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Acute Effects of Ethanol *in vivo* on Neuromuscular Transmission

T. EDWARD REED

Departments of Zoology and Anthropology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

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REED, T. E. *Acute effects of ethanol* in vivo *on neuromuscular transmission.* PHARMAC. BIOCHEM. BEHAV. 13(6) 811–815, 1980.—The effects *in vivo* of acute doses of ethanol on impulse transmission in the region of the neuromuscular junction are poorly known. These effects were studied with a new procedure, using mouse tails in a constant temperature chamber to study the delay in nerve-to-muscle impulse transmission. A saline control and five ethanol doses (0.5, 1.0, 1.4, 2.0, 4.24 g/kg) were used with 96 mice. The interval ("residual latency", RL), between (a) the peak of the compound nerve action potential and (b) the first peak of the associated compound muscle action potential, was measured. Ethanol was given IP and tail nerve stimulations were done at 4 min intervals to 16 min post-ethanol. The mean pre-ethanol RL was 0.93 ± 0.01 (SE) msec; about 25% of this time should be synaptic delay and the remainder is nerve and muscle fiber conduction time. Individual post-ethanol relative RL (RRL) values were calculated for each mouse, based on its pre-ethanol value. With doses of 1.0 g/kg and higher there was a mean increase in RRL; at 16 min this increase was 0.8 to 4.4% (all $p<0.01$). At 0.5 g/kg, and also at higher doses, there was a significantly ($p<0.01$) increased variance in RRL 8 to 16 min post-ethanol. A marked correspondence between mean RRL and ataxia is apparent. This appears to be the first *in vivo* demonstration of acute effects of ethanol on neuromuscular transmission. The methods and mice used may comprise a useful animal model for detecting acute effects of low doses of ethanol on synaptic function.

Acute response Animal model Ataxia Ethanol Mice Neuromuscular junction
Residual latency Synaptic delay

SYNAPTIC delay (SD) is the time interval between the entry of a nerve impulse into a pre-synaptic terminal and the appearance of a resulting post-synaptic potential. Study on the SD at the frog neuromuscular junction showed that the modal value at 20°C was about 0.75 msec [8]. At mammalian junctions the SD is smaller; an estimate at 0.22 msec in the rat at 34–36°C was obtained [5]. It was concluded [8] that SD results primarily from delay in the release of transmitter after arrival of the nerve impulse.

There have been a number of studies of the effects of ethanol (EtOH) on the neuromuscular junction [7], most of them being *in vitro* studies with experimental vertebrates. Some of these used very high EtOH concentrations, above the LD_{50} for mice (about 800 mg/dl or 170 mM; ([18], p. 56)), and are not relevant to the present study. A summary of many studies ([18] pp. 220–222) of EtOH effects on neuromuscular transmission shows that low concentrations (e.g., 10 mM) of EtOH facilitate or potentiate muscle twitches while very high concentrations (over 1% or 220 mM) have the opposite effect. A study [4] of isolated phrenic nerve-diaphragm neuromuscular junctions in rats found increased end-plate potentials with EtOH concentrations of 16–40 mM; it was concluded that EtOH had both presynaptic and postsynaptic effects.

Several human studies have reported on possible effects of EtOH on neuromuscular function. Two studies considered a possible effect on "residual latency" (RL), a delay in muscle response (determined by stimulating a motor nerve and recording the resultant muscle action potential) which includes SD [1]. The first study [9] stimulated the ulnar nerve of the arm and recorded from the hypothenar muscles of the hand; a reduction in RL was claimed after consumption of 4.5 oz of whiskey (which produced blood EtOH concentrations up to 100 mg/dl). A repeat study [11], however, presented evidence that the "reduction" in RL is likely due to EtOH-induced temperature change in the limb. Another study [16] reported that moderate to large doses (0.55–1.65 ml EtOH/kg) produced small but real increases in the latencies of auditory-evoked brainstem potentials. These latencies undoubtedly include synaptic delays but this aspect was not specifically considered.

With the possible exception of the last study [16], the acute *in vivo* effects of EtOH on synaptic transmission are still little known. The present report describes a simple technique for studying impulse transmission at the neuromuscular junction of awake, intact mice in a temperature controlled environment. Evidence that acute, low-to-moderate doses of EtOH affect this transmission will be presented.

