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Ethanol-Induced Depressions in Cerebellar and Hippocampal Neurons of Mice Selectively Bred for Differences in Ethanol Sensitivity: An Electrophysiological Study

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SORENSEN, S., T. DUNWIDDIE, G. McCLEARN, R. FREEDMAN AND B. HOFFER. *Ethanol-induced depressions in cerebellar and hippocampal neurons of mice selectively bred for differences in ethanol sensitivity: An electrophysiological study*. PHARMAC. BIOCHEM. BEHAV. **14**(2) 227–234, 1981.—The recently discovered profound differential sensitivity of cerebellar Purkinje (P) cells in long-sleep (LS) versus short-sleep (SS) mice to the depressant effects of locally applied ethanol was extended in this study. First, the sensitivity of Purkinje neurons from HS mice (an outbred stock of mice from which the LS and SS lines were derived), was found to be almost exactly intermediate between the values for the long-sleep and short-sleep animals. Second, no differential sensitivity in long-sleep versus short-sleep hippocampal pyramidal neurons was observed. This was true using both spontaneous and evoked activity. Third, no differential sensitivity of P cells was seen in long- versus short-sleep mice with local application of halothane. Taken together with previous reports, these data strongly suggest that whatever genetically determined central nervous alterations result in the differential soporific effects of ethanol in the two (LS and SS) mouse lines, such alterations are brain region- and depressant drug-specific rather than generalized.

Ethanol Long-sleep mice Short-sleep mice Hippocampus Cerebellum

THE interaction of ethanol with central nervous elements has been the subject of much recent investigation. Electrophysiological [10, 14, 18, 21, 31] and behavioral [4, 6, 10, 25, 28, 31] studies have suggested that the cerebellum and hippocampus may be particularly sensitive to this drug. Neurophysiological studies of outbred mouse lines which differ in ethanol-induced behaviors offer a unique opportunity to examine the relationship between electrophysiological and behavioral alterations. For example, lines of mice are available which differ markedly in their soporific response to acute ethanol administration [24]. The long-sleep (LS) mice sleep many times longer to a given dose of ethanol than the short-sleep (SS) animals. Pharmacokinetic studies indicate that this difference in sleep times is probably related to differential central nervous sensitivity, rather than peripheral metabolic differences [15,16]. The parental heterogeneous stock (HS), derived from a randomized 8-way cross of various strains [24], shows an intermediate sleep time.

We have recently reported that the spontaneous discharge of cerebellar Purkinje (P) cells is depressed by ethanol at markedly lower doses in LS than in SS animals [29]. When administered locally using pressure ejection, the mean effective dose for LS mice was 29 PSI-sec; for SS mice, it was 888 PSI-sec.

In this communication, we sought to resolve three questions critical to the interpretation of this electrophysiological observation. First, what is the relative sensitivity of Purkinje cells in HS animals, as compared with LS and SS mice [29] to the depressant effects of ethanol? Second, is the differential sensitivity of central neurons in LS and SS mice region-

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specific; do other brain areas in these two lines show similar responses to ethanol? Third, does the differential sensitivity in LS and SS Purkinje cells exist only for ethanol, or is it found also for other depressant agents?

METHOD

Methods for In Situ Recording

Surgery. Long-sleep, short-sleep and HS mice were anesthetized with urethane (1.25 g/kg, IP), intubated, and allowed to breathe spontaneously. Body temperature, monitored by a rectal probe, was maintained at 37°C with a heating pad. Animals were then placed in a stereotaxic frame and, for cerebellar recordings, the skin and muscle over the posterior fossa was removed. After cisternal drainage, the skull and dura over this region were also removed, and the brain surface covered with warm 2% agar in saline. Recordings were made from Purkinje cells in the vermis, lobules VI and VII, identified by anatomical location and the characteristic discharge of complex and single spikes [9]. For hippocampal recordings, one hole was drilled in the skull 2.0 mm posterior to bregma and 1.5 mm lateral to the midline, so as the overlie to CA1 region on one side. Another hole, 2.0 mm posterior to bregma and 3.1 mm lateral to the midline on the contralateral side, was drilled so that the stimulating electrode could be lowered into the CA3 region approximately 2.0 mm ventral to the brain surface. The CA1 pyramidal neurons were identified on the basis of depth, discharge pattern, and the waveform of responses evoked by electrical stimulation of the commissural pathway from the contralateral CA3 region [1]. Histological verification of recording sites was also undertaken using local ejection of fast green dye from an adjacent barrel of the micropipette after the recording [32]. All stimuli were monophasic square waves delivered from a fine concentric metal stimulating electrode with tip separations $<200\ \mu$.

