

ABSTRACT

Title of Document: PHYSIOLOGICAL EFFECTS OF ALCOHOL
ON CRAYFISH ESCAPE CIRCUITRY

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Alcohol is one of the oldest and most widely used drugs on the planet, but the cellular mechanisms by which it affects neural function are still poorly understood. Unlike other drugs of abuse, alcohol has no specific receptor in the nervous system, but is believed to operate through GABAergic and serotonergic neurotransmitter systems. Invertebrate models offer circuits of reduced numerical complexity and involve the same cell types and neurotransmitter systems as vertebrate circuits. The well-understood neural circuits controlling crayfish escape behavior offer neurons that are modulated by GABAergic inhibition, thus making tail-flip circuitry an effective circuit model to study the cellular mechanisms of acute alcohol exposure. Crayfish are capable of two stereotyped, reflexive escape behaviors known as tail-flips that are controlled by two different pairs of giant interneurons, the lateral giants (LG) and the medial giants (MG). The LG circuit has been an established model in the neuroscience field for more than 60 years and is almost completely mapped out.

In contrast, the MG is still poorly understood, but has important behavioral implications in social behavior and value-based decision making. In this dissertation, I show that both crayfish tail-flip circuitry are physiologically sensitive to relevant alcohol concentrations and that this sensitivity is observable on the single cell level. I also show that this ethyl alcohol (EtOH) sensitivity in the LG can be changed by altering the crayfish's recent social experience and by removing descending inputs to the LG. While the MG exhibits similar physiological sensitivity, its inhibitory properties have never been studied before this research. Through the use of electrophysiological and pharmacological techniques, I show that the MG exhibits many similar inhibitory properties as the LG that appear to be the result of GABA-mediated chloride currents. Finally, I present evidence that the EtOH-induced changes in the MG are blocked through pre-treatment of the potent GABA_A receptor agonist, muscimol, which underlines the role of GABA in EtOH's effects on crayfish tail-flip circuitry. The work presented here opens the way for crayfish tail-flip circuitry to be used as an effective model for EtOH's acute effects on aggression and value-based decision making.

PHYSIOLOGICAL EFFECTS OF ALCOHOL ON CRAYFISH ESCAPE
CIRCUITRY

By

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Bulleted Summary

Major findings of my dissertation are as follows:

- The crayfish lateral giant (LG) circuit is physiologically sensitive to ethyl alcohol (EtOH), and this sensitivity is strongly modulated by changes in social experience.
- Although the effects of EtOH are observed locally in the LG circuit, they are affected by inputs from the brain. EtOH's effects generalize to the medial giant (MG) circuit, significantly increasing excitatory post-synaptic potentials elicited by mechanosensory input.
- The MG contains similar physiological and inhibitory mechanisms as the LG including a primarily excitatory early EPSP component and a chloride-mediated inhibitory late component.
- The GABA_A agonist, muscimol, blocks the EtOH-induced increase of MG EPSPs which may suggest competitive binding to the same receptors.

Chapter 1: Introduction

Summary

To put my dissertation research into a broader perspective requires a brief discussion of the significant human-health and economic impact of alcohol as a drug of abuse. Alcohol is characterized across human and non-human animal models by its bi-phasic behavioral symptoms that manifest as an increase in activity at lower doses and a sedated state at higher concentrations. These behavioral symptoms are largely conserved in crayfish, which have significant advantages as a model for this line of study including their well-described escape and social behavior and, most importantly, the ease of which electrophysiological recordings can be made to show alcohol-induced changes in single neurons.

Significance

The human health and societal impacts of alcohol abuse are wide and devastating. A set of statistics taken from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) indicates that approximately 17 million adults (> 18 years of age) in the United States have an alcohol use disorder (AUD). Additionally, 24.6% of adults were reported to have experienced an episode of binge drinking within one month of the survey. Binge drinking, as defined by the NIAAA, is the consumption of enough alcoholic beverages to raise the blood alcohol concentration (BAC) levels to 0.08 g/dL, which is generally the result of consuming 4-5 drinks within a two hour period of time. Alcohol is assessed to be responsible for approximately 88,000 deaths