METHOD

The general procedures were the same as used previously in studying effects of acute EtOH on nerve conduction velocity [14]. The mice used were of the HS strain, a vigorous, genetically heterogenous strain derived from an 8-way cross of pure lines and maintained by random mating [10]. This strain has been used as an animal model in studying certain aspects of responses to acute EtOH, namely the role of heredity in a wide variety of responses [12] and the "normal-

ization" of heart rate [13,15]. Ninety-six HS mice from the investigator's colony, aged 60 to 71 days (mean 61.8 days), were tested. A mouse was restrained on its back, awake, with its tail in a copper trough filled with mineral oil at 37.0° ± 0.1°C. Electrodes were 0.4 mm diameter stainless steel pins 5 mm apart in each of the two pairs. The stimulating pair was 5 mm (to the nearest electrode) from the base of the tail. The recording pair was 15 mm distal to the stimulating pair. Current through the stimulating pair was maintained at just above that required for a maximal response in the compound nerve action potential (CNAP). This condition was monitored throughout the experiment. Pulse length was 0.05 msec and the sweep speed of the storage oscilloscope was 0.05 msec/scale division. The gain of the pre-amplifier was set at 100×; that of the oscilloscope was adjusted so that both the CNAP and the following compound muscle action potential (CMAP) were well displayed. A typical display of CNAP and CMAP is shown in Fig. 1. Recording began six min after placing the tail into the trough and continued every four min to 30 min. Five stimuli (5 sec inter-stimulus interval) were given at each time. The interval between the peak of the CNAP and the first peak of the CMAP was used as a measure of delay, from all causes, at the neuromuscular junction. By analogy with human electromyography (see above) this interval will be termed the residual latency (RL). RL is the sum of SD and nerve and muscle conduction times in the recording region. This peak-to-peak distance was measured on the oscilloscope screen, to the nearest 0.1 mm, with dividers or dial calipers. For each mouse the relative RL (RRL) for each time was calculated by dividing the mean RL at a given time by the mean RL at time zero (14 min after placing tail into the trough and just before giving the test injection). Each mouse, therefore, served as its own control.

In each test a saline injection control, at −4 min (before time zero), preceded the test injection (both were 0.2 ml/g wt.; IP). If the RRL changed more than 1% between −4 min and 0 min, the mouse was rejected. Six different test doses were given, each on a different group of naive mice, immediately after the time zero stimulations: saline; ethanol-saline at concentrations of 0.5, 1.0, 1.4, 2.0, and 4.24 g EtOH/kg body wt. The number of mice per test group was 12 (males) except for that at 1.0 g/kg, which used 36 (24 males, 12 females). Weight, age, tail length, littersize, cage size (no. in cage after weaning), loss of consciousness or not (after EtOH), and degree of ataxia at the end of the test (about 17 min after EtOH) were recorded for each mouse. The possible effects of the first five of these concomitant variables on RRL were tested for by regression before looking for EtOH effects.

RESULTS

Nature and Time Course of the Residual Latency

The general nature of the RL is shown in Fig. 1. This appearance of the CNAP and CMAP peaks has been seen in each of the more than 500 HS mice stimulated and recorded as described above. The mean time between these peaks in the 96 mice of the present study, at 0 min, was 0.931 ± 0.010 msec (mean ± SE), with a range from 0.75 msec to 1.35 msec. There was no sex difference in RL.

That the CMAP is actually due to muscle action potentials was shown in two ways: (1) Injection of a muscle relaxant, succinylcholine, into the base of the tail of two mice abolished the CMAP for four to 30 min without altering the CNAP. (2) Stimulating and recording antidromically (positions of stimulating and recording electrodes interchanged) in the tail of two mice produced a normal CNAP but no CMAP.