Electrophysiology. Action potentials from Purkinje and pyramidal neurons were recorded with the 5 M NaCl-filled barrel of a 3-barreled micropipette. The pipettes were fabricated from Corning 7740 thin-walled (o.d.=1.5 mm; i.d.×1.1 mm) fiber-filled glass capillary tubing. The multibarrel micropipettes were pulled in two stages on a Narishige (Tokyo) vertical puller. First, an initial 180–360° twist, followed by an 0.5–1.5 cm pull, was applied to the pipette; the current to the heating coil was then turned off, and the partially pulled pipette was allowed to cool. Next, the narrowed portion of the glass was recentered in the heating coil, and the pipette was pulled for a second time. This technique produced two usable pipettes from each pull, with a tip diameter of $<1\ \mu$m. To facilitate drug ejection, tips were subsequently bumped to a diameter of approximately 3 μ under a microscope.

The action potentials were monitored on an oscilloscope, separated from background activity and converted to constant voltage pulses with a window discriminator, integrated over 1 sec intervals by a ratemeter, and displayed on a strip chart recorder.

Micropressure ejection was carried out through tubing affixed to the end of one or two barrels of the multibarreled pipette. The pressure applications, ranging from 1–35 pounds per square inch (psi), were controlled by a Medical Systems Corp. (Great Neck, NY) pneumatic pump which regulated the magnitude of pressure delivered and, utilizing a crystal clock, the timing of the pressure pulse. Pressure ejection from glass micropipettes has been reported to release drug in an amount linearly related to ejection pressure and time [23, 26, 27]. Moreover, the pressure and time parameters appear to be interchangeable and thus, dose can be expressed as pounds per square inch × seconds (PSI-sec).

Drug solutions in the pipette were as follows: Ethanol, 750 mM in normal saline; normal saline alone; and sucrose, 670 mM in normal saline. This concentration of sucrose+vehicle is isosmotic with the ethanol+vehicle solution.

For each cell, drug was tested at least twice with consistent effects required. Data from cells which did not show recovery of the response after cessation of drug application were discarded.

Data analysis. The drug-induced responses were quantitated by digital computer. The data from ratemeter displays were digitized using a Tektronix graphics tablet and fed into a Data General NOVA 3/12 computer. The neuronal activity over sequential one-second periods was stored, and subsequently displayed on a Tektronix CRT computer terminal. CRT terminal-generated vertical vectors could then be superimposed over the display on the CRT, and used to analyze neuronal activity before, during and after local drug applications. The area under the curve during drug-induced depressions was analyzed as a percent change from the averaged area (mean spikes per sec) under a control epoch of the curve. The resulting computer output was quantitated as percent depression or excitation elicited by drug applications of various pressures and durations.

In these experiments, a 30 to 70% depression in spontaneous activity was utilized for quantitation because this range insured reliable dose-dependent responses. Since our objective was to compare sensitivity between the strains of mice, we routinely attempted to apply the dose of drug required to produce a 50% depression of the neuronal activity. Using pressure ejection or microiontophoresis, however, it is rarely possible to titrate the dose with sufficient accuracy to elicit exactly a 50% reduction, so the above criteria were established. Moreover, when the depressions produced by the various doses were averaged over the entire population of neurons scored in this fashion, the average depression for every strain was 50% ± 3 SEM. A two-tailed Student's t-test was used to determine the significance of all data, with a value of $p<0.05$ as the criterion.

Methods for In Vitro Recording from Hippocampal Slices

Coronal slices were prepared as described previously [8,30]. Slices were cut at 400 μm on a Sorvall tissue chopper and immediately placed in ice-cold medium consisting of (mM) NaCl 124, KCl 4.9, KH_2PO_4 1.2, $MgSO_4$ 2.4, $CaCl_2$ 2.5, $NaHCO_3$ 25.6, and glucose 10; which was pregassed with 95% O_2 and 5% CO_2. Slices were transferred to a recording chamber maintained at 33°C within 5 min, and the fluid level was maintained at or just below the upper surface of the slice.