in the U.S. annually, 10,322 of those occurring in car accidents. In 2006, the Center for Disease Control and Prevention (CDC) reported the cost of excessive alcohol consumption in the United States to have reached \$223.5 billion. Although the human-health and economic influence of alcohol is wide-reaching and affects a significant percentage of the U.S. population, the mechanisms of alcohol intoxication on the circuit and cellular level are still poorly understood. Unlike many drugs of abuse, alcohol does not appear to have a specific receptor in the brain (Wolf & Heberlein, 2003), but is believed to operate through serotonergic (Barr et al., 2003; Ferraz & Boerngen-Lacerda, 2008) and GABAergic mechanisms (Mehta & Ticku, 1988; Mihic et al., 1997; Lobo & Harris, 2008; Kumar et al., 2009). The demonstrated interaction of alcohol and these neurotransmitter systems make crayfish an effective model to investigate the interactions of alcohol with both GABA and serotonergic neurotransmitter systems. Comprised of the same neuronal cells and neurotransmitter systems as vertebrates, invertebrates can accomplish complex behavioral outputs with a fewer number of total neurons. Working with the crayfish nervous system, a CNS that has fewer than 100,000 neurons (Wine, 1984) allows the experimental use of behaviors controlled by circuits that have been largely mapped out. The escape tail-flip of the crayfish, *Procambarus clarkii*, is one such behavior. Crayfish are capable of two reflexive escape tail-flips that are mediated by two pairs of giant interneurons, the lateral giants (LG) and the medial giants (MG), which are activated differentially based on the type and locus of stimulation and result in tail-flips that take the animal in different directions (Wine & Krasne, 1972; Edwards et al., 1999). The lateral giant (LG) circuit is one of the best understood neural circuits

in the animal kingdom and has been demonstrated to undergo both phasic and tonic GABAergic inhibition (Roberts, 1968; Vu & Krasne, 1993; Vu et al., 1997); it has also been shown to be altered by social experience (Edwards et al., 1999). The MG circuit, while understood to a lesser extent, is relevant to agonistic encounters (Herberholz et al., 2001; Edwards et al., 2003) and value-based decision making (Liden et al., 2010) in crayfish and is activated through visual or tactile stimuli to the head or thorax (Liu & Herberholz, 2010). The work described here will further our understanding of alcohol's influence on GABAergic mechanisms on the cellular level using relatively simple neural circuits controlling escape behavior. Additionally, because of the social factors of alcohol as a drug of abuse and the history of aggression research in crayfish, this work will facilitate the exploration of alcohol's influence on physiological changes mediated by social experience.

Human Alcohol Research

While the practical and ethical limitations of alcohol research in humans necessitate the use of animal models, a background of human-oriented research is useful for ensuring the applicability of these models. As stated previously, alcohol is one of the most widely abused drugs in the world and results in substantial human health and economic costs due to its abuse. Acute alcohol intoxication in humans can be categorized by a number of stereotyped behaviors in accordance with increasing concentrations of blood alcohol concentration (BAC) (Vonghia et al., 2008). In Vonghia et al., (2008), BAC levels of approximately 11 mM typically induce a disinhibition of normal behaviors resulting in an increase in talkativeness and

impairments of certain skill-based tasks that are the first signs of behavioral deficits from alcohol intoxication. Subjects with BACs measuring between 22 and 43 mM exhibit a large degree of deficits including impaired judgment, a lack of coordination, and increased reaction times. As concentrations increased to levels between 43 and 87 mM, subjects presented with more significant symptoms including vomiting and amnesia and finally, BACs exceeding 87 mM were typically associated with depression of respiratory function, coma, or death. Obviously, an increased severity of adverse symptoms with elevated doses of alcohol is not new in terms of drug abuse research, but a distinct bi-phasic response to alcohol intake does appear to be present. Lower acute doses of alcohol generally evoke an increase in excitability or disinhibition (impaired judgment and an increase in talkativeness), while higher, non-fatal, doses evoke more sedative effects (lack of coordination and amnesia) (Vonghia et al., 2008). Interestingly, this bi-phasic dose dependence is also present in crayfish with early intoxicated behaviors which are characterized by increases in activity and later behaviors by increases in sedation.