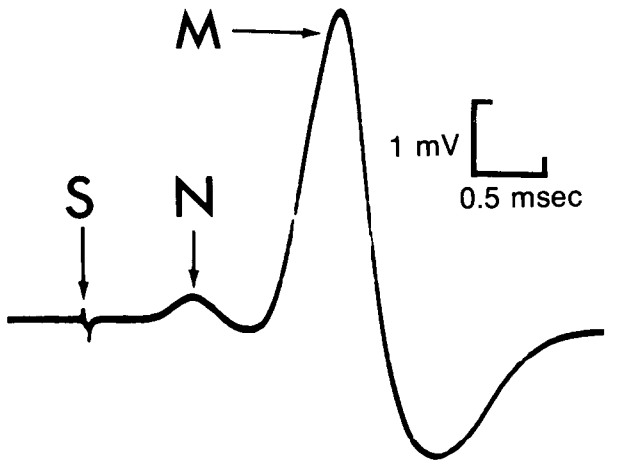

FIG. 1. Typical compound nerve action potential (CNAP) and compound muscle action potential (CMAP). Time base: 0.5 msec/scale div. Gain: 0.5 mV/scale div. *S:* Stimulus, *N:* CNAP, *M:* CMAP (first peak).

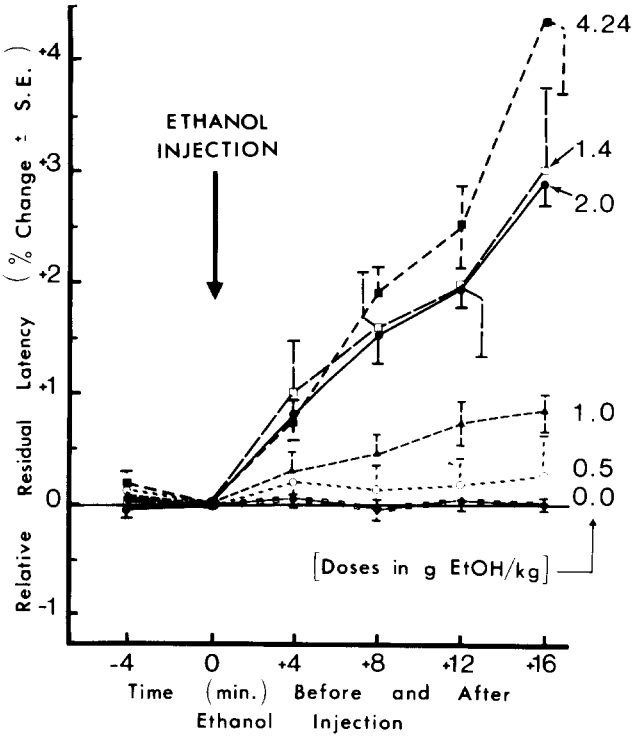

FIG. 2. Mean relative residual latencies for six ethanol doses. N=36 mice for 1.0 g EtOH/kg dose. N=12 mice for other doses.

EtOH Effects on RRL at Different Doses

The mean RRL values over the 30 min test period are shown in Table 1 and Fig. 2. The values at +4 to +16 min for the 0.5 g/kg dose showed a significant effect of tail length on RRL, the regression coefficients at +4, +8, +12, and +16

TABLE 1

CHANGE IN MEAN RELATIVE RESIDUAL LATENCY (RRL) AFTER VARIOUS ETHANOL DOSES

Dose (g/kg)	N	Percent change from baseline in mean RRL (± SE) at indicated time (min)				
		−4	+4	+8	+12	+16
0	12	0.061 ± 0.083	0.59 ± 0.070	−0.048 ± 0.087	0.043 ± 0.094	0.009 ± 0.069
0.5	12	0.144 ± 0.086	0.214 ± 0.107	0.141 ± 0.217	0.163 ± 0.234	0.256 ± 0.352
1.0	36	0.095 ± 0.053	0.299 ± 0.157	0.464 ± 0.170*	0.736 ± 0.202‡	0.844 ± 0.181‡
1.4	12	0.019 ± 0.162	1.018 ± 0.469	1.596 ± 0.535*	1.925 ± 0.599†	3.006 ± 0.761†
2.0	12	−0.032 ± 0.075	0.826 ± 0.127‡	1.531 ± 0.210‡	1.939 ± 0.163‡	2.904 ± 0.206‡
4.24	12	0.211 ± 0.015	0.756 ± 0.167‡	1.908 ± 0.247‡	2.494 ± 0.367‡	4.388 ± 0.694‡

2-tail probabilities: *$p<0.05$; †$p<0.01$; ‡$p<0.001$.

min being significant at probability levels of 0.004, 0.005, 0.015, and 0.03, respectively. At +4 min with this dose littersize was also significant ($p=0.005$). These effects of tail length and littersize were removed by regressing to the mean tail length and mean littersize. The RRL values at other doses required no correction. There were no significant sex differences in RRL for the 1.0 g/kg dose group. It is seen that saline and the 0.5 g/kg dose have no significant average effects on RRL but doses of 1.0, 1.4, 2.0, and 4.24 g/kg do have significant mean effects, causing a mean increase of residual latency at +16 min of from one to four and a half percent from the pre-EtOH baseline. The post-EtOH increases in mean RRL of the 1.4 g/kg and the 2.0 g/kg groups are very nearly equal. If these two groups were pooled, there would be an increase in RRL with dose for the three post-EtOH times, +8 to +16 min, but this increase is not proportional to dose.