Twisted 62 μ nichrome wire stimulation electrodes were placed under visual guidance in the stratum radiatum near the border of CA1-CA2. Stimuli were monophasic 0.1 msec-pulses of 5 to 25 V, delivered via an isolation unit. Stimulating electrodes were lowered into the slice until potentials of maximum amplitude were obtained from a particular recording site, and the voltage was then set so as to evoke a 1 to 5 mV population spike. Recording was done with 2 to 3 MΩ glass microelectrodes filled with 2 M NaCl which were also placed under visual guidance. Responses were recorded

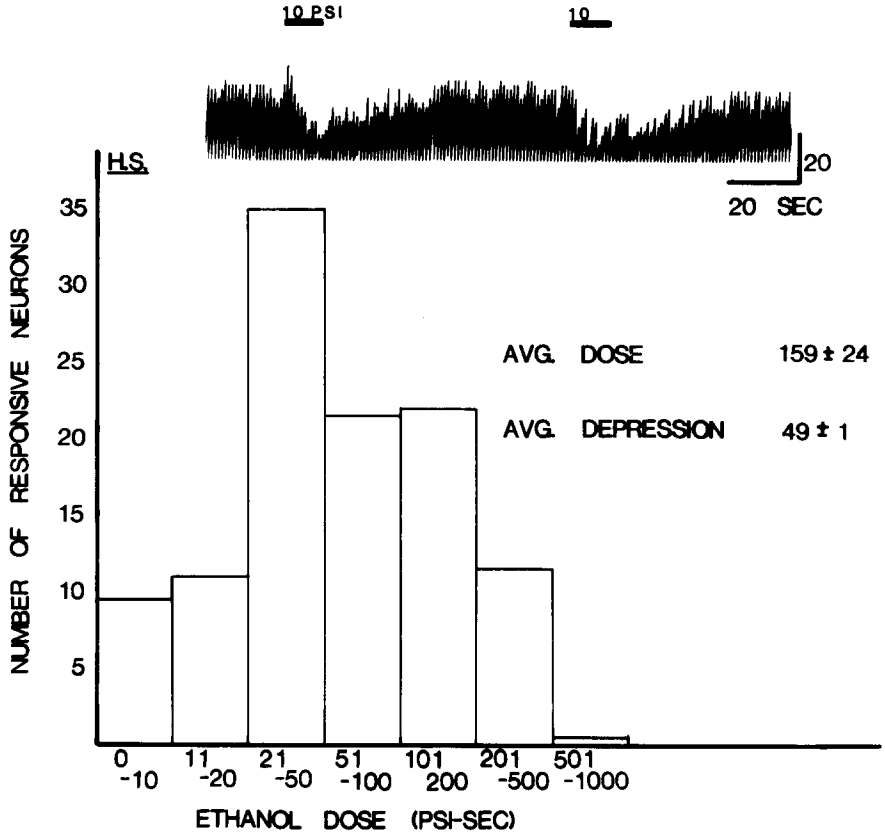

FIG. 1. Depressant responses to locally applied ethanol in P cells from HS mice. Bar graph indicates number of neurons showing a 30–70% reproducible depression at each indicated dose range of ethanol in PSI-seconds (abscissa). Population mean depression is 48%. Inset shows a typical ratemeter record for a single HS Purkinje neuron. In this and all succeeding ratemeters, the bar above the trace indicates duration of ejection, and the overlying number shows the ejection pressure in PSI.

from the CA1 pyramidal cell layer (population spike response), averaged, and entered into the NOVA 3/12 computer in digital form for subsequent analysis [7].

Slices were normally maintained without perfusion until they were to be tested, at which time they were transferred to the test chamber and a constant flow of fresh oxygenated pre-heated medium was initiated at a rate of 2 ml/min. Following an equilibrium period, testing of response amplitude was begun; usually two responses evoked 5 sec apart at 1 min intervals were averaged and displayed throughout the test period. More frequent stimulation was avoided to prevent frequency potentiation in the slice. Ethanol was made up in medium at 100× the desired final concentration, then added to the flow of perfusion fluid with a calibrated Sage Model 355 syringe pump.

RESULTS

Purkinje Cell Recordings from HS Animals

A total of 115 cells, fitting the criteria outlined in METHOD, were recorded from 14 HS animals. Spontaneous discharge rate (34 spikes/sec) did not differ from that seen in LS and SS animals [29]. The average depression in this population of neurons, elicited by a mean ejection of 159 ± 27.4 PSI-sec, was 48 ± 1.0% (Fig. 1). The mean latency to the maximal effect was about 5 sec from onset of drug ejection. In Fig. 2, the ethanol dose-response distribution of HS Purkinje neurons is plotted with our previously reported SS and LS data [29] on the same abscissa. The HS ethanol dose for an approximately 50% mean population inhibition is about 5.4 times greater than the LS dose, and 5.6-fold less than the corresponding SS dose.

