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## Effects of Ethanol on the Functional Properties of Sodium Channels in Brain Synaptosomes

Michael J. Mullin and Walter A. Hunt

**Abstract.** Voltage-sensitive sodium channels in excitable cell membranes are responsible for the rapid increase in permeability to sodium ions that occurs during depolarization. Neurotoxins that bind with high affinity and specificity to voltage-sensitive sodium channels have been widely used to identify and characterize the structure and function of sodium channels in nerve and skeletal muscle. This chapter describes the actions of ethanol on the functional properties of voltage-sensitive sodium channels in mammalian brain nerve endings. The effects of acute and chronic ethanol administration are also reviewed. Alterations in the function of neuronal membrane sodium channels may be involved in the depressant effect of ethanol. *Keywords:*

*nerve transmission; membrane function; reprints; biochemistry; toxins and antitoxins*

### 1. Introduction

Since the turn of the century, general anesthetics have been believed to interact with excitable membranes to produce their depressant effects. In fact, aliphatic alcohols were used to formulate the well-known Meyer-Overton principal of anesthesia, stating that the potencies of anesthetics are directly related to their lipid solubility. (For a complete review of alcohol-membrane interactions, see Hunt.<sup>1</sup>)

An action of aliphatic alcohols on lipids is expected on physicochemical grounds based on the structure of alcohols. Since alcohols, such as ethanol, are molecules consisting of a single hydroxyl group on a hydrocarbon chain, they would be expected to be both lipophilic and hydrophilic, that is, amphiphiles. The concentration of an alcohol in a membrane would depend on its relative solubilities in membranes and water as expressed by the membrane/water partition coefficient.

Alcohols dissolve in membranes and as a result disorder lipid structure. This has been shown in a number of ways. In the presence of alcohols, the surface area of membranes increases, and the temperature at which mem-

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branes undergo transition from a gel to a liquid phase decreases.<sup>2</sup> A more direct indication of lipid order can be determined with molecular probes that can report on the microenvironment in which they reside. Attached spin-labels or fluorescent molecules can be studied with electron paramagnetic resonance or fluorescence spectrometry.

With these methods aliphatic alcohols have been found to disorder or fluidize membranes at concentrations equivalent to those found in intoxicated animals.<sup>3,4</sup> The disordering occurs more in the lipid core of the membrane than at the surface and can be reduced by increasing the concentration of cholesterol in the membrane or by reducing its temperature.<sup>3</sup>

Cell membranes contain integral units that process various activities in support of the function of the cell. For the lipid-disordering effects of alcohols to have relevance to the pharmacological and behavioral effects of alcohols, these functional entities must in some way be modified. Many enzymes and ion channels depend for optimal activity on the lipid environment associated with them. Consequently, the lipid-disordering effect of alcohols may disrupt this environment with possibly substantial physiological consequences.

Neurons generate and transmit electrical impulses by moving various ions across membranes at precisely controlled intervals. As shown in the classic experiments of Hodgkin and Huxley,<sup>5</sup> electrical impulses or action potentials develop in neurons that have a resting membrane potential and result from an initial but transient increase in the inward movement of sodium ions, whose concentration is mostly extracellular. The cell depolarizes until there is a reversal in the polarity, allowing for depolarization of adjacent portions of the membrane. For the cell to repolarize, potassium ions, which are predominantly intracellular, flow out.

Alcohols can alter the movement of ions through membranes, thereby altering the electrical properties of neurons. Several electrophysiological studies have demonstrated that in invertebrates the height of the action potential and maximum sodium conductance are reduced.<sup>7-9</sup> However, high concentrations of ethanol are required to produce these changes. Similar results were observed with electrically stimulated brain slices.<sup>10,11</sup> In the presence of 105 mM ethanol, sodium uptake into intracellular spaces was significantly reduced.

A molecular approach can also be used to study ion movements. Ions move through pores in membranes called channels containing gates that open and close in response to physiological conditions. Recent advances have led to the isolation and characterization of some of these channels. In the case of sodium channels, various neurotoxins that are known from electrophysiological experiments to interact with sodium channels have been used to characterize the toxins in synaptosomes and cultured cells.