Variability among Individuals at a Given Dose

The mean RRL values conceal great individual variability in RRL values. The extent of this variation, and a demonstration that it is caused by individual differences in response to EtOH, is shown in Figs. 3 and 4. The standard deviation of RRL is approximately constant over time for saline, as expected, but for each of the various EtOH doses it increases markedly after the EtOH injection (Fig. 3). Comparing the variance for a given EtOH dose and time with the corresponding variance of the saline control (F test) shows that, with the one exception of the 0.5 g/kg dose at +4 min, all post-EtOH increases in standard deviation are significant. The p values for 2.0 g/kg at +4 and +12 min are 0.03 and 0.04, respectively; those for all other post-EtOH doses and times are well below 0.01. At +16 min, that for 0.5 g/kg is 3×10^{-6}.

There is no linear relation between dose and increase in standard deviation; the largest increase is at the 1.4 g/kg dose. Individual variability in RRL is shown in Fig. 4, illustrating individual curves for six consecutively tested mice (from two litters) at the 1.0 g/kg dose. It is apparent that

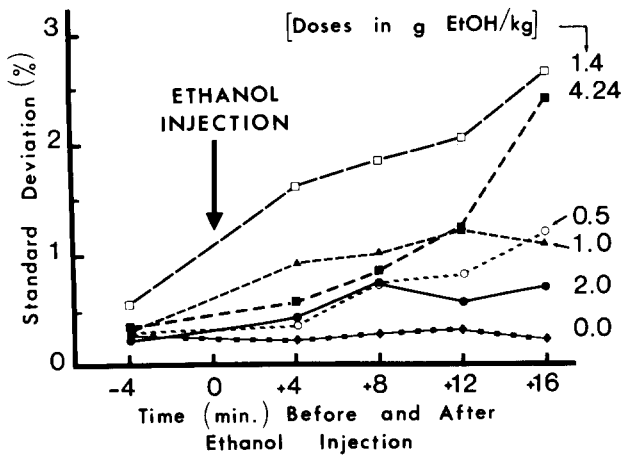

FIG. 3. Standard deviations of relative residual latencies for six ethanol doses. Same mice as in Fig. 2.

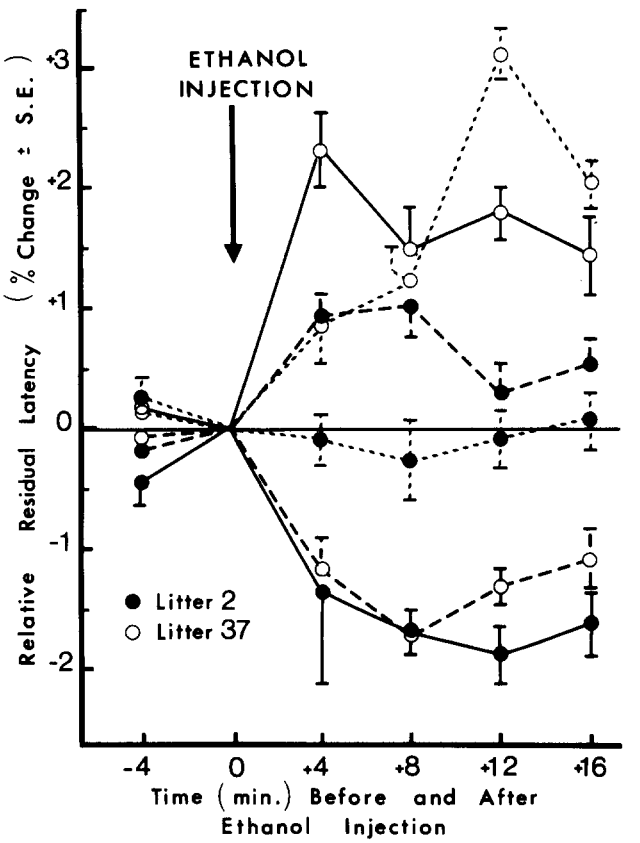

FIG. 4. Indiviudal relative residual latencies for six male HS mice tested with 1.0 g EtOH/kg. Mice were tested consecutively and include three littermates from each of two different families.

some mice consistently, and significantly, show increased RRL while others show *decreased* RRL.