Non-Human Vertebrate Alcohol Research

The behavioral and physiological effects of alcohol in non-human animal models suggest similar functioning mechanisms behind intoxication. A wide range of different vertebrate models have been used to study the behavioral effects of alcohol and to explore more specific aspects of alcohol abuse most befitting the strengths of their given model system. Behavioral effects of alcohol intoxication follow similar patterns to intoxication in humans. When acutely exposed to alcohol in their tank,

zebrafish have been shown to display altered levels of locomotion depending on the alcohol dose (Gerlai et al., 2000). Animals exposed to low to moderate doses of ethanol (EtOH), 50 mM and 100 mM respectively, displayed significantly increased locomotion when compared to non-intoxicated animals. When the dose was increased to 200 mM EtOH, animals displayed a significant hypoactive effect. In addition to behavioral symptoms of toxicity, rodent animal models are widely used to investigate how external factors influence voluntary alcohol intake (Blanchard et al., 1987; Henniger et al., 2002; Sommer et al., 2008). Factors such as anxiety have been shown to alter the amount of self-administration of alcohol in rats (Henniger et al., 2002). Rats have also been used to study effects of social status on alcohol consumption, as in one such study where subordinate males consumed significantly more alcohol than dominant males (Blanchard et al., 1987). Manipulation of the mouse genome allows researchers to manipulate behavioral phenotypes in response to alcohol exposure. Davies & Alkana (2001) found that using two mouse genotypes, short sleep and long sleep mice, they could link a differential phenotypic response to a difference in GABA_A receptor mediated chloride (Cl⁻) uptake between the two lines. While genetic manipulations provide advantages to behavioral work, experiments such as these frequently require *in vitro* techniques that may not always be comparable to *in vivo* scenarios. Unrelated to alcohol work, but important in terms of considering *in vitro* vs. *in vivo* experiments, is some work by Prescott et al. (2008) that showed CA1 hippocampal pyramidal cells in *in vitro* slice preparations significantly changed their functional characteristics when *in vivo* connectivity was simulated. The authors showed, through computational modeling and experimental data, how the cells shifted

from class 1 to class 2 cells as defined by Hodgkin's classification (Hodgkin, 1947). This work draws attention to the issue of how *in vitro* experiments may not accurately represent the functioning of neural circuits in a living, breathing, and behaving animal. The crayfish model allows the use of relatively simple neural circuits that can be manipulated pharmacologically in semi-intact, *in vivo* preparations in a complete nervous system and not a much more restricted slice preparation.

Invertebrate Alcohol Research

Invertebrates, primarily in the fruit fly, *Drosophila melanogaster*, have been used to study the effects of alcohol on behavior and genetics for several decades and have been shown to exhibit similar symptoms of alcohol intoxication compared to human and other vertebrate models (van der Linde & Lyons, 2011; Devineni & Heberlein, 2013; Robinson & Atkinson, 2013). Behavioral experiments revealed a potentiated locomotor response when acutely exposed to alcohol (Wolf et al., 2002). Research using the fruit fly, like the mouse model, emphasizes the use of genetic manipulation to explore the interplay of alcohol, genetics, and behavior, but generally ignore more specific circuit mechanisms that cause alcohol's intoxicating effects. The limited exploration of alcohol's cellular mechanisms in *Drosophila* is a direct consequence of the difficulty of linking behavior and genetics with electrophysiological investigation in such a small animal. While lacking a sequenced genome to permit genetic manipulations, the crayfish is an excellent model for studying the neurophysiological effects of alcohol exposure due to their large,

identifiable neurons. The electrical activity of these cells can be easily recorded using intracellular electrodes, something that is more difficult in *Drosophila*.

Like research in the fruit fly, crayfish have also been previously used for alcohol research. Alcohol research in crayfish has focused on behavioral and physiological effects. Friedman et al. (1988) used *Procambarus clarkii* to demonstrate the intoxicating effect of acute exposure on righting response, the time required for an animal to return to an upright posture from an upturned posture, and the building of tolerance with a chronic application of 75 mM EtOH in their housing tank. Increased righting times were recorded during the first week of exposure, but by the end of the second week righting times returned to baseline in animals chronically exposed to 75 mM implying behavioral tolerance. The authors also investigated the physiological effects through the use of the opener excitor muscle system of the claw in a reduced preparation. In this work, Friedman et al. found that higher EtOH concentrations (300-600 mM) produced consistent inhibitory effect on synaptic potentials in the opener muscle while lower concentrations (10-100 mM) increased excitatory potentials, albeit more variably than the effect of high doses. These results indicate a bi-phasic influence of alcohol on inhibitory mechanisms. Several years later, Blundon & Bittner (1992) continued their physiological exploration of EtOH's effects in crayfish using an *in vivo* preparation of the neuromuscular junction (NMJ) of the claw opener muscle using a wider range of concentrations over 10 minute periods of exposure. Concentrations of 60-90 mM EtOH were shown to reduce presynaptic inhibition while not significantly influencing excitatory transmission. More intermediate concentrations, between 120-180 mM, reduced excitatory