The sodium channel is a glycoprotein with multiple polypeptide subunits that traverses the neuronal membrane.<sup>12</sup> Studies in which purified channels were reconstituted in artificial membranes have demonstrated an absolute requirement of lipids for normal channel function.<sup>13</sup> At least three functional

**Table 1. Neurotoxin Binding Sites in the Sodium Channel**

| Site | Neurotoxin                          | Effect on ion influx                                | Effect of ethanol |
|------|-------------------------------------|-----------------------------------------------------|-------------------|
| I    | Saxitoxin<br>Tetrodotoxin           | Inhibits ion flux                                   | None              |
| II   | Batrachotoxin<br>Veratridine        | Promotes ion influx                                 | Inhibits          |
| III  | Scorpion venom<br>Sea anemone venom | Potentiated effect of neurotoxins acting at site II | None              |

sites within sodium channels have been identified<sup>14</sup> (Table I). Site I, located on the external surface of the neuronal membrane, binds tetrodotoxin and saxitoxin, drugs that block the generation of action potentials. Site II, located in the lipid core of the membrane, binds batrachotoxin and veratridine, lipid-soluble drugs that activate the sodium channels. And site III, located on the membrane surface but with projections down to site II, binds scorpion and sea anemone toxins that enhance the actions of toxins acting on site II but have no intrinsic activity of their own.

With these latest advances it has been possible to examine further the effects of alcohols at the molecular level on sodium channels in the mammalian brain. The research to date has explored the effects of ethanol on the uptake of radioactive sodium ions into synaptosomes that are stimulated with the neurotoxins batrachotoxin or veratridine. Synaptosomal preparations are incubated in a low-sodium or sodium-free physiological medium.<sup>15,16</sup> A preincubation in which ethanol and the toxin are added together or the toxin is added 2 min later lasts for 10 min after the toxin is added. Uptake of the labeled ion is then determined in incubations generally lasting 2-5 sec. Blanks to assess passive movement of sodium ions are performed similarly, except tetrodotoxin may be added to inhibit toxin-stimulated uptake.

## 2. Effects of Ethanol *in Vitro* on Neurotoxin-Stimulated Sodium Influx and Neurotoxin Binding

As mentioned previously, the lipid-disordering action of ethanol should result in changes in the functional properties of neuronal membranes. In recent years, a number of studies have examined the effects of ethanol on calcium,<sup>17,18</sup> potassium,<sup>19</sup> and sodium channels<sup>20,21</sup> in synaptosomes. This section reviews the changes in the properties of voltage-sensitive sodium channels that occur after incubation of ethanol with synaptosomes *in vitro*.

Initially, it was demonstrated that when whole rat brain synaptosomes were incubated with ethanol *in vitro* there was a concentration-dependent inhibition of veratridine-stimulated  $^{22}\text{Na}^+$  influx.<sup>20</sup> This effect of ethanol oc-



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curred at sublethal, pharmacologically relevant concentrations and was fully reversible when the ethanol was removed from the membranes by centrifugation and washing. Pentobarbital (0.45 mM) also reduced the veratridine-stimulated  $^{22}\text{Na}^+$  influx. In addition, Harris<sup>21</sup> demonstrated that ethanol, ether, enflurane, halothane, pentobarbital, and phenytoin reduced the veratridine-dependent uptake of  $^{24}\text{Na}^+$  in a mouse brain synaptosomal preparation. Each of these intoxicant-anesthetic agents also increased the fluidity of mouse brain synaptic plasma membranes. Thus, it seemed reasonable to conclude that an increase in membrane fluidity could alter the functional properties of voltage-sensitive sodium channels in the brain. Since ion channels in the brain play an important role in the processing of information and the control of neuroexcitability, it was necessary to characterize further the effect of ethanol on sodium channel function.

