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# Ethanol increases resting potential magnitude and increases EJP amplitude in the presence of serotonin

COLIN HIGGINS, PETER BLATTNER, and ALEX ROSEN
Department of Biology, Grinnell College, Grinnell, Iowa

## ABSTRACT

Serotonin has been shown to modulate synapses, and ethanol has been shown to increase serotonin levels in the synaptic cleft. In this experiment, the contribution of ethanol to resting membrane potential (RMP) and excitatory junction potentials (EJP) in crayfish neuromuscular junctions was explored by manipulating the concentrations of serotonin and ethanol in the extracellular fluid and comparing these RMP and EJP values. Results showed that ethanol polarized RMP, an unforeseen effect, and increased EJP amplitude, supporting the hypothesis that ethanol acts to increase serotonin concentration in the synapse, in a concentration dependent manner.

## INTRODUCTION

Due to ethanol's (EtOH) prevalent use in modern society, it is important to identify and understand the mechanisms by which it acts on our brains. In brain tissue, EtOH acts partially by inhibiting serotonin (5-HT) clearance from the synaptic cleft; re-uptake is the primary means by which 5-HT is cleared from the synaptic cleft (Daws, et al 2006).

5-HT has been shown to modulate synapses in human brains and is responsible for brain functions such as emotion (Daws et al 2006). It has been shown that 5-HT superfusion alone enhances excitatory junction potentials, EJPs, by 310% at a concentration of 300 nM (at 14-17°C) and does not further enhance EJP amplitude at greater concentrations (Beaumont 2000).

Through its effect on 5-HT uptake, EtOH should have a profound influence on synaptic modulation (enhancement) at serotonergic neurons. Presently, though, testing the effects of EtOH on brain signaling is costly and complicated due to the stringent requirements on scientific testing in mammals.

Determining the similarities or differences in ethanol's effect on crayfish neuromuscular junctions compared to mammalian brain tissue could afford a simpler alternative model organism for EtOH mechanistic research if the overall effects are found to be similar. This is important because crayfish are invertebrates and have fewer and less stringent regulations for their treatment compared to the costly, complicated, and heavily regulated testing on mammals. (OLAW 2007) It is therefore easier and less costly to use crayfish or other invertebrates for mechanistic studies.

The goal of this research is to determine if EtOH inhibits or enhances the effects of 5-HT on the neuromuscular junction in crayfish. If our hypothesis is correct, addition of EtOH to the extracellular fluid should result in a potentiation of serotonergic enhancement of EJP response.

Our research showed that EtOH potentiates 5-HT EJP enhancement in crayfish neuromuscular junctions. This was tested by examining the EJP amplitude while submerged in saline and perifused with differing amounts of 5-HT and EtOH. EtOH also polarized the membrane of the post-synaptic muscle cell, an unforeseen response.

## MATERIALS AND METHODS

*Background*

Previous study showed that maximal 5-HT enhancement occurred 30 minutes after addition to solution (Beaumont 2000). Another study showed that EJP amplitude in a serotonergic lobster (closely related to crayfish) neuromuscular junction was maximal at 18°C (Hamilton 2007).

*Environment*

The EJPs of crayfish neuromuscular junctions were measured under different conditions: baseline—regular crayfish ringer saline (formulated to mimic the natural ion concentrations of crayfish extra-cellular fluid); crayfish saline with added 5-HT (0.5μM and 1.0μM in separate experiments) and incremental additions of EtOH ($[EtOH]_{saline}$=5mM (labeled EtOH), 10mM (labeled EtOH 2), and 15mM (labeled EtOH 3) in each 5-HT concentration). For convenience in measurement, 100mL of standard saline solution was used, refreshing 20mL of saline every 15 minutes via pipette.

*Dissection*

A live adult crayfish was submerged in ice and dissected dorsoventrally between the abdomen and walking legs. The ventral portion (carapace and muscle tissue) of the abdomen was removed to expose superficial extensor muscles, and the dorsal portion (dorsal side down) was secured in a dissection dish with pins in the anterior and posterior ends.