potentials, indicating different effects based on EtOH doses (Blundon & Bittner, 1992). Further exploring the interaction of EtOH and GABA systems, GABA agonists (baclofen, GABA, and muscimol) and antagonists (bicuculline and picrotoxin) were used to determine the role of inhibition in this *in vivo* circuit. Through this work, the authors made the inference that presynaptic inhibition played a major role in this circuit and that the disinhibiting effects observed were likely the result of EtOH interactions with GABAergic inhibition. Exposing inhibited excitatory post-synaptic currents (EPSCs) to EtOH increased EPSC amplitudes suggesting disinhibition. Additionally, behavioral responses to EtOH were observed over the course of these experiments. Animals were acutely exposed for a shorter amount of time (0.5-2 hours) to a higher concentration (434 mM) and experienced an increased responsivity to tail stimulations hours after exposure. This responsivity was recorded as the number of escape tail-flips that were performed when presented with mechanosensory taps to the abdomen. However, manual mechanosensory stimulation is highly variable and any differences for this experiment could also be explained by variability in the application of the stimulus. Perhaps more importantly for my research, Blundon & Bittner (1992) also describes internal alcohol concentrations when the animals are bath exposed to 434 mM EtOH, recording hemolymph (crayfish blood) concentrations after one hour of approximately 60 mM, or roughly 14% of the bath concentration. Other research has shown internal concentrations rising to values approximately 15-25% of the external concentration (Macmillan et al., 1991; Hausknecht et al., 1992). This is important as behavioral experiments performed in the Crustacean Neurobiology and Behavior Laboratory (CNBL; PI: Herberholz)

exposed crayfish to one hour of 1M EtOH. Based on the data from these studies, the internal concentration after the hour of exposure should be approximately 100-150 mM.

Previous behavioral work in the CNBL has focused on using the latency to alcohol-induced behavioral changes as a metric for alcohol intoxication in crayfish. The results matched stages of intoxication displayed by many other species (Swierzbinski et al., in preparation). Crayfish immersed in 1M EtOH (23 g of 100% Ethyl Alcohol mixed with 500 mL of de-ionized water) experienced several distinct and stereotyped behaviors over the course of EtOH exposure. Many animals began exhibiting a period of elevated posture, a posture normally associated with a crayfish holding dominant status in a dominance hierarchy (Livingstone et al., 1980; Edwards et al., 2003). This behavior, normally performed in the presence of another crayfish, was the first spontaneous behavior to be observed in intoxicated crayfish, but was not present in all animals. Next, as exposure time increased, crayfish experienced a bout of unprovoked, spontaneous tail-flipping. Under normal circumstances, tail-flipping behavior is reserved for responding to particularly threatening stimuli, but the crayfish continuously performed these tail-flips when sufficiently intoxicated in the absence of any apparent threat. Following this increase in excitation, crayfish entered a state of sedation where they ceased tail-flipping and were unable to right themselves from a supine posture. In addition to these behavioral observations, neurophysiological experiments were conducted. Extracellular stimulating electrodes were implanted on afferents to the lateral giant (LG), a command neuron whose activation initiates a reflexive tail-flip escape response, and the influence of acute

alcohol exposure on LG activation threshold was studied over the course of one hour in freely behaving animals. Animals placed in water for one hour showed a slight increase in LG activation threshold, but when animals were placed in a 1M EtOH bath for one hour, the threshold significantly decreased evidenced by a reduction in the amount of voltage required to activate a tail-flip. Interestingly, the social history of a crayfish affected its behavioral and physiological sensitivity to alcohol. Animals placed in social isolation for 7 days from a larger tank housing approximately 30-50 crayfish (communal tank) displayed decreased sensitivity to alcohol evidenced by increased latencies of intoxicated behaviors and a reduced EtOH-mediated shift in LG activation as compared to animals taken directly from a communal tank.