Relation Between Change in RRL and Ataxia

Ataxia was measured after removing the mouse from the restraining frame at the end of the test (about 17 min after injecting EtOH). A seven-point scale for degree of ataxia was used, where *0* indicates no ataxia and *6* is inability of the mouse to right itself (unconsciousness). A horizontal wire grill (3 mm diameter parallel rods, 2 cm between rods, mouse walking at right angles to rods) was used for determining ataxia states: *1* = walks on grill well but without usual ("sober") caution; *2* = walks on grill with some stumbling; *3* = walks on grill with much stumbling; *4* = doesn't walk on grill, does walk on flat surface; *5* = doesn't walk on flat surface, does right self. The following mean ataxia scores, with approximate standard errors, were obtained for the six doses; the mean percent change in RRL(± SE) at +16 min is also shown:

Dose	RRL Change	Ataxia Score
0.0	0.01 ± 0.07	0.00 (±0.00)
0.5	0.26 ± 0.35	0.00 (±0.00)
1.0	0.84 ± 0.18	0.11 (±0.05)
1.4	3.01 ± 0.76	1.17 (±0.17)
2.0	2.90 ± 0.21	1.50 (±0.23)
4.24	4.39 ± 0.69	5.08 (±0.26)

A marked, significant increase in mean ataxia score, increasing from 0.11 to 1.17, occurs when the dose is increased from 1.0 to 1.4 g/kg and this increase in ataxia is paralleled by a correspondingly large and significant increase in mean RRL change, from 0.84 to 3.01. Loss of consciousness (for any duration) was not a useful indicator of EtOH effect on the CNS here since at the highest dose, 4.24 g/kg, all mice lost consciousness but at 2.0 g/kg and lower doses no mice did.

DISCUSSION

The technique employed and its resulting clear display of nerve and muscle potentials may be new in neurophysiology. It is therefore of interest to note that the nerve action potential seen in these mice, just preceding the much larger muscle action potential (Fig. 1), is similar to nerve action potentials seen in clinical electromyography [3,17]. Apparently there has been no clinical or experimental use of this nerve potential.

Since the test parameter is the time between the peak of the compound nerve action potential and the peak of the subsequent compound muscle action potential, it is useful to consider briefly the several components of this time (here termed the "residual latency"). The CNAP results from many nerve fibers, both sensory and motor, in the ventral tail nerve in the region of the recording electrodes. Muscle fiber potentials are also recorded from many motor units in the region of the recording electrodes. Each motor nerve fiber subdivides terminally into many finer fibers, each serving a single muscle fiber of the motor unit. These finer nerve fibers will conduct more slowly than the undivided fiber because of their smaller diameter and also because they become unmyelinated a few μm before the pre-synaptic terminal. This slower nerve fiber conduction velocity (CV), and also the relatively slow muscle fiber CV (one-tenth or less than the motor nerve CV, [2]), should constitute a major part of the RL. Since the true mammalian SD is about 0.22 msec [5], the mean terminal nerve fiber conduction time (CT) plus the mean muscle fiber CT should be about 0.93–0.22 or about 0.7 msec. SD is about 0.22/0.93 = 24% of the RL.

A complication for interpreting RL is that the electrode recording region is not well-defined. Most nerve fibers recorded by the two electrodes are not motor nerves terminating in the immediate region. Further, muscle fiber potentials will be recorded from some local region around the electrode. Differences among nerve fibers in magnitudes of CV, according to their diameters, and differences in distances of the stimulated nerve and muscle fibers from the recording electrodes, will cause the familiar spreading out of the compound action potential illustrated in Fig. 1. The nerve peak-to-muscle peak measurement of RL employed here cannot separate these individual delay times. Even so, RL is a relatively simple and objective measure of impulse conduction delay around the neuromuscular junction and three major components of this delay (SD, nerve CT, muscle CT) are known to exist.

