Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2825–2829, March 1995
Pharmacology

Cutoff in potency implicates alcohol inhibition of *N*-methyl-D-aspartate receptors in alcohol intoxication

(glutamate receptor/ion channel/neurotransmitter)

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Communicated by William A. Catterall, University of Washington, Seattle, WA, December 23, 1994

ABSTRACT **As the number of carbon atoms in an aliphatic *n*-alcohol is increased from one to five, intoxicating potency, lipid solubility, and membrane lipid disordering potency all increase in a similar exponential manner. However, the potency of aliphatic *n*-alcohols for producing intoxication reaches a maximum at six to eight carbon atoms and then decreases. The molecular basis of this "cutoff" effect is not understood, as it is not correlated with either the lipid solubility or the membrane disordering potency of the alcohols, which continue to increase exponentially. Since it has been suggested that inhibition of *N*-methyl-D-aspartate (NMDA) receptors by alcohols may play a role in alcohol intoxication, we investigated whether a series of aliphatic *n*-alcohols would exhibit a cutoff in potency for inhibition of NMDA receptors. We found that although potency for inhibition of NMDA receptors increased exponentially for alcohols with one to five carbon atoms, potency for inhibition of NMDA receptors reached a maximum at six to eight carbon atoms and then abruptly disappeared. This cutoff for alcohol inhibition of NMDA receptors is consistent with an interaction of the alcohols with a hydrophobic pocket on the receptor protein. In addition, the similarity of the cutoffs for alcohol inhibition of NMDA receptors and alcohol intoxication suggests that the cutoff for NMDA receptor inhibition may contribute to the cutoff for alcohol intoxication, which is consistent with an important role of NMDA receptors in alcohol intoxication.**

The molecular basis of alcohol's behavioral effects is poorly understood. Since the turn of the century, it has been widely accepted that alcohols produce their central nervous system effects by acting on the lipids of neuronal membranes (1–8). According to this "lipid theory," the effects of alcohols on the function of membrane proteins, such as receptors and ion channels, are secondary to their perturbation of membrane lipids. Consistent with this hypothesis, the intoxicating potency of aliphatic *n*-alcohols with up to five carbon atoms is correlated with both their lipid solubility (membrane/buffer partition coefficient) and their membrane lipid disordering potency (9, 10). However, as the number of carbon atoms is increased from six to eight, the intoxicating potency of aliphatic *n*-alcohols reaches a plateau and then decreases (9, 10). This cutoff effect for intoxication is difficult to reconcile with the lipid theory of alcohol action, because it is not correlated with either membrane/buffer partition coefficient or potency for disordering membrane lipids, which both continue to increase exponentially with increasing carbon chain length (9–12).

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. *N*-Methyl-D-aspartate (NMDA) receptors are a subtype of glutamate receptors that appear to be involved in a variety of neural phenomena including nervous system excitability, cognitive function, and motor coordination (13–15). Since NMDA receptor function is inhibited by ethanol at concentrations that are associated with intoxication, it has been suggested that NMDA receptors may play a role in alcohol intoxication (16, 17). We reasoned that if NMDA receptors are involved in intoxication, a cutoff in the potency of alcohols for inhibition of NMDA receptors might underlie the cutoff in the potency of alcohols for producing intoxication. To assess this possibility, we investigated whether a series of aliphatic *n*-alcohols exhibits a cutoff in potency for inhibition of NMDA receptor function. Some of this work has been presented previously in preliminary form (18).

