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Sedative-Hypnotic Anomalies Related to Dose of Pentobarbital in Long-Sleep and Short-Sleep Selectively-Bred Mice

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ALPERN, H. P. AND T. D. MCINTYRE. *Sedative-hypnotic anomalies related to dose of pentobarbital in long-sleep and short-sleep selectively-bred mice.* PHARMACOL BIOCHEM BEHAV **25**(2) 333–336, 1986.—Hypnotic effects following administration of three doses of pentobarbital were evaluated in mice selectively-bred for differential hypnotic sensitivity to ethanol. Although the ethanol-sensitive Long-Sleep (LS) line displays greater sedation to a wide variety of CNS depressants (alcohols, barbiturates, benzodiazepines, general anesthetics), when compared to the ethanol-insensitive Short-Sleep (SS) line, the response pattern to pentobarbital remains equivocal. Thus, to clarify the effect of pentobarbital, certain variables (dose, sex, circadian rhythmicity) believed to be important in the expression of sleep time were evaluated. For all doses examined "sex" and "time of day tested" impacted on sleep time. With these provisos, 40 mg/kg consistently induced shorter sleep time in SS mice. The 60 mg/kg dose either failed to distinguish these two lines, or induced greater sleep times in the SS mice. The 80 mg/kg dose tended to have the same effect as the 60 mg/kg dose, but to a greater degree. Overall, it appears that for each line the dose response curve for pentobarbital is sigmoidal, but that the slope of the curve for the middle range of doses is greater for the SS line. Since pentobarbital has a unique effect on these lines of mice that is dissimilar to those reported for other barbiturates, the implication is that an additional factor, that is unimportant for other barbiturates, is essential for pentobarbital-induced hypnosis. Factors that could be responsible for this effect include differential metabolism or Gabaergic receptor dynamics.

Pharmacogenetics Selected lines Alcohol Pentobarbital Sedation/hypnosis Circadian rhythms Sex differences

THE LS and SS lines of mice that were selectively-bred for different hypnotic reactions to a sedative dose of ethanol are interesting in that the most recent evidence suggests that they also display similar patterns of response to a wide variety of CNS depressants. For instance, the LS line has been shown to be more sensitive to alcohols [5], barbiturates [1,11], benzodiazepines [11], general anesthetics [9,13], and other miscellaneous agents such as L-phenylisopropyl adenosine [4]. Nonetheless, certain findings with pentobarbital and alcohols are used to support the idea that these lines are uniquely sensitive to the hypnotic effects of alcohol [3,12]. The conclusion that the hypnotic reactions displayed by these lines are alcohol-specific depends almost entirely on a study which examined how these lines responded to the hypnotic effects of ethanol, methanol, n-butanol, pentobarbital, paraldehyde, chloral hydrate and trichloroethanol [5]. In that report it was concluded that only the aliphatic alcohols were able to separate the mouse lines. Shortly thereafter it was reported that SS mice are more sensitive than LS mice to the hypnotic effects of pentobarbital [14]. Others, however, did not replicate this finding [13]; but, more recently, certain evidence again indicates that SS mice are more sensitive to pentobarbital than LS mice [3, 8, 12]. Hence, some have hypothesized that concomitant selection for pentobarbital hypnotic sensitivity occurred, but in a direction opposite to that of ethanol [12]. On the other hand, it has also been reported that LS mice are more sensitive to pentobarbital than SS mice [1].