Alcohol Treatment Programs

Understandably, alcohol treatment programs focus on long-term alcohol abuse, with little attention paid to the immediate effects of acute alcohol intoxication. Possibly the most widely-known method of treatment of chronic alcohol problems would be participation in a group such as Alcoholics Anonymous (AA). While popular, this form of treatment has been shown to have varying levels of effectiveness (Ferri et al., 2006; Humphreys et al., 2014). While AA may be better known culturally, a wide array of pharmacotherapies have been used to treat alcohol abuse. The many different targets of these drug treatments is further evidence of the complicated nature of alcohol's cellular mechanisms. Clinical trials have shown evidence that therapies targeting *N*-methyl-D-aspartate (NMDA) receptors (acamprosate), GABA receptors (baclofen), opioid receptors (naltrexone and

nalmefene), and acetaldehyde dehydrogenase (disulfiram) have proven effective (Franck and Jayaram-Lindström, 2013). Disulfiram prevents the metabolism of acetaldehyde, the primary metabolite of alcohol, resulting in aversive symptoms of headaches and nausea and vomiting among others when alcohol is ingested during treatment. In a study, it was found that disulfiram enhanced the ability of patients to prevent relapse compared to placebo when patients complied with treatment, but only 20% of the 577 participants were able to comply. Additionally, patients that continued to ingest alcohol despite the disulfiram treatment ran the risk of liver damage (Franck and Jayaram-Lindström, 2013). Naltrexone has been shown to decrease alcohol consumption in rats (Stromberg et al., 2002), reduces craving and increases the time to relapse (Franck and Jayaram-Lindström, 2013). Its usefulness in the treatment of alcoholism appears to be in preventing alcohol relapse. While baclofen targets GABA_B receptors and has recently been shown to effectively treat alcohol abuse at higher doses in some trials, there is comparatively little evidence for it as a treatment when compared to the previously described treatment options. Though many therapies appear to treat alcohol abuse, these treatments have highly variable outcomes across patients, have significant drawbacks, and may require strict adherence to unpleasant protocols. An improved understanding of the cellular mechanisms of alcohol exposure would lead to drug treatments that better target these specific mechanisms. Importantly, despite the array of existing treatments, social history of an individual is typically not a component considered in treatment programs. Given the body of research focused on the interaction of alcohol abuse and

social environments, it seems likely that incorporating the effects of social history could have dramatic effects on treatment outcomes.

Alcohol and γ -Aminobutyric Acid (GABA)

While there remains much work to elucidate the specific mechanisms of alcohol on the nervous system, it is known to interact with GABAergic neurotransmitter systems in numerous ways (Lobo & Harris, 2008; Enoch, 2008; Kumar et al., 2009). GABA is the primary inhibitory neurotransmitter in the brain of both vertebrates and invertebrates. Genotyping of human alcoholics have implicated a link between the GABA_A α 2 receptor gene, a gene that is known to modulate the response to stress, anxiety, and alcoholism (Enoch et al., 2006). The α 2 subunit of GABA_A receptors has also been shown to be involved in the sedative effects of alcohol as α 2-GABA_A receptor knockout mice show decreased prevalence of loss of righting reflex (Kumar et al., 2010). Other research discussed in the review by Kumar et al. (2010) implicates EtOH in facilitation of pre-synaptic GABA release throughout different regions of the vertebrate brain, including the spinal cord, amygdala, hippocampus, and cerebellum. Chronic, but not acute exposure to EtOH has been shown to alter GABA_A receptor subunit expression, increasing the prevalence of the α 4 subunit while decreasing α 1 subunit expression (Kumar et al., 2003). The α 1 subunit is present in approximately 50% of GABA_A receptors in the brain and is usually present in synaptic GABA_A receptors while the α 4 subunit is mostly present in extrasynaptic GABA_A receptors.

EtOH has also been shown to either inhibit or facilitate GABAergic neurotransmission through a variety of mechanisms. In addition to their role in anxiety and depression, corticotropin-releasing factor receptor 1 (CRFR1) has been implicated in EtOH-induced enhancement of GABAergic inhibition in the central amygdala (Nie et al., 2004). Disrupting CRFR1s through antagonists and knockout mice reduces GABAergic inhibitory post-synaptic currents (IPSCs) suggesting a possible mechanism for EtOH's enhancement of GABA. In the rat cerebellum, EtOH has been shown to enhance GABAergic vesicular release (Kaplan et al., 2013). The same study also showed that EtOH can suppress GABAergic action by directly acting on post-synaptic GABA receptors.