Recently, it was shown that the concentration of ethanol required to inhibit neurotoxin-stimulated sodium influx by 50% ( $\text{IC}_{50}$ ) was dependent on which neurotoxin was used to activate the channel.<sup>22</sup> Batrachotoxin is a full agonist that activates nearly all sodium channels present in synaptosomes. In contrast, veratridine, a partial agonist, activates about 50% of the sodium channels present in rat brain synaptosomes. The  $\text{IC}_{50}$  values for ethanol were 345 and 583 mM for veratridine- and batrachotoxin-stimulated sodium influx, respectively. In an independent study, Harris and Bruno<sup>23</sup> described a similar difference in the potency of ethanol using mouse brain synaptosomes. Thus, although batrachotoxin and veratridine bind to the same site in the channel, there may be major differences in the manner in which each toxin affects the channel after binding occurs. It is interesting to note that the veratridine-stimulated cation influx in reconstituted sodium channels from skeletal muscle is sensitive to changes in temperature, whereas batrachotoxin-stimulated cation influx is rather insensitive to changes in temperature.<sup>24</sup>

When the effect of ethanol on sodium influx was measured over a range of concentrations of veratridine or batrachotoxin, we found that ethanol reduced the maximum effect of the toxin ( $V_{\text{max}}$ ) with no significant change in the affinity ( $K_{0.5}$ ) of the toxin for its receptor, as measured by modified Michaelis-Menten equations. In contrast, Harris and Bruno<sup>23</sup> reported that although ethanol reduced the maximum effect of both batrachotoxin and veratridine, the apparent affinity of veratridine for its receptor was also affected by ethanol in a temperature-dependent fashion. This difference may be caused by the different membrane preparations and assay conditions used in the two studies.

Whereas the neurotoxin-dependent influx of sodium is inhibited by ethanol, the influx of sodium in the presence of veratridine and tetrodotoxin is not affected by ethanol. In addition, the passive influx of sodium into synaptosomes in the absence of any neurotoxins is unchanged by ethanol. Thus, it appears that ethanol reduces the influx of sodium ions that occurs specifically through the voltage-sensitive sodium channel. Ethanol *in vitro* also has no effect on the inhibition of batrachotoxin-stimulated sodium influx by te-

trodotoxin. The presence of ethanol (400 mM) *in vitro* has no effect on the binding of [<sup>3</sup>H]saxitoxin to its receptor in the channel.<sup>25</sup> This may be because the saxitoxin receptor is located at the extracellular side of the membrane, where the fluidizing effect of ethanol is rather weak. Ethanol may only affect certain specific areas in the channel. In addition to this apparent selectivity for different binding sites in the channel, specific brain areas also differ in sensitivity to the inhibitory effect of ethanol on sodium influx, with rat cortex being more sensitive than synaptosomes derived from the cerebellum.<sup>25</sup>

### 3. Effects of Acute and Chronic Ethanol Treatment on Sodium Channel Function

Most of the literature concerning the effects of ethanol on the properties of sodium channels deals with the direct effects of ethanol *in vitro*. However, it was also of interest to determine if acute or chronic ethanol treatment would alter the properties of voltage-sensitive sodium channels in rat brain synaptosomes. To study this question in some detail, dose-response and time-course studies were performed after a single dose of ethanol.<sup>26</sup>

In the time-course study, male Sprague-Dawley rats received a single dose of ethanol (4.5 g/kg) given orally, and cortical synaptosomes were prepared and blood ethanol concentrations were determined at 1, 2, 6, 18, and 24 hr after the dose of ethanol. For the first 6 hr after the single dose of ethanol, the blood ethanol concentrations were between 250 and 325 mg/dl. At 18 and 24 hr after the dose of ethanol, the blood ethanol concentrations were below 25 mg/dl. To assess the function of the channel, the cortical synaptosomes were incubated with batrachotoxin only, batrachotoxin plus ethanol (400 mM), and batrachotoxin plus tetrodotoxin to define the nonspecific influx of sodium. Acute ethanol treatment did not significantly alter the influx of sodium in the presence of batrachotoxin only or the nonspecific influx in the presence of batrachotoxin and tetrodotoxin at any of the time points examined. Thus, it appeared that the acute ethanol treatment did not impair the binding of the neurotoxins or affect the response (sodium influx) to the toxins in the absence of ethanol *in vitro*.