A number of controls for pressure ejection artifacts were carried out in HS animals. These consisted of ejection of isotonic saline (154 mM) or isosmotic sucrose (670 mM) from barrels of the multibarreled micropipette adjacent to those containing ethanol. Ejection of saline or sucrose had little or no effect on P neuron discharge, whereas ethanol ejected with the same pressure and time parameters elicited a marked inhibition of activity. This was true in all three groups of animals [29].

Recording From Hippocampal Pyramidal Neurons In Situ and In Vitro

Using the electrophysiological and histological criteria for

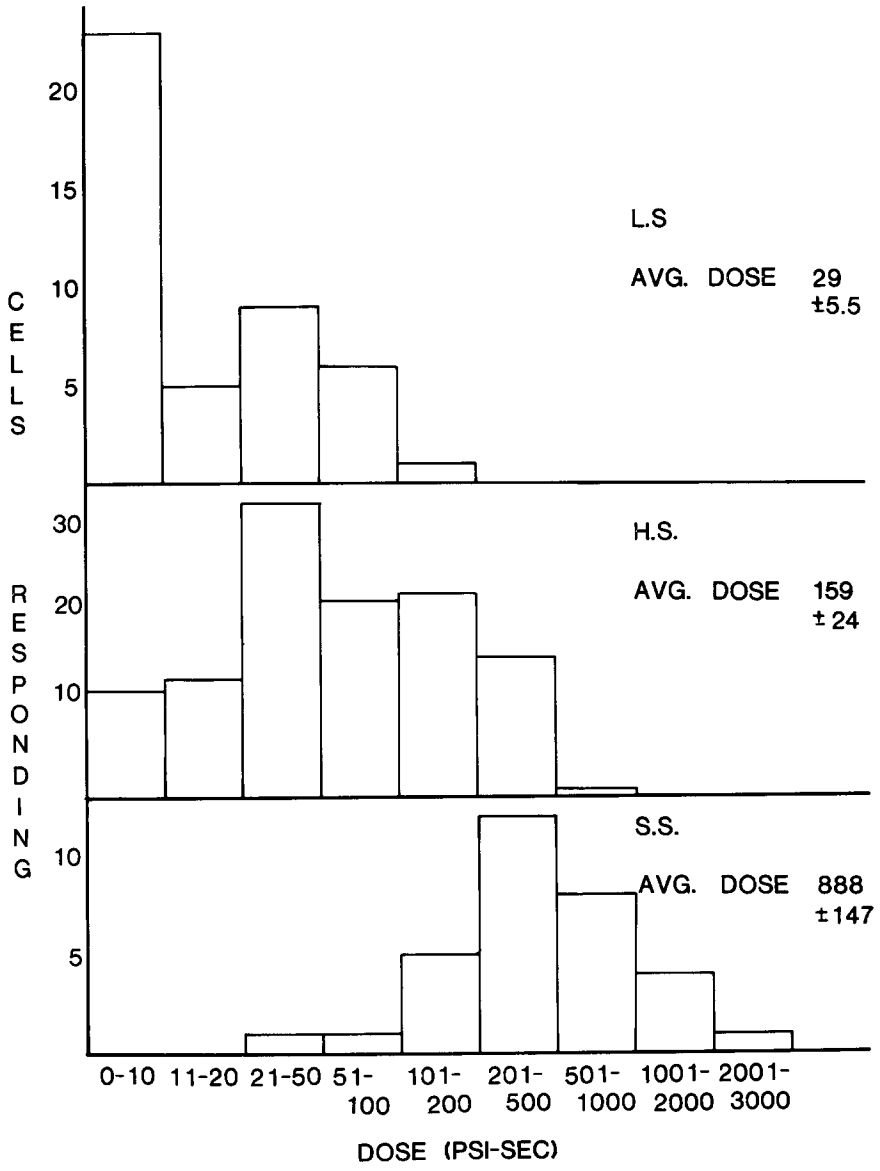

FIG. 2. Bar graphs comparing ethanol-induced P cell depression in HS mice with previously reported data on LS and SS mice. Height of each bar represents number of neurons showing a 30–70% depression at each dose range of ethanol indicated on abscissa. Population mean depression for LS and SS mice were 54 and 52%, respectively [29]. Note that HS population mean ethanol dose is almost exactly intermediate between similarly calculated LS and SS doses.