Chapter 2: Crayfish Escape Behavior

Summary

In order to effectively build on the behavioral and freely-behaving physiological experiments already performed in the CNBL, a more detailed background of crayfish escape behavior is necessary. This chapter will provide necessary background on crayfish escape behavior, the underlying neural circuitry, and the known GABAergic inhibition involved in these circuits. Additionally, because of the nature of GABAergic inhibition in the crayfish as *depolarizing* inhibition, the mechanisms by which depolarizations to the membrane potential of a neuron can be inhibitory will be briefly discussed.

Tail-flip Escape Behavior

Crayfish use reflexive escape tail-flips in order to avoid sudden threatening stimuli. Two of these tail-flips are mediated through the use of two pairs of giant interneurons, the lateral giants (LG) and the medial giants (MG). The lateral giant tail-flip is initiated when the animal receives strong, phasic mechanosensory stimuli to the abdomen (Wine & Krasne, 1972; Wine, 1984). The medial giant tail-flip is activated through mechanosensory stimulation to the head or thorax or in response to threatening visual stimuli (Liden & Herberholz, 2008). Activation of a giant neuron subsequently activates motor giant (MoGs) neurons, which activate phasic flexor muscles leading to flexion of the tail in accordance with which pair of giants is

activated (Wine & Krasne, 1972; Wine, 1984; Edwards et al., 1999; Krasne et al., 2015). In addition to the MoGs, both LG and MG indirectly activate another group of flexor motor neurons called the “fast flexors” through the segmental giant (SG) interneuron. The LG activates only the three most rostral MoGs, causing contraction of muscles in only those segments. The behavioral output of this specific MoG activation is that the crayfish jackknifes upward and forward, away from the stimulus directed at the rear of the animal (Wine, 1984). In contrast, activation of the MG activates MoGs in all abdominal ganglia, resulting in the contraction of all segments of the tail and thrusting the animal directly backward and away from a rostral stimulus. Activation of either one MG or one LG is sufficient to drive the tail-flip escape behavior. The inputs to the LG are very well-understood and the LG is governed by both phasic and tonic GABAergic inhibition.

In addition to the two giant-mediated tail-flips, crayfish are also capable of tail-flipping through a non-giant (NG) circuit. This tail-flip is activated in response to more gradual stimuli than its giant-mediated counterparts, and does not make use of the MoGs or the SGs (Herberholz et al., 2001; Krasne et al., 2015). While the LG and MG tail-flips are stereotypical in their behavioral output, the NG can vary widely across tail-flips and requires significantly more time, believed to be the result of making minute postural changes to change the resultant tail-flip’s trajectory (Krasne et al., 2015). The spontaneous tail-flips induced in juvenile crayfish through EtOH bath exposure are believed to be the result of non-giant tail-flips (Herberholz, unpublished).

Depolarizing Inhibition in the Crayfish

A major difference between the crayfish, *Procambarus clarkii*, and vertebrate model systems is the manifestation of GABAergic inhibition. This inhibition is depolarizing due to the relation of LG's resting membrane potential (approximately -80 mV) to chloride's reversal potential (roughly -55 mV) which is a result of the increased internal concentration of Cl^- ions of the LG (Roberts, 1968). The result is a depolarizing inhibitory post-synaptic potential (dIPSP) that shunts depolarizing excitatory currents. This dIPSP inhibits cells through several different mechanisms that also hint at the advantage of depolarizing inhibition over hyperpolarizing inhibition. As shown by Donald Edwards (1990), this depolarization is believed to inactivate voltage-sensitive inward currents, thought to be sodium ions that would normally work to bring the cell to threshold. This form of inhibition may be advantageous compared to hyperpolarizing inhibition as it would reduce the post-inhibitory rebound that would come with hyperpolarizing potentials as they would lead to increased activation of sodium channels. This depolarizing inhibition complicates the study of GABAergic inhibition in the crayfish as it must be determined if depolarizing potentials are excitatory or inhibitory. For this reason, depolarizing the membrane potential through current injection near chloride's reversal potential can be a valuable tool for determining the presence of GABAergic inhibition in the crayfish circuit.