Examination of the reports which found that SS mice are more sensitive than LS mice to the hypnotic effects of pentobarbital indicates that a dose, circadian and/or sex effect could have influenced the data. For example, it is well documented that barbiturates show circadian rhythmicity with respect to the potency of their anesthetic effects and with these mouse lines circadian effects on blood ethanol elimination rates have been reported [6]. Specifically, in one report investigators used only 50 mg/kg of pentobarbital, began testing at 1600 hr and combined data from both sexes [14]. In another report other investigators began testing males at 0730 hr and found opposite results for 50 mg/kg of pentobarbital [1]. When testing began at 0600 hr with 60 mg/kg of pentobarbital (again just one dose was used) the mouse lines were not different [13]. Where several doses of pentobarbital were employed, SS mice appeared to be the more sensitive line [12]. Unfortunately, in this report, the time of day that testing began was not reported and each drug group contained a mixture of male and female mice. Further, the greatest difference between the two lines was found at 62 and 78 mg/kg doses. With the two lower doses employed (39 mg/kg and 49 mg/kg), which is approximately

TABLE 1

MEDIAN SLEEP TIME (MIN) FOR 40 mg/kg OF PENTOBARBITAL SODIUM ADMINISTERED TO LONG-SLEEP AND SHORT-SLEEP MALE AND FEMALE MICE AT 0800, 1600 AND 2400 HR

		Time		
		0800	1600	2400
LS	Males	17.0[a]	27.0[b]	0[c]
	Females	21.0[d]	12.5[e]	5.5[f]
SS	Males	0[g]	0[h]	0[j]
	Females	0[k]	0[m]	0[n]

Significant group comparisons referred to in text: a–g ($p<0.01$), b–h ($p<0.001$), d–k ($p<0.001$), e–m ($p<0.05$), f–n ($p<0.05$), b–e ($p<0.01$), c–f ($p<0.05$), b–c ($p<0.001$), e–f ($p<0.05$).

TABLE 2

MEAN SLEEP TIME (MIN) ± S.E. FOR 60 mg/kg OF PENTOBARBITAL SODIUM ADMINISTERED TO LONG SLEEP AND SHORT SLEEP MALE AND FEMALE MICE AT 0800, 1600 AND 2400 HR

		Time		
		0800	1600	2400
LS	Males	24.3 ± 5.3	43.9 ± 3.3	51.4 ± 10.7
	Females	35.0 ± 7.4	51.8 ± 4.3	26.9 ± 3.8
SS	Males	56.3 ± 5.4	52.9 ± 5.7	53.4 ± 2.6
	Females	36.0 ± 6.9	47.3 ± 7.9	43.5 ± 11.0

TABLE 3

MEAN SLEEP TIME (MIN) ± S.E. for 80 mg/kg OF PENTOBARBITAL SODIUM ADMINISTERED TO LONG SLEEP AND SHORT SLEEP MALE AND FEMALE MICE AT 0800, 1600 AND 2400 HR

		Time		
		0800	1600	2400
LS	Males	87.1 ± 6.1	99.4 ± 3.5	94.9 ± 9.4
	Females	102.4 ± 15.0	92.3 ± 10.8	81.3 ± 8.7
SS	Males	107.3 ± 12.1	176.6 ± 12.4	133.0 ± 14.8
	Females	147.5 ± 11.4	102.5 ± 14.1	107.9 ± 16.6

the doses where we [1] found our greatest effect (35, 40 and 50 mg/kg), the magnitude of the line differences was greatly attenuated. In the last of these reports [3], the authors used a sample of male mice but did not report the time of day that testing was conducted and employed only a single dose (65 mg/kg) of pentobarbital.

The above notwithstanding, we believe that perhaps the most critical finding concerns the results of the seminal paper of this field, where it was reported that the two mouse lines could only be differentiated by aliphatic alcohols [5]. If, however, one analyzes the data in that paper with *t*-tests, using the summary statistics provided, it can be shown that every CNS depressant employed actually differentiated the two lines of mice [1]. Thus, not only did aliphatic alcohols separate the lines, but as long ago as the fourteenth generation LS mice were more sensitive than SS mice to the hypnotic effects of chloral hydrate, paraldehyde, and trichloroethanol. Interestingly, LS mice were less sensitive than SS mice to pentobarbital. Other investigators have confirmed the results of the reanalyzed data for paraldehyde and trichloroethanol using animals from generations 19–21 [13]. The conclusion, therefore, that these lines are uniquely distinguishable by aliphatic alcohols is clearly not supported. Not only can it be shown that they are different with respect to many hypnotic-depressants [1, 4, 9, 11], the reanalyzed data from the 14th generation demonstrate that this is not a recent phenomenon; and thus, supports the notion that these lines of mice were selected for more general aspects of neural functioning than specific alcohol sensitivity.