identification of hippocampal pyramidal cells, as outlined in METHOD, a total of 25 such neurons from six SS animals and 23 neurons from five LS animals were studied *in situ*. In contrast to cerebellar Purkinje neurons, the pyramidal cells from SS animals were somewhat, but not significantly, more sensitive to ethanol than cells in the LS animals (Fig. 3). Mean latencies to maximal depressions for LS animals (19.6 sec) and SS animals (19.4 sec) were also similar, but both were considerably greater than that observed in cerebellar Purkinje cells. Ejection of isotonic saline or isosmotic sucrose from adjacent barrels of the same pipette again elicited little or no change in pyramidal neuron discharge.

Since physical or chemical barriers could exist in the hippocampus *in situ* and underly such negative data, a second approach to studies of the electrophysiological properties of hippocampus utilized perfusion in the *in vitro* slice. The amplitude of the CA1 pyramidal cell population spike, evoked by Schaffer-commissural stimulation, was used as an index of excitability. The population spike is caused by the extracellular currents generated by the near-synchronous firing of

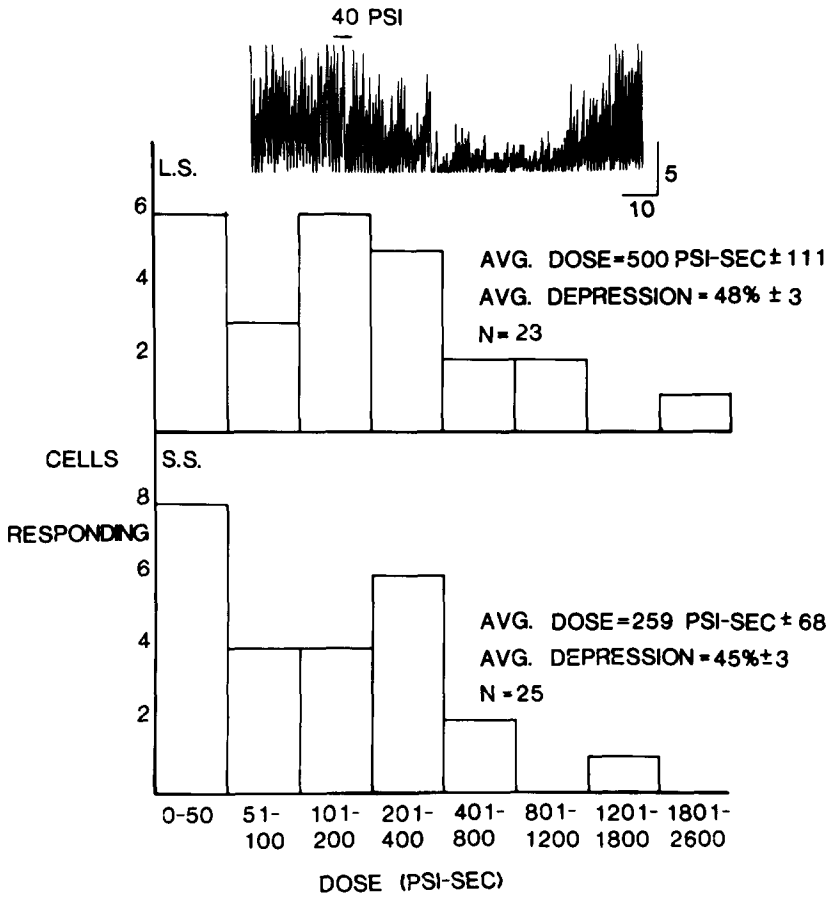

FIG. 3. Effects of locally applied ethanol on hippocampal pyramidal neurons *in situ*. Bar graph showing numbers of neurons manifesting a 30–70% depression in response to ethanol, is shown for LS (upper graph) and SS (lower graph) cells. Typical ratemeter record for an LS neuron is shown above bar graph. Pyramidal neurons from SS animals were slightly, but not significantly, more sensitive.

the pyramidal neurons; changes in its amplitude thus reflect changes in the intrinsic excitability of these neurons [1, 2, 3, 13, 22]. In initial experiments, perfusion of ethanol over the dose range of 100–900 mM revealed little difference in the response of the two lines. Threshold population spike depressions were seen at 75–100 mM, and perfusion of 600–900 mM often yielded profound depressions which did not readily recover. Hence, in a second series of experiments, the effects of a perfusion of 300 mM, which elicited approximately half-maximal depressions, were studied in slices from 13 animals. As shown in Fig. 4, no statistically significant differential effect was seen in the two lines. As observed *in situ*, ethanol appeared to have, if anything, a slightly greater depressant effect in the SS mice.