The most notable result of the present study is the demonstration, under controlled conditions, of acute *in vivo* effects of ethanol on RL. Earlier *in vivo* studies on RL in humans were not temperature controlled and consequently the results were not conclusive. Since this effect was found at each of four different doses (1.0, 1.4, 2.0, 4.24 g/kg), it seems established. In order to relate these doses to humans, it is useful to note that 3 g/kg is about the ED_{50} for sleep in HS mice [14] and that 1 g/kg produces almost no detectable ataxia (present study).

The reported greater sensitivity of neuromuscular transmission to ethanol, relative to nerve conduction effects ([4], [18] pp. 220–222), was confirmed by the present findings. Not only was a mean effect found with a dose of 1.0 g/kg, but a very significantly increased *variance* in RRL after EtOH was found with a dose of only 0.5 g/kg.

The differential action of EtOH on the three major components of RL is an important question which, to date, seems to have no *in vivo* answer. It might be expected that there could be an effect (probably decreased velocity) on terminal nerve fiber CV at doses as low as 1.0 g/kg even though effects on (larger, myelinated) non-terminal nerve fibers were found only at doses of 2.5 g/kg and higher [12]. Different effects of EtOH on SD and on muscle fiber CV might be expected. The effect of low EtOH doses on SD could be decreased delay time since the *in vitro* studies reviewed above indicate a potentiating effect of low doses on neuromuscular transmission.

At each dose there is considerable variability in individual RRL value (Fig. 3) and at lower doses some mice show *decreased* RRL values (Fig. 4). Increased variance reveals EtOH effects on RRL even when there is no mean effect. It is conceivable that the decreased RRL of some mice at low EtOH doses results from a predominating effect on SD (relative to nerve and muscle fiber effects) but this remains to be demonstrated.

The apparent non-linearity of mean RRL with increasing dose contrasts with the relatively linear EtOH dose-response of nerve conduction time (reciprocal of velocity; [14]). If this nonlinearity is real, it might be explained by the different effects of EtOH on the three major components of residual latency.

It is noteworthy that the EtOH responses of both RRL and ataxia seem to pass a threshold with the small increase in dose from 1.0 g/kg to 1.4 g/kg. In the present study ataxia was just becoming detectable (about 17 min post-EtOH) at a dose of 1.4 g/kg. An extensive earlier study on HS mice at this dose noted much more apparent ataxia at three min post-EtOH [12]. Although all mice were ambulant at this time, about half had a noticeable ataxia in an open-field. This similarity in dose response of RRL and ataxia suggests that the observed effects of EtOH on synaptic transmission in the tail may have important parallels in the central nervous system, at least in the parts concerned with locomotion and equilibrium.

In summary, acute effects of ethanol on neuromuscular transmission have been demonstrated in intact mice at three sub-hypnotic doses and at one hypnotic dose. These results may be very relevant to humans since there is reason to believe that similar effects would occur in humans at lower EtOH doses. The LD_{50} for mice is a blood EtOH level of about 800 mg/dl while that for humans is about 500 mg/dl ([18] p. 56). ED_{50} for sleep in HS mice is a blood level of about 400 mg/dl since a 3.0 g/kg dose, which is very near the ED_{50} [14], produces a mean blood level of about 372 mg/dl eight min after injection (T. E. Reed, unpublished). In contrast, ED_{50} for sleep in alcohol-naive humans is probably around 200 mg/dl (H. Kalant, personal communication). It is not known how closely peripheral synapses, such as in mouse tails, may model those in the central nervous system (CNS) but there may be some similarity in their responses to EtOH because of (a) the similarity in observed dose responses of RRL and ataxia (see above) and (b) the theoretical consideration of general similarity of mammalian chemical synapses (excitatory cholinergic synapses in the CNS should, of course, be very similar) and the similarity of EtOH concentrations in peripheral blood and brain fluids [6]. If there is such similarity, it is then interesting to note that even the lowest dose used, 0.5 g/kg, produced individual differences in RRL. This dose should make a blood EtOH level of about 65 mg/dl, which is less than the legal limit for human intoxication in many areas. These considerations suggest that the procedure and the mice used in this study may be a useful animal model in several ways for studying effects of low doses of EtOH on transmission of impulses across synapses.

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