*Testing*

Testing consisted of voltage measurements (in reference to the surrounding saline solution) using a glass micro electrode (with a resistance of 5-20MΩ) pulled from a 1.2mm capillary tube filled with KCl (3.0M) inserted into the extensor muscle cell. An axon bundle of an excitatory nerve was captured using a suction electrode. Voltage to induce an action potential in the axon was supplied through the suction electrode. The measuring electrode registered the resulting EJP. Ten EJP measurements at each concentration were collected.

First, baseline readings were taken. Then 5-HT was added. As prior studies indicate, 5-HT modulation is highest 25-35 minutes after it is introduced into the solution so our 5-HT readings were taken at 30 minutes after addition, and immediately afterwards EtOH was added and tested incrementally. The same axon bundle was used throughout each experiment to ensure minimum biological variability. All trials were concluded within the 35 minute window.

Temperature has a known effect on EJP amplitude and we regulated it to 18°C (±1°C).

*Explanation of Curve-Slope Analysis*

In some trials at the highest 5-HT concentration, 1 µM, action potentials (AP) were elicited in the post-synaptic muscle cell (Figure **1** peak marked c). In most others, an EJP (Figure **1** peak marked b) was elicited. All EJP responses reached maximum voltage below -20 mV. Conversely, all AP responses had an increase in slope just above -20 mV. By visually overlaying a slightly enlarged (but proportionally constrained) EJP response trace and an AP response trace, the first portion of both traces (up to point a) is seen to be identical. After (a) there is a dramatic difference. Through comparison of the ratio of EJP point (a) to EJP maximum amplitude (b) and the ratio of AP slope shift point (a) and AP maximum amplitude (c), the initial stimulating EJP response "underneath" each AP trace is isolated. The values for a:c and a:b were calculated from averages of each measurement.

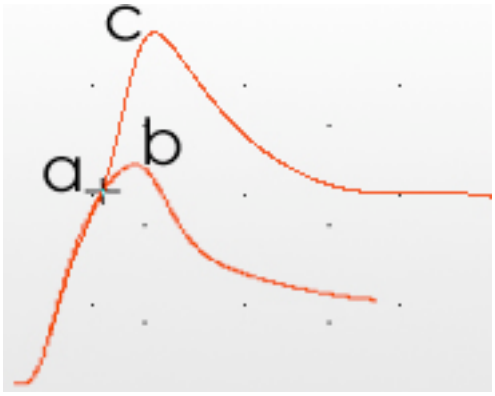


**Figure 1.** Sample EJP trace (taken from EtOH 3 group in Figure **5**) and AP trace (taken from EtOH 2 group in Figure **5**) overlaid to show correlation. Y-axis is voltage (mV), x-axis is time (sec). 5-HT concentration 1µM.

## RESULTS

We investigated EtOH's effect on neuromuscular EJP amplitude and RMP by manipulating concentrations of EtOH and 5-HT in extracellular fluid.

*Resting Membrane Potential*

The effect of EtOH on resting membrane potential (RMP) under control conditions is a concentration dependent decrease in voltage from baseline. (Figure **2**)

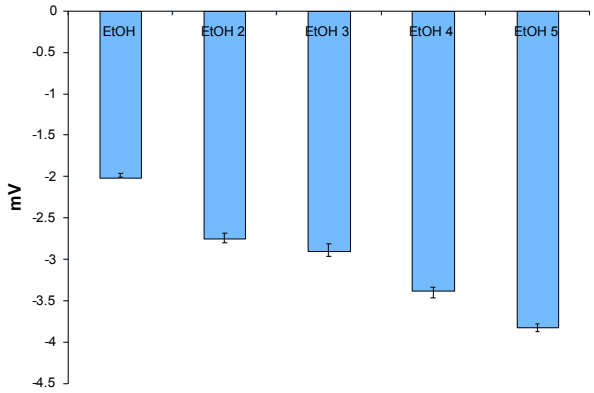


**Figure 2.** Height of bars represent average RMP compared to baseline (0) and adjusted for experimentally determined time dependent baseline degradation. Each average consists of 20 values. 5-HT at normal physiological concentration (no additional added). EtOH concentrations 5,10,15,20,25 mM. Error bars are ±1 s.e.

Slightly different effects are seen, however, when the cells are perifused with the neurostransmitter 5-HT, making the concentration of 5-HT much higher than biological conditions.