Nevertheless, reasons accounting for the ambiguous findings with pentobarbital require further inquiry. Consequently, in this experiment three doses of pentobarbital were evaluated. The lowest dose (40 mg/kg) was selected because it had been shown to produce hypnotic response patterns similar to other depressants [1]. The second dose (60 mg/kg) was chosen because it either had failed to distinguish the two lines [13], or had differentiated them in a direction opposite to that of other depressants ([5], see also [1]). The last dose (80 mg/kg) was chosen in order to ascertain the upper end of the dose-response function. Moreover, sex and time of day that the drug was administered were systematically manipulated, since there are reasons to believe that these factors contributed to the ambiguous results cited above.

METHOD

Forty-eight LS (24 male and 24 female) mice and 48 SS (24 male and 24 female) mice were divided equally into independent groups that were administered 40 mg/kg of pentobarbital sodium at 0800 hr, 1600 hr and 2400 hr. Employing the same design, 96 animals were administered 60 mg/kg pentobarbital sodium and 96 animals were administered 80 mg/kg pentobarbital sodium. The animals were tested at 150 days of age and were descendants of the 39th production generation which were obtained from the Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309. Littermates were housed together, but randomly distributed across experimental groups. The animals had continuous access to food and water and were maintained on a 12 hr light/dark cycle. With respect to the latter, it should be noted animals were tested under low-intensity red light at 2400 hr. Pentobarbital sodium dissolved in 0.9% saline was injected intraperitoneally in a volume of 0.1 ml/g body weight. After injection an animal was placed on its back in a V-shaped (90° angle) Plexiglas sleep trough until it was not able to right itself four times within 60 seconds, at which time it was considered to have lost its righting reflex. An animal regained its righting reflex when it was able to right itself four times within 60 seconds. Duration from loss to reacquisition of the righting reflex was considered its sleep time. It should be noted that no animal died from the experimental protocol.

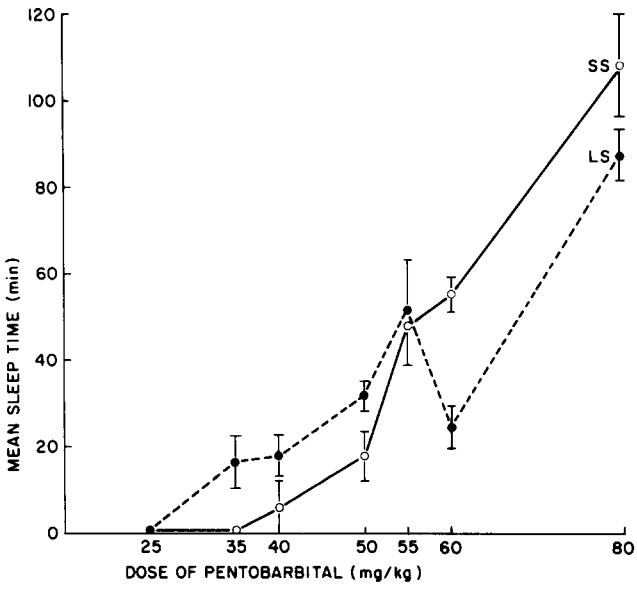

FIG. 1. Mean sleep time (min) in LS and SS male mice as a function of dose of pentobarbital sodium administered at 0800 hr. Data for the 60 and 80 mg/kg doses are from this experiment (n=8 for each point), whereas the other data points (n=5) come from a previous report [1]. Although the 40 mg/kg data points from this experiment are not included in the figure their means (for SS males, 3.37±2.52; for LS males, 21.00±4.69) emphasize the replicability of the data. Both linear correlations between sleep time and dose are highly significant (for LS: r(39)=.82, $p<0.001$; for SS: r(39)=.88, $p<0.001$). Additionally, the slopes of the regression lines (1.46 for LS and 2.15 for SS) are significantly different from each other ($t(78)=2.46$, $p<0.01$ for a one-tailed test).