MATERIALS AND METHODS

Cultures of hippocampal neurons on glial feeder layers were prepared from 15- to 17-day fetal mice by a method modified from that of Goslin and Banker (19). Neurons were cultured for at least 4 days prior to use in experiments. Patch-clamp recording of whole-cell currents was performed at room temperature with an Axopatch-1D (Axon Instruments, Foster City, CA) patch-clamp amplifier. Gigaohm seals were formed by using electrodes with tip resistances of 2–4 MΩ, and series resistances of 4–10 MΩ were compensated by 80%. Data were filtered at 2 kHz and recorded by using a microcomputer (Compaq 386/20e) with a Labmaster TL-1 interface and AXOTAPE software (Axon Instruments). Neurons were superfused at 1–2 ml/min in an extracellular medium containing 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, 0.0002 mM tetrodotoxin, 0.1 mM picrotoxin; pH was adjusted to 7.4 with NaOH and osmolality was adjusted to 340 mosmol/kg with sucrose. In some cases, bicuculline methiodide (0.05 mM) was also added to the medium. The pipette (internal) solution contained 140 mM CsCl, 2 mM $MgCl_2$, 10 mM bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate, 2 mM Mg_4ATP, 10 mM Hepes; pH was adjusted to 7.4 with CsOH and osmolality was adjusted to 310 mosmol/kg with sucrose. Solutions of NMDA (Sigma) and alcohols (Aldrich) were prepared daily in extracellular medium. To ensure dissolution, alcohols with six or more carbon atoms were added to extracellular medium in tightly sealed glass or Teflon containers, vigorously shaken, and sonicated for ≥30 min. Loss of long-chain alcohols from solutions due to evaporation or adsorption to the experimental apparatus was minimized by using a closed solution application apparatus composed entirely of Teflon and fused silica. Flexible Teflon reservoirs were connected via Teflon tubing and valves to a linear multibarrel array of fused silica tubing (diameter of each pipette, ≈200 μm) placed within 50 μm of the cell body to

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Abbreviations: NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid.

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allow for rapid solution changes. Cells were constantly superfused by extracellular medium flowing from one pipette barrel (flow rate, 2–3 μl/s), and treatment solutions were applied by opening a valve and moving the barrel array so that the desired solution bathed the cell. Unless noted otherwise, solutions of NMDA and alcohols were applied at intervals of at least 90 s. Statistical analysis of concentration–response data was performed using the nonlinear curve-fitting program ALLFIT (20). Values reported for slope factors (n) and alcohol concentrations producing half-maximal inhibition (IC_{50}) are those obtained by fitting the data to the logistic equation

$$y = \{(E_{max} - E_{min})/(1 + [x/IC_{50}]^{-n})\} + E_{min},$$

where x is alcohol concentration, y is response (i.e., percentage inhibition), E_{max} is maximal response, and E_{min} is minimal response. In these studies, E_{min} and E_{max} were constrained to 0% and 100% inhibition, respectively; this did not affect the quality of fits for alcohols with two to five carbons and was necessary for fitting curves for alcohols with more than five carbons, because the maximal obtainable concentrations of those alcohols fell in the linear portion of the concentration–response curves. In addition, the concentrations of alcohols with more than five carbons that could be tested were limited by their tendency to disrupt the pipette–membrane seal at concentrations approaching 50% of aqueous saturation. Data were statistically compared by the paired t test or ANOVA, as noted. Average values are expressed as the mean ± SE.

RESULTS

Fig. 1 illustrates the inhibition of NMDA-activated ion current by a series of aliphatic n-alcohols from methanol to 1-octanol. Note that as the carbon chain length increased, decreasing concentrations of the alcohols produced roughly similar magnitudes of inhibition. Fig. 2, however, illustrates that 0.5 mM 1-nonanol (Fig. 2*A Left*) and 0.1 mM 1-decanol (Fig. 2*B Left*) did not inhibit NMDA-activated current, even though these concentrations would result in membrane alcohol concentrations equivalent to those produced by over 3 and 1.9 M ethanol, respectively (9). In view of this, we also tested the effect of 1-nonanol and 1-decanol on γ-aminobutyric acid (GABA)-activated current in the same neurons. Although NMDA-activated current was not significantly affected by 0.5 mM 1-nonanol or 0.1 mM 1-decanol, GABA-activated current was greatly increased in amplitude by both 1-nonanol (Fig. 2*A Right*) and 1-decanol (Fig. 2*B Right*) at these concentrations. On average, 0.5 mM 1-nonanol potentiated GABA-activated current to 1322% ± 247% of control (paired t test; $P < 0.05$; $n = 5$) and 0.1 mM 1-decanol potentiated GABA-activated current to 861% ± 232% of control (paired t test; $P < 0.001$; $n = 4$); however, 1-nonanol and 1-decanol at these concentrations did not inhibit NMDA-activated current in the same neurons (106% ± 2% of control and 102% ± 2% of control, respectively). These observations indicate that the lack of effect of 1-nonanol and 1-decanol on NMDA-activated current was not due to technical problems, such as incomplete dissolution or evaporative loss of the alcohols. In addition, there appear to be differences between NMDA- and GABA-gated ion channels with respect to the molecular mechanism and/or site of action of the alcohols.