At a concentration of 1 µM, 5-HT addition caused an increase in the voltage of the RMP from baseline (see Figure **2**) which was expected (Hamilton, et al 2007). On addition of EtOH ($[EtOH]_{saline}$= 5mM), RMP decreased to near baseline. At $[EtOH]_{saline}$= 10mM RMP

showed no change from 5mM, but on the third addition ($[EtOH]_{saline}$= 15mM) RMP levels below baseline (Figure **3**).

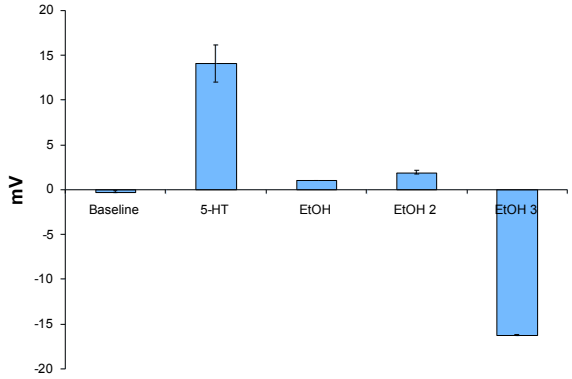


**Figure 3.** Height of bars represent average RMP compared to baseline (0) and adjusted for time dependent baseline degradation. Each average consists of 40 values. 5-HT concentration 1µM. Error bars are ±1 s.e.

Similar response was found at [5-HT] = 0.5µM. A wash of the preparation with standard saline shows partial reversal of effects (see Figure **4**)

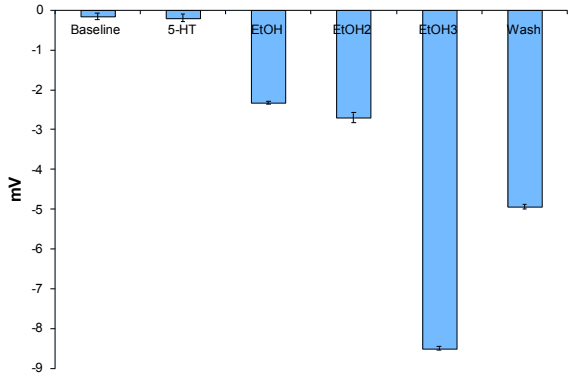


**Figure 4.** Height of bars represent average RMP compared to baseline (0) and adjusted for experimentally determined time dependent baseline degradation. Each average consists of 40 values. 5-HT concentration 0.5µM. Error bars are ±1 s.e.

*Excitatory Junction Potentials*

When 5-HT (1 µM) was added to the crayfish ringer solution the post-synaptic muscle cell elicited an AP upon stimulation. The approximate membrane threshold for creating an AP was found to be -20mV. When EtOH was added to reach a concentration of 15mM the RMP fell such that the EJP elicited by the electrical stimulus did not reach the -20mV threshold, and no action potential was observed; only the EJP was observed (Figure **5**).

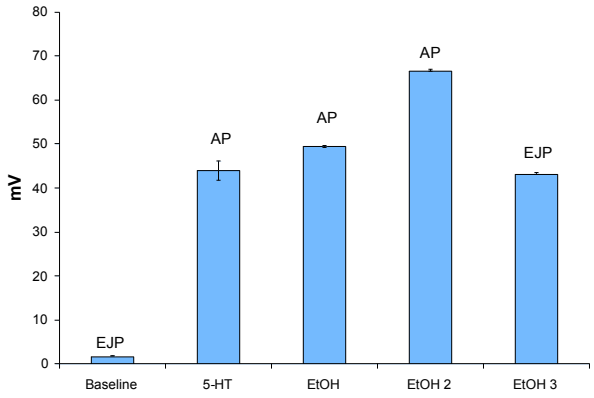


**Figure 5.** Height of bars is average response amplitude adjusted for time dependent baseline degradation and AP to EJP amplitude conversion. Each average consists of 40 values. 5-HT concentration 1µM. Error bars are ±1 s.e.

By the curve-slope comparison described in the methods, the amplitude of the initial EJP stimulating each action potential was approximated, and the following data was resolved (Figure **6**).