RESULTS

The major finding, with certain key exceptions, is that hypnotic sensitivities of the two mouse lines to 40 mg/kg of pentobarbital is opposite to that displayed to 80 mg/kg, and it appears that 60 mg/kg is close to the actual dose at which this reversal occurs. The exceptions to this generalization are that the effects do not occur at all of the hours tested for both sexes.

For 40 mg/kg of pentobarbital (see Table 1), it is obvious that the LS line displays greater median sleep times in comparison to the SS line, with the exception of males tested at 2400 hr, where there is no difference. Further, both male and female LS groups tested at 2400 hr are different than their respective counterparts tested at 0800 hr and 1600 hr. Since variances across groups were not homogenous (i.e., within particular groups a majority of the scores were identical), these data were statistically analyzed with the Rank Sum test, which is the non-parametric equivalent of the t-test for two independent groups [2]. For the same reason, in Table 1, medians rather than means are given, and significant individual comparisons are presented. Note that for a few of the comparisons an individual group was used more than once, which has an effect on level of significance. Unfortunately, there is no non-parametric statistical procedure permitting all possible group comparisons. Nevertheless, the bias with respect to level of significance is in the failure to detect significant differences, where in fact, real differences exist. This bias, therefore, has the most minimal, if any, consequence for our data analysis.

For 60 mg/kg of pentobarbital (see Table 2) the major finding is that males and females of both lines display similar sleep times, except that: SS males tested at 0800 hr sleep longer than LS males and at 2400 hr SS females sleep longer than LS females. A three-way analysis of variance (Line × Hour Tested × Sex) was used to analyzed these data. Line was the only significant effect found, $F(1,84)=4.64$, $p<0.05$. Protected t-tests were used to make group comparisons and LS males tested at 2400 hr slept significantly more than LS females ($p<0.05$). Further, LS males tested at 0800 slept less ($p<0.05$) than LS males tested at 1600 hr, and those tested at 2400 hr ($p<0.05$). LS females, when compared to SS females tested at 2400 hr, only approached statistical significance. Nevertheless, LS females tested at 2400 hr sleep significantly less than LS females tested at 1600 hr ($p<0.05$).

For 80 mg/kg of pentobarbital (see Table 3) the overall difference in sleep time between the LS and SS line (again with the SS line more sensitive) is even more marked than for the 60 mg/kg dose. For LS males and females mean sleep times are more or less constant for the three times of day tested, but such is not the case for the SS line. Further, it appears that there are no sex differences for the LS line, but notable ones for the SS line. An analysis of variance (Line × Hour Tested × Sex) was used to evaluate these data and significant effects were found for Line, $F(1,84)=28.38$, $p<0.001$, Hour Tested × Sex, $F(2,84)=8.76$, $p<0.01$, and Line × Hour Tested × Sex, $F(2,84)=3.78$, $p<0.05$. Protected t-tests used to compare individual groups confirm that for the LS line there were no sex differences at any time of day tested, but for the SS line there was a sex difference at 0800 hr ($p<0.05$) and at 1600 hr ($p<0.05$). Similarly, for the LS line, hour tested had no effect on sleep time. On the other hand, SS males had the greatest mean sleep time at 1600 hr ($p<0.05$) when compared to the means of 0800 hr and 2400 hr, and SS females had the greatest mean sleep time at 0800 hr ($p<0.05$) when compared to means at 1600 hr and 2400 hr.