The graph in Fig. 3 shows the concentration–response curves for inhibition of NMDA-activated current by aliphatic n-alcohols from methanol to 1-decanol. As the carbon chain length increased from methanol to 1-hexanol, the curves successively shifted to the left in a parallel manner. However, the curve for 1-heptanol was only slightly to the left of the curve for 1-hexanol, and the curve for 1-octanol shifted to the right. The concentration producing 50% inhibition (IC_{50}) for each alcohol was 350 mM methanol, 130 mM ethanol, 59 mM 1-propanol, 24 mM 1-butanol, 8.3 mM 1-pentanol, 4.2 mM 1-hexanol, 3.4 mM 1-heptanol, and 5.8 mM 1-octanol. It was not possible to determine the IC_{50} value for octanol without extrapolation, as the highest concentration of octanol that could be tested produced <50% inhibition. The IC_{50} values for

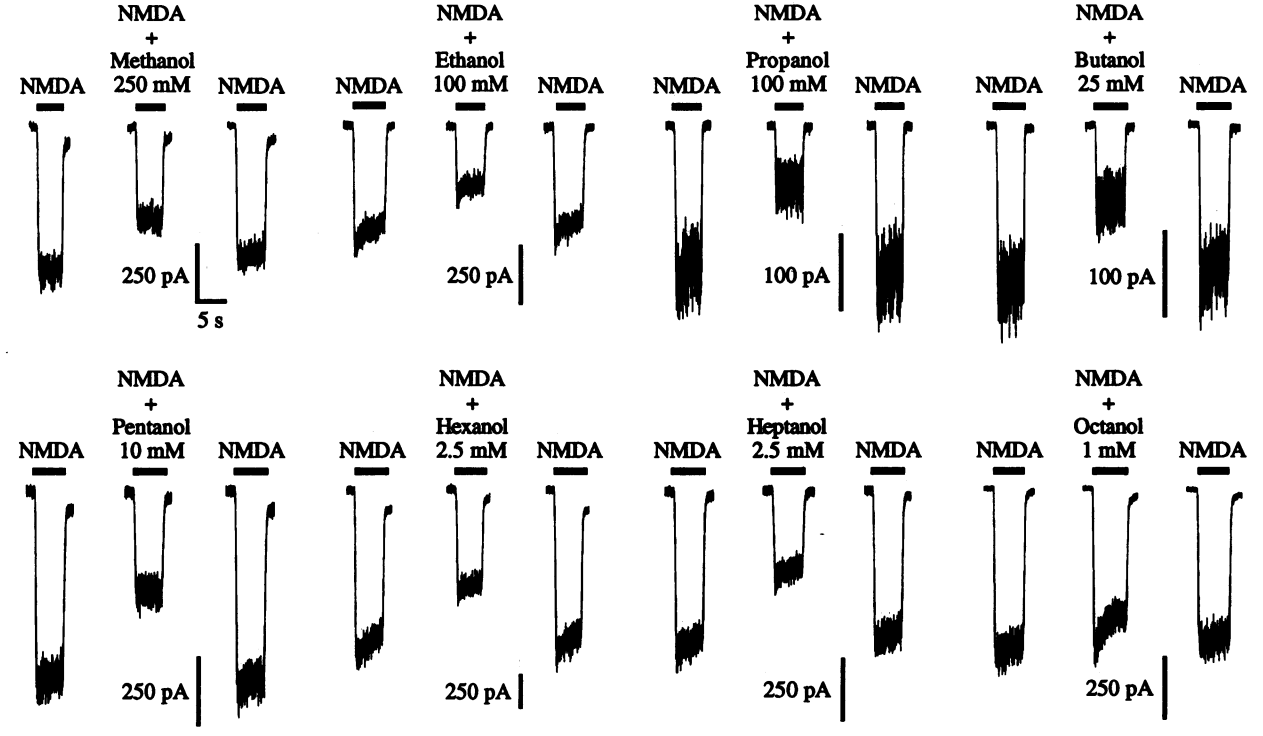

FIG. 1. Inhibition of NMDA-activated ion currents in hippocampal neurons by aliphatic n-alcohols from methanol to 1-octanol. Currents were activated by 25 μM NMDA in the presence of 1 μM glycine. Each set of records is from a different hippocampal neuron. The time calibration in the first set of records applies to all records.