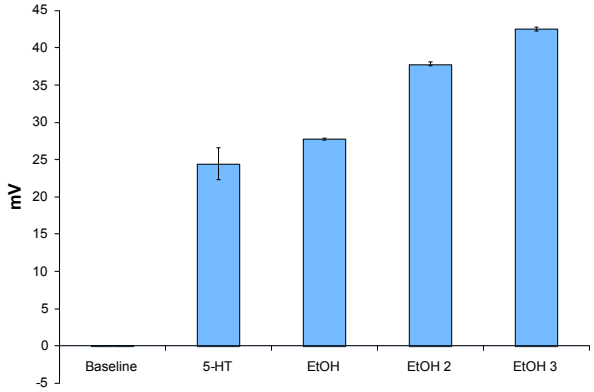


**Figure 6.** Height of bars is average EJP amplitude adjusted for time dependent baseline degradation and AP to EJP amplitude conversion. Each average consists of 40 values. 5-HT concentration 1µM. Error bars are ±1 s.e.

As can be seen in Figure **6**, after isolation of the EJP data, EtOH's effect is seen as a concentration dependent increase in EJP amplitude.

Lower concentrations of 5-HT (physiological and 0.5µM) did not exhibit significant 5-HT or EtOH potentiation.

## DISCUSSION

*Resting Membrane Potential*

The data of RMP from varied concentrations of EtOH (5mM, 10mM, 15mM) and varied concentrations of 5-HT (physiological, 0.5µM, 1.0µM) (See Figures **2**, **3, 4**) suggest that crayfish neuromuscular junctions exhibit polarizing sensitivity to EtOH, and the effect is modulated when 5-HT levels are elevated above physiological

(control) conditions. This modulation appears to follow a dose-response relationship with the threshold-dose between 10mM and 15mM EtOH (Figures **3**, **4**), but further and more exhaustive study is required to verify this relationship.

*Excitatory Junction Potential*

The effect of EtOH at 1.0μM 5-HT, after isolation of data, on EJP amplitude is shown to be a concentration dependent increase. Similar to modulatory effects on RMP, the threshold dose for this increase appears to be between 0.5μM and 1.0μM 5-HT.

*Competition between RMP and EJP influences*

At [5-HT] = 1.0μM, EtOH's two effects, potentiation of EJP and polarization of RMP, are competing (Figure **5**), and at this concentration EtOH's overall behavior is indicative of hormesis, a phenomenon when toxic chemicals elicit opposite responses at low and high doses (Figure **7**).

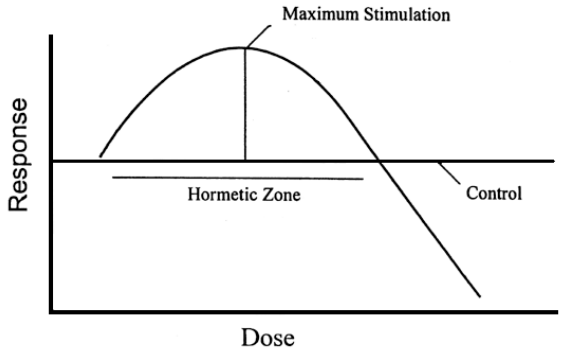


**Figure 7.** Shows relationship of typical hometic dose-response. Modified from (Calabrese, Baldwin 2000).

Previous studies have shown EtOH to have hormetic effects in cases of heart disease and stroke (Cook, Calabrese 2006) so a hormetic relationship in neuronal transmission would not be unprecedented. Further study in this area is required to better establish such a relationship though. Better understanding of neural response to various doses of EtOH by establishing a titrated response curve would help to better define the risks and benefits of alcohol consumption by humans, a contentious issue.

To put these results in a human perspective, the lowest concentration used, 5mM (marked EtOH) corresponds to approximately the blood alcohol content of a 180 lb man ingesting one standard alcoholic drink within one hour, 0.022% (using the United States definition of Blood Alcohol Content). The responses labeled EtOH 3 correspond to a BAC of approximately 0.07%, just under the legal impairment level for driving.

The combined results of this study show the merit of using the crayfish neuromuscular junction as an alternative to mammalian brain tissue to study alcohol response. While this study can not conclusively determine if the response is identical, observed potentiation of EJP amplitude is an indication that further study will be informative at a fraction of the cost and difficulty of mammalian study.

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