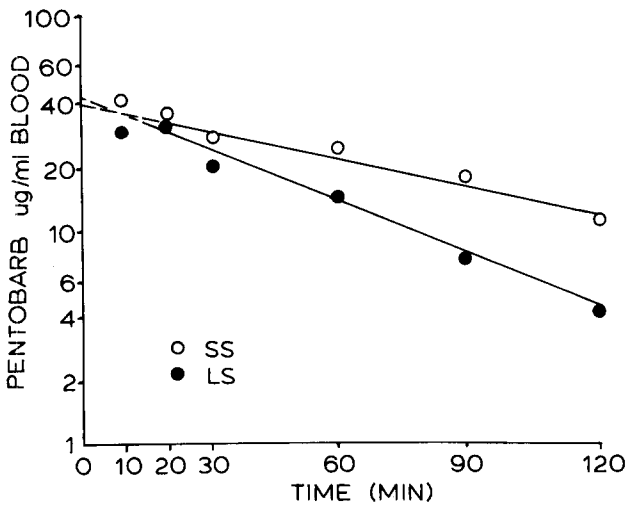

FIG. 4. Time course of ^{14}C-pentobarbital disappearence from blood of LS and SS mice. Each point represents the mean of 5–6 mice. Linear regression lines (solid lines) were calculated from 20–120 min after injection and extrapolated (dashed lines) to zero time. The rate constants (k) and the correlation coefficients (r) for the LS line were −0.0200 and 0.9905, respectively; for the SS line, they were −0.0099 and 0.9874.

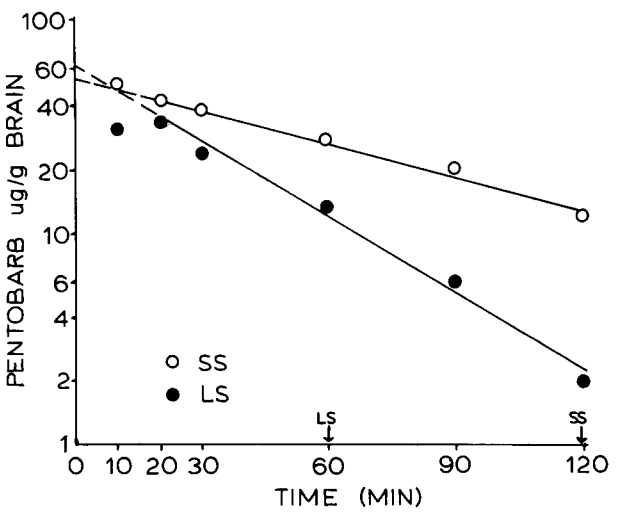

FIG. 5. The course of ^{14}C-pentobarbital disappearence from brain tissue of LS and SS mice. Arrows indicate average sleep times of LS and SS mice in the 28th–31st generations (taken from Fig. 1) for estimation of brain pentobarbital concentrations at time of regaining the righting reflex. The remaining information is the same as for Fig. 4, except that k and r were as follows: for the LS line, −0.0275 and 0.9931; for the SS line, −0.0111 and 0.9878.

tively). Of note is the fact that the half-life for pentobarbital in blood of LS mice (34.7 min was very similar to that found earlier (33 min), whereas the corresponding value for SS mice (70 min) is double the previously reported estimate of 34 min [9].

DISCUSSION

With the exception of generations 6, 7 and 19–24, the LS and SS lines have been continually subjected to selection pressure. For the greater portion of this time, the ethanol challenge dose was 4.2 g/kg. When selection was reinitiated in generation 25, it was observed that a majority of SS mice failed to lose the righting reflex, while the LS mice exhibited toxic effects of ethanol (i.e., the most sensitive animals died). In order to obtain maximal sensitivity differences, the selection dose was changed in generation 26. The dose for the LS line was reduced to 3.8 g/kg, while that for the SS mice was increased to 4.7 g/kg. This change seems to have resulted in a number of changes in the characteristics of the LS and SS lines. For example, Heston *et al.* [4] failed to detect a difference in the rate of ethanol elimination between the two lines of mice. This observation had been replicated countless times in our laboratory. However, recent studies [3,5] have detected a difference between the lines, with the SS mice exhibiting a slightly faster rate of ethanol metabolism than the LS. Thus, it appears that the change in selection pressure may be extracting additional genes which influence responsiveness to ethanol.

Since additional selection pressure seems to be bringing out ethanol elimination differences in the LS and SS mice, it is not surprising to see a difference in pentobarbital elimination rate between the lines. What is surprising is the observation that LS mice eliminate pentobarbital more rapidly than do SS mice, thereby explaining the shorter pentobarbital-induced sleep time seen in the LS mice in the present experiment. The difference in sleep time does not appear to be related to a differential CNS sensitivity to pentobarbital. This conclusion is drawn from the observation that mean brain pentobarbital concentrations, as extrapolated from the disappearance curves (Fig. 5), are nearly identical at the times the two lines regain the righting reflex. Siemens and Chan [9] noted a modest pentobarbital-induced sleep time difference between the LS and SS lines using generation 18 animals, but this difference did not seem to be due to differences in the rate of elimination of pentobarbital. That study also failed to detect CNS sensitivity differences for pentobarbital. It may be that those factors which are leading to increased ethanol metabolism rate in the SS mice are also leading to decreased pentobarbital elimination.

French *et al.* [2] examined a number of hepatic enzymes related to drug metabolism in generation 21 LS and SS mice. These investigators reported greater concentrations of cytochrome P-450 in LS mice, as well as greater activities of aniline hydroxylase, ethylmorphine-N-demethylase, and benzphetamine-N-demethylase. It seems reasonable to suggest that the faster rate of pentobarbital elimination in the LS mice may be due to this difference in cytochrome P-450 or in the activities of oxidase enzymes linked to this cytochrome. Whether the altered selection pressure has influenced cytochrome P-450 levels or drug metabolizing enzyme activities has not been studied. Our findings suggest that such studies might produce interesting results. Similarly, investigators of the cytochrome P-450 hepatic microsomal ethanol oxidizing system (MEOS) have failed to observe a difference between the lines in basal activity of this system [2,5]. Our ethanol and pentobarbital elimination studies suggest that further investigation of the MEOS in the LS and SS mice may be useful not only in explaining the metabolism differences between the two lines, but also in assessing the relationship between ethanol metabolism and the metabolism of barbiturates or other drugs.

In addition, our studies provide further data which argue that pentobarbital and ethanol have different mechanisms or sites of action. This conclusion follows from the observation the LS and SS mice regain the righting reflex following pentobarbital at nearly identical brain concentrations, while the two lines differ markedly in blood and brain ethanol concentrations at time of regaining the righting reflex. Further studies are currently in progress to describe more completely the genetic and biochemical influences on pentobarbital and ethanol actions in these selected mouse lines.

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