## Brain Sensitivity to Alcohol in Inbred Mouse Strains

Abstract. *The voluntary selection of aqueous ethanol in preference to water has been studied in a number of animal species. A pharmacological phenomenon observed in all species, including humans, is depression of the central nervous system which eventually leads to "sleep." The sleeping time at a given concentration of alcohol in the brain depends on strain sensitivity.*

In a number of animal species, including several strains of inbred mice (*1*), voluntary selection of aqueous ethanol in preference to water has been studied from genetic, sensory, metabolic, pharmacologic, and behavioral perspectives (*2*). A pharmacological phenomenon observed in all species, including humans, that absorb an adequate dose of alcohol is depression of the central nervous system leading to sleep. This anesthetic effect of alcohol is irregular and unpredictable in humans, and depends on such variables as dose, beverage, food intake, and habituation (*3*). Physiological differences such as brain sensitivity, brain-alcohol level, and metabolic capacity are factors which must be considered in evaluating differences in the response to standard doses under controlled conditions. Some investigators ascribe the differences to tissue sensitivity and others to metabolic variations in absorption, distribution, and detoxification of ethanol.

An opportunity to test for a factor responsible for the differences in sleeping time is afforded by comparison of brain-alcohol levels in strains of inbred mice that show different sleeping time after intraperitoneal injections of ethanol. McClearn (*4*) demonstrated that with the injection of a standard dose of alcohol the time of onset of sleep is the same in both the C57BL/Crgl and BALB/cCrgl mouse strains, but the duration of sleep is almost three times as long for BALB/cCrgl mice as for C57BL/Crgl mice.

The present study was initiated to investigate further the relationship between genotype, ethanol tolerance, and sleeping. If sleeping is a function of blood-alcohol level, animals of both strains should awaken at the same blood-alcohol level. If sleeping is a function of brain sensitivity, the blood- and brain-alcohol levels of the awakened animals of each strain should be different, showing different sleeping times.

Two experiments are reported. In the first experiment, the animals were tested for sleeping time, and then their blood-alcohol levels were determined. In the second experiment, the brain-alcohol was determined. Fifteen C57BL/Crgl and 15 BALB/cCrgl male mice (196 to 203 days of age) were used in the first experiment. Twenty-three C57BL/Crgl and 26 BALB/cCrgl male mice (205 to 215 days of age) were used in the second experiment. The mice were maintained on ad lib food and water until the experiment.

The sleeping time test was designed to measure the alcohol tolerance of the animal by determining the length of time required to regain the righting response (awakening) under standard conditions after the intraperitoneal injection of 0.014 ml of 30 percent ethanol (in saline) per gram of body weight. As soon as injection was completed, the animal was hung from a wire mesh. The time until the animal fell to a foam rubber pad was measured. This constitutes "fall time." The animal was then placed on its back in a trough; the interval of time until the animal righted itself is defined as "sleeping time." A detailed description of the procedure is given in a previous study (5). In the present study, an arbitrary cut-off time was set for sleeping time; if an animal had not righted itself within 2½ hours after injection, a score of 150 minutes was assigned as its sleeping time.

Three samples of blood (0.2 ml) were taken from the tip of each animal's tail in the first experiment. A sample was taken when the mouse awakened, and again 3 and 4 hours after injection of ethanol. The blood was analyzed for ethanol by a micromodification of the gas chromatographic method of Parker *et al.* (*5, 6*).

In the second experiment in which brain-alcohol was determined, the mice were injected with the standard dose, and then returned to the cage. The animals were sacrificed 40, 100, and 140 minutes after injection by severing the spinal cord at the neck; the brains were dissected immediately. These times were chosen because the results of the first study showed the median sleeping times of C57BL/Crgl and BALB/cCrgl mice to be approximately 40 and 140 minutes, respectively, at this age. The brains were homogenized in a cold 0.15 percent 2-butanone solution (ten times their weight) and injected into the gas chromatograph. The brain analysis was completed in less than 3 minutes.

Table 1. Fall time (seconds) and sleeping time (minutes) of C57BL/Crgl and BALB/cCrgl mice.

| Strain | No. | Fall time (Mean ± SD) | Sleeping time median |
|---|---|---|---|
| C57BL/Crgl | 15 | 111 ± 21 | 38 |
| BALB/cCrgl | 15 | 103 ± 23 | 138 |

The fall time and sleeping time of C57BL/Crgl and BALB/cCrgl mice are presented (Table 1). The difference in fall time between the strains is not statistically significant by *t*-test. This is taken to mean that the rate of absorption is approximately the same for the two strains. On the other hand, the difference in sleeping times is significant ($p < 0.01$) by the Composite-rank method of Wilcoxin (*7*). (Note: $R = 122$. If $R = 170$, $p = 0.01$ when $n = 15$, and significance increases as $R$ decreases.) This is consistent with the findings of McClearn (*4*) in which BALB/cCrgl mice were shown to sleep three times as long as the C57BL/Crgl mice. Five of the 15 BALB/cCrgl mice had not awakened in 2½ hours, and were assigned the maximum sleeping time of 150 minutes. The results of the sleeping time test also confirm the findings of a previous study (*8*) in which sleep time was shown to increase

Table 2. Blood alcohol (milligrams per 100 ml of blood) in C57BL/Crgl and BALB/cCrgl mice at "waking," at 3, and at 4 hours after injection.

| No. | At waking* | 3 hours | 4 hours |
|---|---|---|---|
| | *C57BL/Crgl* | | |
| 14 | 473 ± 50 | 286 ± 32 | 183 ± 36 |
| | *BALB/cCrgl* | | |
| 13 | 378 ± 40 | 318 ± 46 | 189 ± 47 |

* Median sleeping time for C57BL/Crgl, 38 minutes; for BALB/cCrgl, 138 minutes.