However, the inhibitory effect of ethanol *in vitro* was significantly reduced by the single dose of ethanol *in vivo*. The results shown in Table II demonstrate the reduction in the inhibitory effect of ethanol *in vitro* over the course of the experiment. Although the response to the *in vitro* addition of ethanol was reduced at 18 hr, recovery was essentially complete at 24 hr. The sodium channels present in the membranes from the ethanol-treated rats were tolerant to the inhibitory effect of ethanol on the neurotoxin-stimulated influx of sodium ions. The tolerance developed rapidly, was maximal 6 hr after the single dose of ethanol, and was still evident 18 hr after the acute treatment, a time when the blood ethanol concentrations in the whole animal were 25 mg/dl or less.

Table II. Effect of Acute Ethanol (4.5 g/kg, p.o.) Treatment on Neurotoxin-Stimulated Sodium Uptake

| Group         | Batrachotoxin-stimulated $^{22}\text{Na}^+$ uptake <sup>a</sup> |                                |
|---------------|-----------------------------------------------------------------|--------------------------------|
|               | BTX <sup>b</sup> only                                           | BTX + ETOH <sup>b</sup> 400 mM |
| Control       | 7.61 $\pm$ 0.21                                                 | 4.91 $\pm$ 0.21                |
| Ethanol 1 hr  | 7.16 $\pm$ 0.21                                                 | 6.02 $\pm$ 0.21*               |
| Ethanol 2 hr  | 6.78 $\pm$ 0.33                                                 | 5.94 $\pm$ 0.29*               |
| Ethanol 6 hr  | 8.00 $\pm$ 0.33                                                 | 8.11 $\pm$ 0.22**              |
| Ethanol 18 hr | 6.73 $\pm$ 0.48                                                 | 5.55 $\pm$ 0.38                |
| Ethanol 24 hr | 7.34 $\pm$ 0.10                                                 | 5.00 $\pm$ 0.34                |

<sup>a</sup> Expressed in nanomoles per milligram protein; \* $P < 0.05$ , \*\* $P < 0.01$  versus corresponding control value.

<sup>b</sup> BTX, batrachotoxin; ETOH, ethanol.

To characterize this effect further, a dose-response study was performed. Animals received a single intubation of saline (controls) or ethanol at a dose of 1.5, 3.0, 4.5, or 6.0 g/kg, and synaptosomes were prepared from the cortex at 2 hr after the intubation. Blood ethanol concentrations were also determined at the same time. The influx of sodium in the presence of batrachotoxin only was unaffected by any dose of ethanol. The inhibition of the batrachotoxin-stimulated sodium influx by ethanol *in vitro* was significantly reduced by the acute ethanol treatment at doses of 3.0, 4.5, and 6.0 g/kg. The mechanism responsible for the acute tolerance is currently under study. It may be that the acute ethanol treatment *in vivo* affects the partitioning of ethanol added *in vitro* into a crucial site of the membrane. It is clear that the treatment of the whole animal with a single dose of ethanol exerts a dramatic effect on the action of ethanol added *in vitro* to the synaptosomes. The functional properties of sodium channels in nerve membranes are known to be regulated by a variety of factors including lipid composition and fluidity, protein phosphorylation, and the levels of calcium in the membrane. The elucidation of the mechanisms responsible for the acute tolerance that occurs could lead to a better understanding of the phenomenon of tolerance in general.

The administration of a liquid diet containing ethanol to mice for 7 days has been reported to have no significant effect on the resting or veratridine-stimulated influx of sodium or the ability of ethanol *in vitro* to inhibit the neurotoxin-stimulated influx of sodium.<sup>23</sup> This treatment regimen is known to produce tolerance, physical dependence, and signs of withdrawal when ethanol is removed from the diet. In contrast to these findings, the chronic administration of ethanol to rats by multiple intubations for a period of 4 days produced tolerance to the inhibitory effects of ethanol *in vitro* on the neurotoxin-stimulated influx of sodium ions. The reduction in the inhibitory effect of ethanol (400 mM) *in vitro* on the batrachotoxin-stimulated influx of sodium ions was apparent after 2 days of chronic ethanol treatment and lasted for at least 20 days after withdrawal. Thirty-five days after withdrawal, the effect

of ethanol *in vitro* was essentially identical in synaptosomes derived from control and chronically treated rats.<sup>25</sup>