Effects of Local Application of Halothane on Purkinje Cell Discharge

In view of the behavioral data showing an equal soporific effect of halothane in LS and SS mice [5], this drug was also studied electrophysiologically. Because of the volatility of this substance, a 10 mM solution was made fresh just before filling the pipette, and the barrel was sealed until it was attached to the pressure line. Recordings were made from 22 Purkinje cells in four SS animals, and 23 cells in four LS animals. As shown in Fig. 5, the SS and LS neurons were not significantly different in their depressant responses to halothane. Inhibition of activity was usually maximal within 3 sec in both lines. Again, ejection of isotonic saline or isosmotic sucrose from adjacent barrels had little or no effect.

DISCUSSION

In long-sleep and short-sleep mice, major differences exist in the behavioral sensitivity to ethanol, as demonstrated by radically different sleep times when challenged with this drug. Since "sleep time" is defined as the time elapsed between the loss and recovery of the righting reflex, which has been shown to involve cerebellar function [9,10], we have used cerebellar Purkinje cells as a target neuron for examining ethanol effects. Two lines of evidence from this study suggest that our previously observed differences in the depressant effects of ethanol on Purkinje cell spontaneous

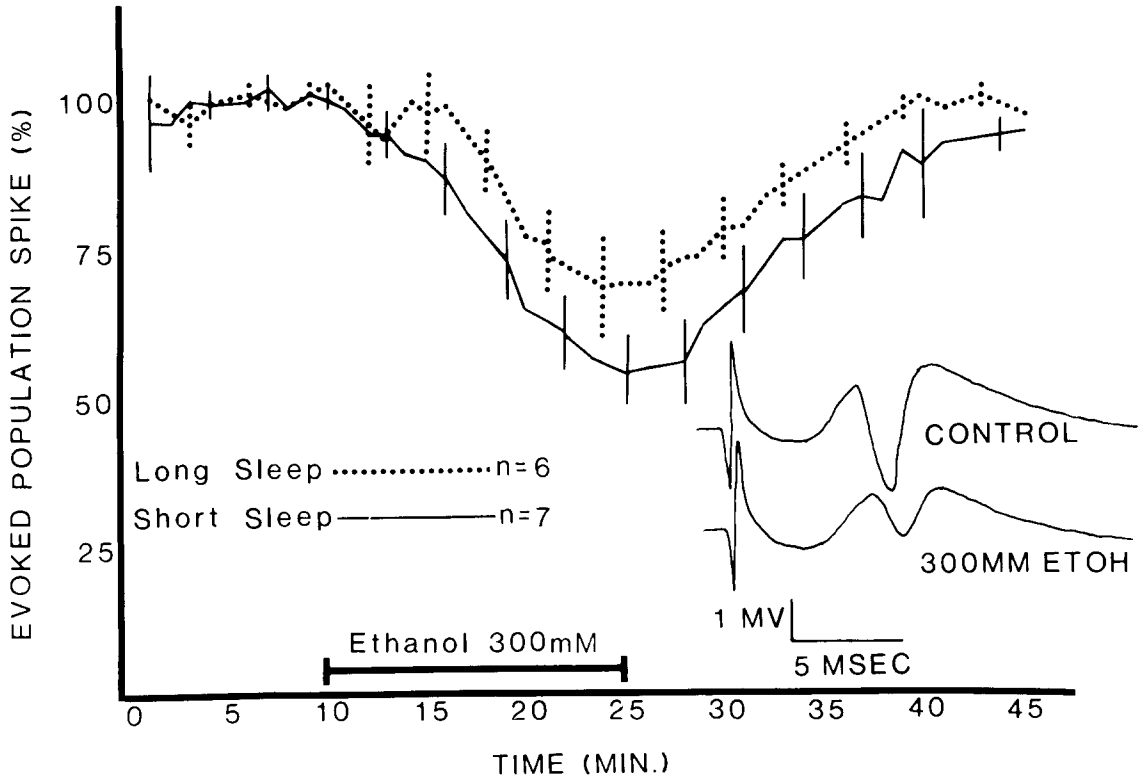

FIG. 4. Effects of superfusion of 300 mM ethanol on the size of the pyramidal neuron population spike evoked by stimulation of Schaffer-commissural afferents in the *in vitro* hippocampal slice. Curves show mean normalized spike amplitude with SEM displayed for every fourth point. Stimuli were pairs of shocks at 5 sec intervals delivered once per minute. There was no significant difference between LS and SS slices. Typical responses during control and ethanol perfusion are shown in lower right. First deflection is the stimulus artifact; the negative-going (downward) part of the waveform with the roughly 5 msec latency to onset is the population spike.