Lateral Giant Escape Tail-flips

Anatomy

As mentioned previously, the LG circuit has been studied extensively for many years and a great deal is known regarding its anatomy. While the LGs consist of a pair of giant interneurons that are visually identifiable using a dissection microscope, each LG consists of a series of segments connected together through septate gap junctions. The crayfish abdomen is divided into six segments, each of which contains an abdominal ganglion housing the cell bodies of neurons in the abdomen. Each LG has a cell body in each abdominal ganglion, where it receives excitatory input from abdominal mechanosensory receptors. In addition to the septate junctions bridging each axonal segment, each LG is also electrically coupled to the other member of its pair, causing activation of one LG to subsequently activate the other. The result is a circuit with numerous redundancies to allow it to continue functioning despite damage to one segment. Damaged axonal segments can be bypassed as long as the other LG axon remains intact in that segment. While much is known regarding the anatomy of the LG including its sensory inputs and outputs to initiate a tail-flip, the LG also ascends to the crayfish brain for reasons still unknown.

Excitatory Sensory Inputs

The behavioral output of the LG tail-flip is highly stereotyped and the underlying neural circuit is one of the best understood circuits in the animal kingdom. The LG escape circuit can be activated through mechanosensory input to any of the six abdominal segments of the tail (Wine & Krasne, 1972), but also receives some

subthreshold excitatory input from descending interneurons from the brain that are activated through antennal or visual stimulation (Liu & Herberholz, 2010). Excitation of mechanosensory afferents in the tail produce a bi-phasic electrical and chemical component of the LG excitatory post-synaptic potential (EPSP) (Krasne, 1969; Edwards et al., 1999). A highly phasic mechanosensory stimulus to the abdomen activates a set of primary afferents which make electrical synapses directly on the LG interneuron and sensory interneurons that receive input from sensory afferents via chemical synapses (**Fig. 2.1**). The primary afferents therefore make up what has been called the alpha (α) component of the EPSP while the contribution from the sensory interneurons has been termed the beta (β) component (Krasne, 1969). This electrical α component typically occurs approximately 1 ms following the stimulus. The chemical β component is always slower, a consequence of the extra chemical synapse onto mechanosensory interneurons (MSIs) before synapsing on the LG, and it typically occurs approximately 3 ms after stimulus. This results in a bi-phasic input, which when sufficient, drives an action potential in the LG that typically rises from the β component.