It is interesting to note that the reduction in the inhibitory effect of ethanol is greatest during the induction period, while the rats are intoxicated and have a blood ethanol concentration of 225 mg/dl or more. After withdrawal, the inhibitory effect of ethanol *in vitro* recovers somewhat, although the degree of inhibition in the treated animals is still significantly smaller than the inhibition in synaptosomes derived from control rats. During the induction period, the effect of ethanol added *in vitro* closely resembles the effect in rats treated with a large single dose of ethanol and sacrificed 1 or 2 hr later. After withdrawal, ethanol added *in vitro* has an effect similar to the results seen at 18 hr after acute ethanol in the time-course study.

#### 4. Effects of Other Agents on Sodium Channels

In addition to ethanol, a number of physical and chemical agents have been studied to determine their effects on the properties of sodium channels using ion flux and receptor-binding techniques.

General anesthetics<sup>21</sup> have been shown to cause a significant inhibition of neurotoxin-stimulated sodium influx at or near the concentration of the drug that is required to produce anesthesia *in vivo*.<sup>23</sup> Additionally, in a series of chemically diverse anesthetics, there was a strong correlation between the potency for decreasing neurotoxin-stimulated sodium influx and the increase in membrane fluidity produced deep in the core of the membrane.<sup>26</sup> Local anesthetics<sup>27,28</sup> have also been shown to be competitive inhibitors of the binding of batrachotoxin and of the membrane depolarization caused by batrachotoxin. Alterations in the function of sodium channels may be related to the pharmacological actions of these agents.

Clinically effective concentrations of the antiepileptic drugs carbamazepine and phenytoin have been shown to inhibit neurotoxin-stimulated sodium influx and the binding of [<sup>3</sup>H]batrachotoxin in neuroblastoma cells and rat brain synaptosomes.<sup>29,30</sup> Sodium valproate, diazepam, and phenobarbital were without effect at concentrations that are effective *in vivo*.

Ultraviolet radiation also inhibits neurotoxin-stimulated sodium movements and has been shown to reduce the binding of [<sup>3</sup>H]saxitoxin to the channel.<sup>31</sup> Ionizing radiation has been reported to inhibit neurotoxin-stimulated sodium influx in a manner remarkably similar to the observed effects of ethanol.<sup>32</sup> Different mechanisms of action are probably involved, as ionizing radiation has no effect on the fluidity of rat brain synaptic plasma membranes. Thus, a number of chemical and physical agents can alter the functional properties of sodium channels, apparently through a variety of mechanisms of action. The sodium channel in excitable membranes may represent a common target for a variety of chemical and physical agents that depress the central nervous system.

### 5. Summary

The direct effects of ethanol added *in vitro* on the voltage-sensitive sodium channels in brain synaptosomes are concentration dependent and fully reversible. Potency for inhibition of neurotoxin-stimulated sodium influx was correlated with lipid solubility and the degree of membrane disordering. Brain regions also vary in sensitivity to the inhibitory effects of ethanol. In addition, ethanol appeared to have an effect only on the channel site for activation, where the toxins batrachotoxin and veratridine bind.

It seems likely that the direct effects of ethanol *in vitro* are a result of the membrane-disordering effect of ethanol. Also, acute and chronic administration of ethanol results in the development of tolerance to the inhibitory effect of ethanol *in vitro*. The tolerance develops rapidly and is long lasting. The mechanisms responsible for the membrane adaptation are not yet known but are currently under study.

In addition to ethanol a number of chemical agents including general and local anesthetics, some antiepileptics, and certain antiarrhythmics alter the function of brain sodium channels. Physical agents such as ultraviolet and ionizing radiation also have dramatic effects on the sodium channel. Further studies should improve our understanding of how the chemical and physical agents work and increase our knowledge of the structure and function of sodium channels in the brain.

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