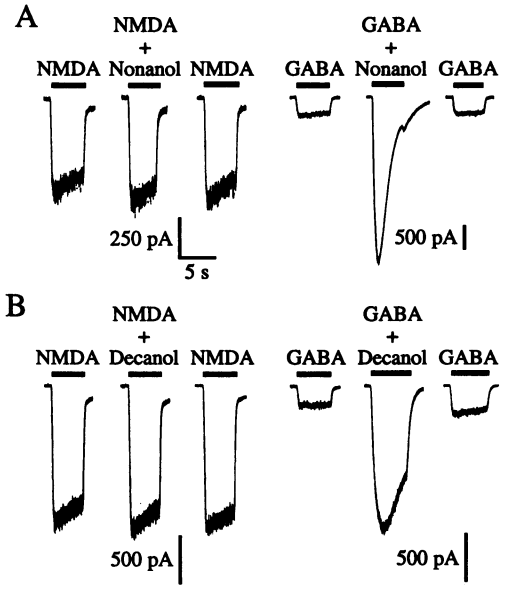

FIG. 2. Effects of 1-nonanol and 1-decanol on NMDA- and GABA-activated currents. Currents were activated by 25 μM NMDA in the presence of 1 μM glycine (*Left*) or 1 μM GABA (*Right*). Alcohol concentrations were 0.5 mM 1-nonanol (*A*) and 0.1 mM 1-decanol (*B*). The records of NMDA- and GABA-activated current for each alcohol were obtained in the same hippocampal neurons. In experiments on GABA-activated currents, extracellular solutions did not contain picrotoxin or bicuculline methiodide. The increased decay rate associated with nonanol and decanol potentiation of GABA-activated current is probably attributable to $GABA_A$ receptor desensitization, as has been reported for other alcohols (21). Solutions of GABA or GABA and alcohols were applied at intervals of at least 3 min. Time calibration in the first set of records applies to all records.

1-nonanol and 1-decanol could not be determined, as maximal attainable concentrations of these alcohols did not inhibit NMDA-activated current.

Fig. 4*A* plots the relative potency of aliphatic *n*-alcohols from methanol to 1-decanol for inhibiting NMDA receptors (ethanol IC_{50}/alcohol IC_{50}) as a function of their membrane/buffer partition coefficient. The dashed line in the graph indicates the membrane disordering potency of the alcohols as a function of their membrane/buffer partition coefficient. For alcohols with up to five carbon atoms, NMDA receptor inhibitory potency increases exponentially as carbon chain length is increased. NMDA receptor inhibitory potency is significantly correlated with both the membrane/buffer partition coefficient ($r = 0.9951$; $P < 0.001$), as has been reported (16, 22, 23), and the membrane disordering potency (r =

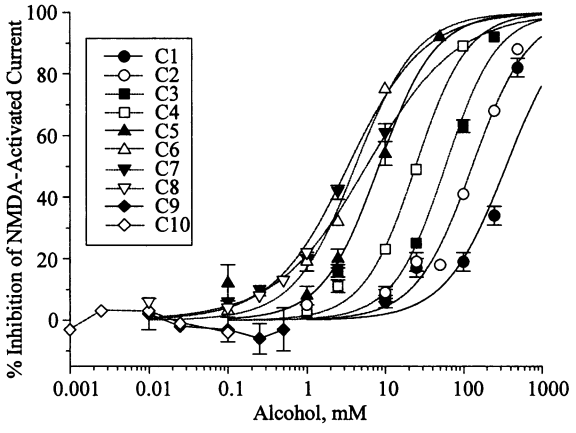

FIG. 3. Concentration–response curves for inhibition of NMDA-activated current by aliphatic *n*-alcohols from methanol (C1) to 1-decanol (C10). Data points are means ± SE of five to nine neurons; error bars not visible are smaller than the size of the symbols. Curves for alcohols from methanol to 1-octanol were obtained by fitting the data to the logistic equation given in *Materials and Methods*. Curves for 1-nonanol and 1-decanol are point-to-point fits.