DISCUSSION

Conclusions derived from this experiment accomodate many of the disparate findings associated with pentobarbital-induced narcosis in LS and SS mice. First, the results with 40 mg/kg of pentobarbital replicate previous findings [1] showing that LS mice are more sensitive than SS mice. Of particular importance is the finding that SS males and females were resistant to the hypnotic effects of pentobarbital at all times tested, whereas both sexes of the LS line displayed a circadian effect. Specifically, LS males and females exhibited significant sensitivity during the light-phase of the daily cycle, while becoming resistant to pentobarbital's effects at 2400 hr. Second, 60 mg/kg of pentobarbital was chosen because previous reports indicated that this dose does not separate the two lines [13], or that it distinguishes them in a direction opposite that of the 40 mg/kg dose ([5], see also [1]). The present results confirm both of these findings. For the most part, LS and SS mice are not different except that LS males tested at 0800 and LS females tested at 2400 hr were less sensitive in comparison to their respective SS counterparts. It is important to note that a circadian effect was not evident for males and females of the SS line, and that the differences described above are due solely to circadian shifts in hypnotic sensitivity in LS mice.

Third, the results for 80 mg/kg of pentobarbital demonstrate again that under certain conditions the lines are either not different, or that the SS line is more sensitive than the LS line. To illustrate this, males from both lines are not different when tested at 0800 hr, but are different at the other two times examined. On the other hand, females from both lines were only different when tested at 0800 hr. In contrast to what was found at the two lower doses, however, it was the SS and not the LS line that exhibited a circadian effect. Interestingly, as with males and females of the LS line at the two lower doses, peak hypnotic sensitivity for SS males and females occurred at different times of day. Thus, as is clear from the foregoing, not controlling for sex or the time of day that a drug is administered can lead to conflicting conclusions about how these mice respond to sedative-hypnotics.

In order to account for the above results, we hypothesize that hypnotic sensitivity in each line is best described by a sigmoidal dose-response function, but that the slope of the accelerating portion of the function is greater for the SS line. Support for this hypothesis can be seen when results from this experiment are combined with those from a prior report [1]. Since the data in the previous report were collected from both lines of mice tested between 0730–1130 hr, only the data from males tested at 0800 hr in this experiment are used. Figure 1 displays the combined data and illustrates differences in slope between the envisioned regression lines that can be fitted to the data, as well as the point (55 mg/kg) at which the hypothesized functions intersect. Only one data point (60 mg/kg) appears to be somewhat displaced from the inferred sigmoidal curve. We believe that this is due to chance variation, and that when one considers that these data were collected at different times, a variation such as this does not seem unlikely. Further, we believe that if functions were developed for both sexes of each line at different times of day, that they would have characteristics similar to those illustrated in Fig. 1. Of course the dose of pentobarbital at which the functions intersect would vary with experimental condition. It should be noted that certain findings of the current experiment do not totally agree with results showing SS mice more sensitive than LS mice at 40 mg/kg [12] and 50 mg/kg [14]. In the latter case, since the dose used is close to the point at which the dose-response functions intersect (further, sex was not controlled and testing began at 1600 hr), it is likely that procedural variables could have accounted for the discrepant findings. The former report cannot be fully evaluated because time that testing began was not noted and male and female data were combined. Nevertheless, the data reported here for males and females tested at 0800 hr and 1600 hr with 40 mg/kg pentobarbital are almost identical to data in a previous report [1] showing that the LS line was exceedingly more sensitive than the SS line.

Overall, the anomalous findings with respect to pentobarbital in these lines of mice are particularly interesting, because the fact that the dose-response functions are different than those for other depressants [1,11], indicates that there is a unique property related to drug-induced narcosis that can be analyzed with pentobarbital. For instance, recent evidence indicates that these lines eliminate pentobarbital differentially [12]. Although a circadian effect was not evaluated, such an effect was found for ethanol in another experiment [6]. On the other hand, we have suggested [10] that most depressant actions in these lines are related to GABA activity. Hence it is plausible that interactive effects among factors such as these may account for the unique pentobarbital dose-response functions in LS and SS mice.

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