discharge in LS and SS mice may be related to the differential soporific actions of this drug [29]. First, the mean dose needed to elicit equivalent depressions of P cell activity in the parental stock HS mouse was intermediate between the mean LS and SS values. This agrees well with the intermediate ethanol-induced sleep time in HS animals [11]. Second, no differential effects were seen with local halothane application in LS and SS lines. Again, this agrees with behavioral data showing that halothane-induced sleep time is equivalent in the LS and SS lines [5].

Pharmacokinetic studies suggest that the differential behavioral effects of ethanol in the various mouse lines are due to a differential central neuron sensitivity rather than pharmacokinetic differences. The time course of blood level changes are identical in LS and SS mice after parenteral injections, and these blood levels are much higher for SS than LS at their respective times of awakening [15,16]. In any case, the amount of ethanol administered by micropressure ejection (see below) would be insufficient to influence peripheral metabolism.

Although micropressure ejection is uniquely suitable for studying the direct effects of alcohol on neurons, use of the technique raises an important methodological question, i.e., the validity of pressure ejection as a quantitative technique for local ethanol administration. Using isotonic saline, or isosmotic sucrose in isotonic saline, ejected from an adjacent barrel, we have shown that Purkinje or pyramidal neuron discharge is little altered by the application of pressure per se at ejection parameters that elicit marked slowing when ethanol is used [29]. Moreover, we [26] and others [23,27] have shown that drug release with pressure is reproducible and linearly related to time and pressure amplitude. Furthermore, our studies indicate that the time and pressure amplitude parameters are interchangeable for a broad range of values and that little leakage occurs between ejection trials [26].

A second difficulty derives from an inability to calculate dose at the receptor site after pressure ejection. It is possible to measure amount of drug released using either measurement of the droplet diameter [23,27] or dpm's from radioactive tracers released *in vitro*. With our pipettes, an ejection of 50 PSI-sec releases about 10^{-5} μl of solution [26]. Yet, since the distance between the pipette and the neuron is unknown, and local diffusional barriers may exist, drug concentration and hence the relationship between locally and parenterally administered ethanol effects is problematic.

Our choice of a "criterion" of 30–70% depression for each cell was made to avoid problems with the "law of initial values". If maximal or supramaximal responses are used, it is conceivable that lower doses of ethanol could yield similar