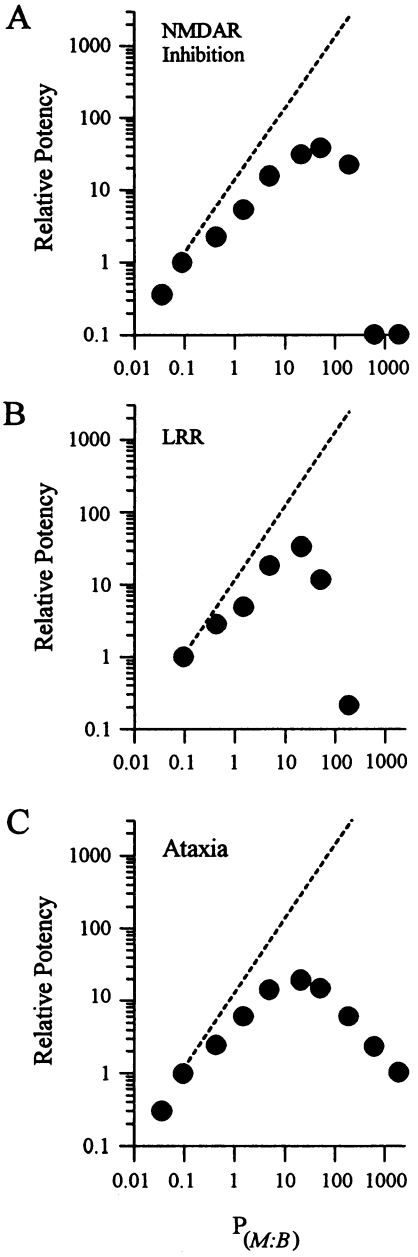

FIG. 4. Cutoff in the potency of aliphatic *n*-alcohols for inhibition of NMDA receptors is similar to the cutoff in their potency for producing intoxication. Relative potency of aliphatic alcohols for inhibiting NMDA receptors (NMDAR) (ethanol IC_{50}/alcohol IC_{50}; *A*), producing loss of righting reflex (LRR) (ethanol ED_{50}/alcohol ED_{50}; *B*), and eliciting ataxia (ethanol ED_3/alcohol ED_3, *C*), plotted as a function of their membrane/buffer partition coefficients ($P_{M:B}$). Dashed line in each plot indicates the membrane disordering potency for aliphatic *n*-alcohols from ethanol to 1-octanol, reported as the reduction in order parameter per 100 mM alcohol in the aqueous phase relative to that of ethanol. Membrane disordering potency data, from Lyon *et al.* (10), were obtained by electron paramagnetic resonance in mouse brain synaptic membranes labeled with the spin probe 5-doxylstearic acid. Data points for 1-nonanol and 1-decanol in *A* are shown on the *x* axis because IC_{50} values could not be obtained for these alcohols. Loss of righting reflex potency data in *B* are from Lyon *et al.* (10), and ataxic potency data in *C* are from McCreery and Hunt (9).

0.9969; $P < 0.005$) of these alcohols. However, alcohol potency for inhibiting NMDA receptors reached a maximum as the number of carbon atoms was increased from six to eight, and alcohols with 9 and 10 carbon atoms did not inhibit NMDA-activated current, despite increased lipid solubility and membrane disordering potency (9, 12, 24).

DISCUSSION

The lipid theory of alcohol action postulates that alcohols produce their actions on neuronal membrane proteins indirectly by perturbing membrane lipids. Fig. 4 illustrates the characteristics of this membrane-perturbing effect of alcohols; the dashed line in each of the plots in Fig. 4 shows the results obtained by Lyon *et al.* (10) on the disorder produced in mouse brain synaptic membranes by *n*-alcohols from ethanol to octanol. As can be seen, membrane disordering potency of alcohols increases exponentially with increasing hydrophobicity. The lipid theory predicts that the behavioral effects of alcohols should be correlated with their perturbation of membrane lipids—namely, their membrane disordering potency (1–6, 9, 10). This prediction holds true for aliphatic *n*-alcohols with up to five carbon atoms (9, 10), but for *n*-alcohols with more than five carbon atoms, although lipid solubility and membrane lipid disordering potency continue to increase exponentially with increases in carbon chain length, intoxicating potency reaches a maximum and then declines. This cutoff in intoxicating potency is thus difficult to reconcile with the lipid theory of alcohol action.