Table 3. Brain alcohol (milligrams per 100 ml of blood) in C57BL/Crgl and BALB/cCrgl mice at 40, 100, and 140 minutes after injection.

| 40 minutes | | 100 minutes | | 140 minutes | |
|---|---|---|---|---|---|
| No. | Mean | No. | Mean | No. | Mean |
| | | *C57BL/Crgl* | | | |
| 9 | 430 ± 29 | 7 | 375 ± 29 | 7 | 275 ± 12 |
| | | *BALB/cCrgl* | | | |
| 11 | 430 ± 40 | 8 | 367 ± 35 | 7 | 287 ± 17 |

with age from 4 to 16 weeks. The mice used in the present experiment were about 30 weeks of age. Their sleeping times were increased, apparently as a function of age, from 90 minutes at 16 weeks to 130 minutes at 30 weeks in the BALB/cCrgl mice, and from 25 minutes to 45 minutes in the C57BL/Crgl mice.

The results of the blood-alcohol analysis are presented in Table 2. The difference between the blood-alcohol levels in the two strains at waking, when the righting response is regained, is significant by the Mann-Whitney U test ($p < .001$). The values obtained 3 and 4 hours after injection are not statistically different for the two strains (by the *t*-test), indicating that the rates of alcohol metabolism are not significantly different during this period. The level of brain-alcohol is the same in both strains at 40, 100, and 140 minutes (Table 3). However, the difference in brain-alcohol levels of C57BL/Crgl mice at 40 minutes after injection and BALB/cCrgl mice at 140 minutes after injection is highly significant ($p < .001$) when each regains its righting response.

The results of this study confirm the findings of McClearn (*4*) and Kakihana *et al.* (*8*) that genotype and age of the animals are important determiners of response to ethanol in mice. The finding of the present study that the C57BL/Crgl and BALB/cCrgl strains awaken at different blood-alcohol levels suggests that brain sensitivity to ethanol is different in the two strains. This hypothesis is further supported by the result that C57BL/Crgl mice awoke at significantly higher brain-alcohol levels than did BALB/cCrgl mice. The hypothesis that differences in brain sensitivity to ethanol underlie the difference in sleeping time is thus strengthened.

It is of interest that the strains that showed the greatest preference for alcohol in choice studies also showed the greatest resistance to the effect of injected alcohol in sleeping time studies. In enzyme studies (*9*) with C57BL/Crgl mice (the strain preferring alcohol) and BALB/cCrgl mice (the strain preferring water), the former showed a significantly higher alcohol dehydrogenase activity. However, the difference in hepatic enzyme activity seems too small to be the entire physiological basis for the difference in strain responses to alcohol. We found no significant difference between the two strains in their rates of ethanol metabolism. The explanation may be that the dose of alcohol used to induce sleeping was so large as to mask the slight difference in metabolic rates. Another factor which may influence metabolic degradation of alcohol is the rate of blood circulation. Even if a difference exists in the circulation of blood in these two strains, the brain-alcohol levels during the time period observed were identical, indicating that the brains of the two strains are differentially affected by the same level of alcohol.

It would appear that at least three phenomena are based on genetic differences in these two strains of mice: (i) the preference for alcohol, (ii) the level of liver alcohol dehydrogenase activity, and (iii) the sleeping time. This last phenomenon is shown to be due to brain sensitivity. Other studies have shown an increased behavioral tolerance to injected alcohol as a result of prolonged drinking, and a decreased tolerance with age in certain mouse strains (*5*, *8*). This suggests that the response to alcohol is influenced by environmental and developmental factors, as well as by inborn susceptibility.

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### References and Notes

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## Cytochemistry of Synapses: Selective Staining for Electron Microscopy

Abstract. *Material at synapses and in some synaptic vesicles becomes selectively stained when glutaraldehyde-fixed rat brain that has not been treated with osmium is stained with phosphotungstic acid. The material stained at synapses is distinct from the adjacent unstained synaptic membranes and has cytochemical properties of protein. The specialized spatial arrangement exhibited by this synaptic material suggests its close involvement in synaptic function.*

The fine structural details of the specialized interneuronal synaptic contacts have traditionally been observed in brains fixed with osmium tetroxide (*1*). Fixation with an aldehyde (*2*) before exposure to the osmium reveals similar synaptic morphology (*3*) (Fig. 1). One distinguishing feature of the synapse in tissue fixed or stained with osmium is the presence of electron-opaque material associated with the presynaptic and postsynaptic membranes. However, there has been virtually no cytochemical characterization of this unknown cellular material that distinguishes synaptic sites from other apposing neuronal membranes. Although the synaptic material does not react with $KMnO_4$, Gray (*4*) has emphasized that enhanced electron-opacity of synaptic membrane components occurs when osmium-fixed brain is stained with phosphotungstic acid. The mechanism of this enhancement is unknown.

In this study, we have departed from the usual preparative procedure for electron microscopy: rat brain was fixed by glutaraldehyde perfusion and stained with alcoholic phosphotungstic acid (PTA) without being exposed to osmium tetroxide. Although membranes are not positively stained by this procedure, electron-opacity is pronounced at synaptic sites (Fig. 2). In fact, electron-opacity in the neuropil is, with one exception, observed only at synaptic sites. Four fine structural details are demonstrable: (i) a highly electron-opaque band beneath the postsynaptic membrane, parallel to the synaptic cleft, and 150 to 250 Å wide; (ii) several clumps of less intense, electron-opaque material projecting from the area of the presynaptic membrane into the terminal bouton; (iii) a discontinuous electron-opaque line (50 to 150 Å) occupying the synaptic cleft separated