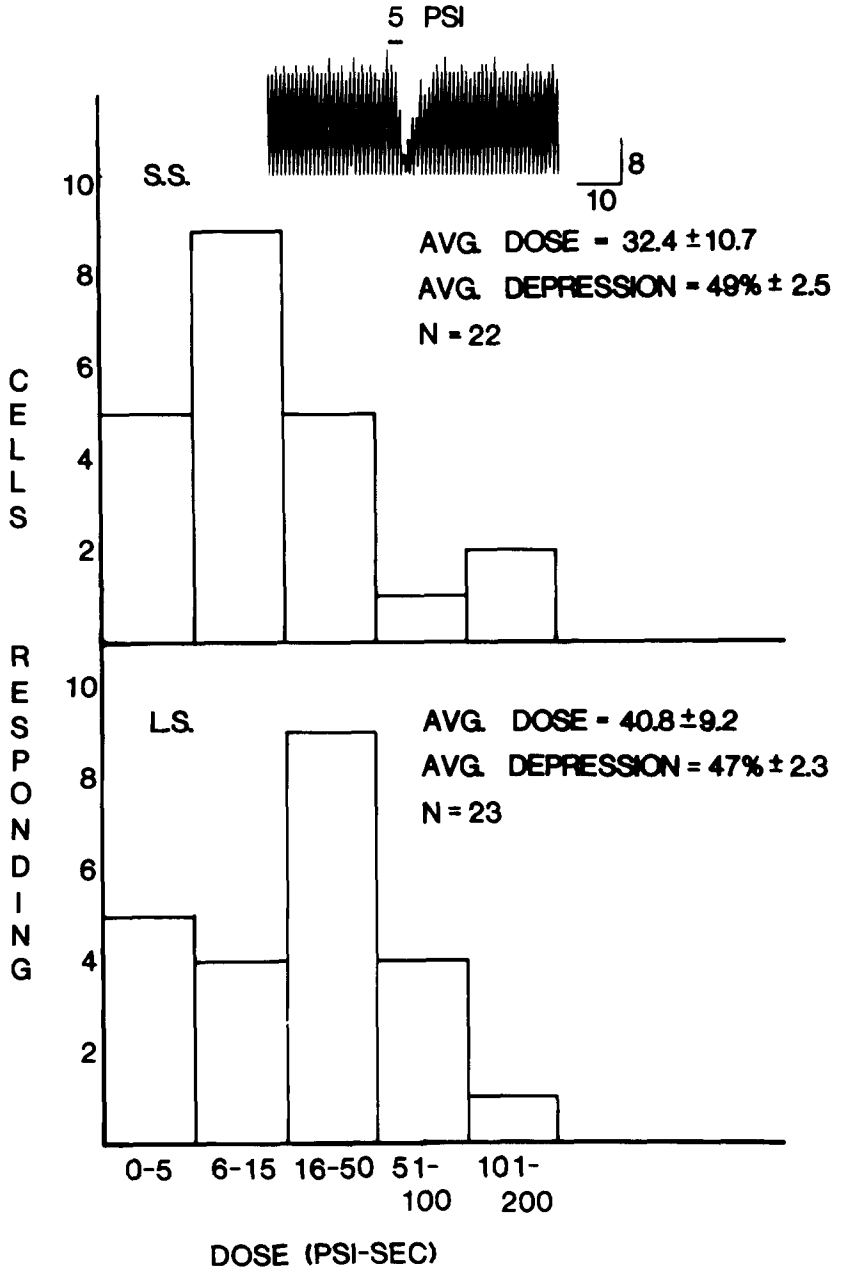

FIG. 5. Effects of locally applied halothane on spontaneous P cell discharge in SS versus LS mice. Bar graphs of numbers of neurons showing a 30–70% depression to a given halothane dose (PSI-sec) is shown for SS cells (upper graph) and LS cells (lower graph). Typical ratemeter record for a single SS Purkinje neuron is shown above the bar graph. No difference was seen in the two lines.

effects. Similarly, if threshold responses are studied, it is possible that larger doses would yield an effect of similar magnitude. The 30–70% criterion was used since it could be assumed that one were at a point on the dose-response curve where response would reliably change with dose. Again, in this as in our previous work, the mean response magnitude for the entire population of cells in each study approximated 50% [29].

There have been several electrophysiological studies on Purkinje [10, 12, 18, 21, 31] and pyramidal neurons [14, 18, 20, 21, 31] after parenteral administration of ethanol. A recent review of these studies reveals that there is considerable diversity in the data, due in part to the variety of techniques employed. Depressions are generally seen at higher doses, with some excitations at lower doses. It is, however, difficult to relate these experiments to our own data for several reasons. First, the time course of ethanol absorption and distribution in the brain may produce changes in the response of the cell as a function of the time between recording and injection. Second, changes in electrical activity after par-

enteral injection may be due to both direct and indirect changes in the region under study. For example, we have recently found that parenteral administration of 1 g/kg of ethanol elicits a transient increase in Purkinje cell discharge (Sorensen, Carter, Marwaha, Baker and Freedman, in preparation). This is probably due to removal of the tonic inhibitory noradrenergic input from the locus coeruleus [17] and subsequent disinhibition of P cell discharge. Third, there are no clearcut relationships between EEG [19,20], evoked potentials [1, 2, 3, 13, 22], "multiunit recording" [12, 19, 20] and the single unit recording which we have employed.

The differences between cerebellum and hippocampus in the LS and SS lines was a most surprising finding. No differential effect on hippocampal pyramidal excitability was seen, using either spontaneous discharge and local administration of ethanol *in situ*, or evoked discharge and perfusion of ethanol *in vitro*. These data suggest that whatever genetically determined membrane macromolecules are altered in the two lines to produce the behavioral differences, such biochemical changes are region-specific or neuron-specific in the central nervous system. Biochemical studies focusing on differences between cerebellum and hippocampus in the LS and SS lines may thus provide important information on the mechanism of ethanol action at the membrane level.

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