In contrast, our observations indicate that inhibition of NMDA receptors by aliphatic *n*-alcohols exhibits a cutoff very similar to that observed for intoxicating potency. A cutoff in the potency of a series of alcohols for inhibiting purified firefly luciferase has been interpreted as evidence that the alcohols act by binding in a hydrophobic protein pocket of fixed dimensions (25). Our observations are consistent with the alcohols inhibiting NMDA-gated ion channels by interacting with a hydrophobic pocket of circumscribed dimensions on these receptors. We estimate that the molecular volume of the putative hydrophobic pocket on NMDA receptors is less than the molecular volume of 1-nonanol, which is 103.5 cm^3/mol (26). However, the molecular basis for the cutoff phenomenon on NMDA receptors appears to differ from that on firefly luciferase. In the case of firefly luciferase, when the volume of the alcohol exceeds the size of the pocket, the additional carbon atoms apparently remain in the aqueous environment and thus do not influence binding in the pocket (25). In contrast, in the case of NMDA receptors, when the volume of the alcohol exceeds the size of the pocket, the alcohol appears unable to bind in the pocket and thus does not inhibit the function of the receptor.

Possible alternative explanations for the cutoff phenomenon for NMDA receptors should also be considered. The lack of inhibitory effect of the 9- and 10-carbon alcohols on NMDA-activated current might result from a concurrent potentiating effect of these alcohols. However, for such a masking effect to occur, the EC_{50} and the slope factor for the potentiating effect would have to equal the IC_{50} and the slope factor for the inhibitory effect, which is highly improbable. The lack of inhibition of NMDA-activated current by nonanol and decanol might also result from technical problems related to the physical properties of these alcohols, such as incomplete dissolution or loss of the alcohols through evaporation or adsorption to the tubing of the application apparatus. In the present experiments, rigorous measures were taken to minimize these possible sources of error, as described above. Furthermore, the observation that nonanol and decanol markedly potentiated GABA-activated current but did not inhibit NMDA-activated current in the same neurons demonstrates that the lack of effect of these alcohols on NMDA receptors cannot be explained by such factors.

Although the behavioral effects of alcohols are thought to result from alterations in the function of proteins in the central nervous system, the proteins responsible for those effects have not been determined. Fig. 4 *B* and *C* illustrates the potency of a series of aliphatic *n*-alcohols for producing intoxication, using either loss of righting reflex (Fig. 4*B*) or ataxia (Fig. 4*C*) as the experimental index of intoxication. Note that as the number of carbon atoms is increased from six to eight, the intoxicating potency of the alcohols reaches a maximum and then declines, despite increased membrane/buffer partition coefficient and membrane disordering potency (Fig. 4 *B* and *C*). Moreover, the cutoffs for alcohol intoxication are similar to the cutoff for alcohol inhibition of NMDA receptors (compare Fig. 4 *B* and *C* with Fig. 4*A*). By contrast, recent experiments in our laboratory indicate that alcohol actions on ATP-gated ion channels (27) and 5-hydroxytryptamine$_3$ receptor-ion channels (28) exhibit cutoffs that differ from the cutoff for alcohol inhibition of NMDA receptors reported here. In addition, the potency vs. hydrophobicity plot for alcohol potentiation of $GABA_A$ receptor-ion channels is linear for alcohols with up to 10 carbon atoms (refs. 21 and 29; unpublished observations), and it has recently been reported that alcohol potentiation of nicotinic acetylcholine-gated ion channels does not exhibit a cutoff for alcohols with up to 9 carbon atoms (30). Thus, the cutoff for alcohol inhibition of NMDA receptors, but not for alcohol effects on other receptors tested to date, closely resembles the cutoff for alcohol intoxication. This does not negate possible roles of other receptors and ion channels in contributing to alcohol intoxication, but it does suggest that NMDA receptors play an important role in this behavioral phenomenon.

We thank C. Li and J. M. Wright for comments on the manuscript and A.-M. Vasquez for technical assistance. R.W.P. was supported by an Intramural Research Training Award from the National Institute on Alcohol Abuse and Alcoholism.

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