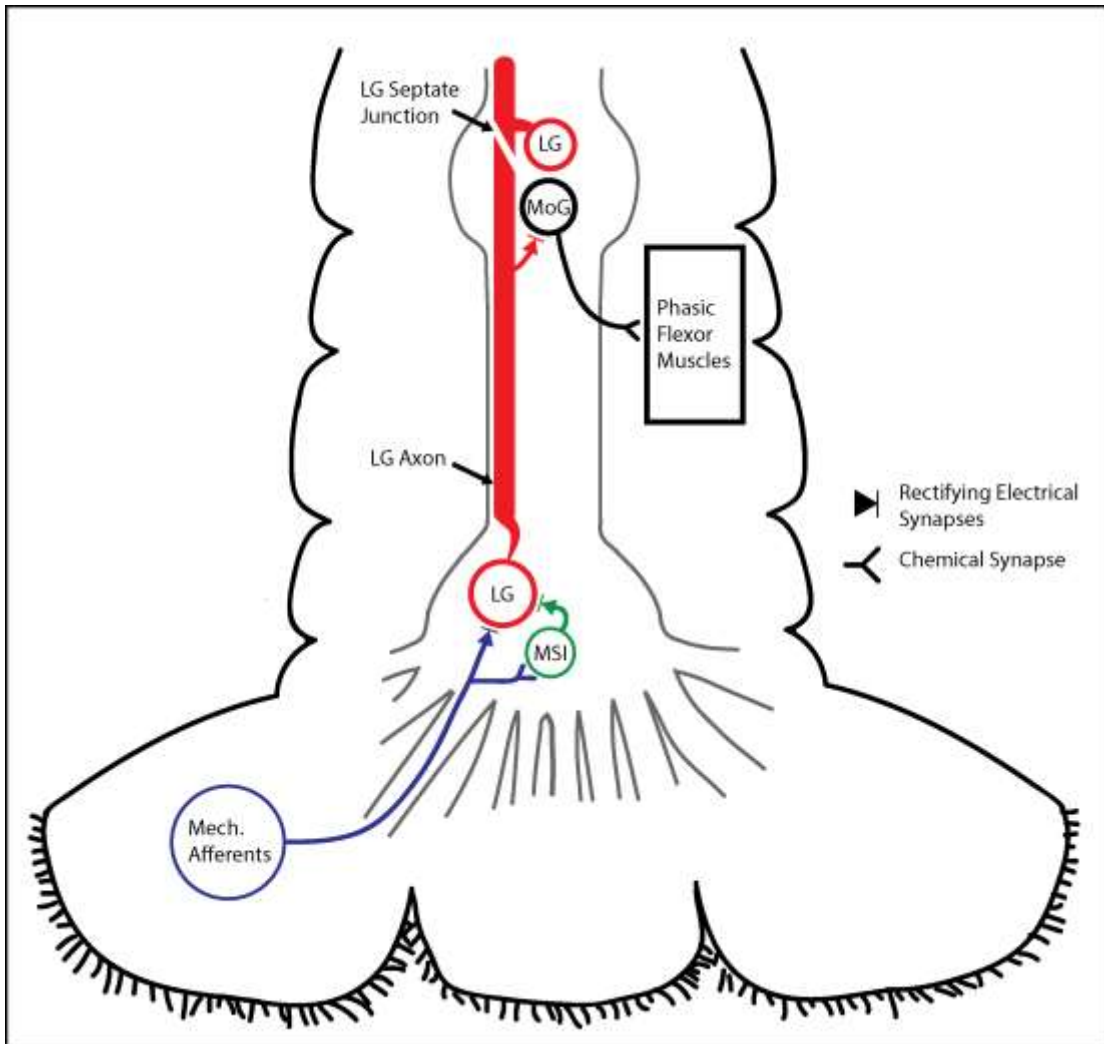


Figure 2.1: Simplified LG circuit diagram. Excitatory inputs shown for LG of the terminal abdominal ganglion (A6). Primary mechanosensory afferents of the crayfish abdomen synapse directly on the LG dendrites through electrical gap junctions in each abdominal ganglia. These direct electrical synapses make up the alpha (α) excitatory post-synaptic potential (EPSP) component. Mechanosensory interneurons (MSI) are activated by mechanosensory afferents through cholinergic chemical synapses that connect to the LG via electrical synapses and make up the LG EPSP beta (β) component. LG cell bodies are located in each abdominal ganglion and LG axons are connected through septate junctions. LG action potentials activate

motorgiant neurons (MoGs) that activate phasic flexor muscles in the three rostral segments of the crayfish abdomen. Size of ganglia relative to crayfish tail exaggerated.

The electrical synapses of the LG circuit have significant side effects for the excitatory input to the giant neuron. In addition to the general property of electrical synapses being faster than chemical synapses, the electrical junctions of the LG are rectifying. When a phasic stimulus is presented, the spike reaches the pre-synaptic terminal before it synapses on the LG. Current flow is increased at this point as the junction is forward-biased. Following this, the activated and inactivated junctions become back-biased and conductance is decreased substantially (Edwards et al., 1998; Krasne et al., 2015). This leads to the selectivity for summated excitation when the inputs reach the LG within 0.2 ms. Inputs that arrive within this window summate very effectively while inputs that arrive outside of this window do not. Because of this, the circuit is specialized for coincidence detection of phasic stimuli.

Additionally, LG depolarization increases the excitability of its own primary afferent and primary interneuronal inputs in a lateral excitatory network that can operate to modulate the escape to a given stimuli (Herberholz et al., 2002). Primary afferents are electrically coupled with each other and when excitation from one afferent induces the current backwash from the LG neuron through both activated and inactivated synapses, these two inputs can excite unstimulated afferents, thereby increasing the excitatory input to the LG. Because there is a slight delay with this

lateral excitation, its input will summate with excitatory input produced by the MSIs compounding with the β component of the LG.

Postexcitatory Inhibition

As an escape reflex, the LG is most responsive to strong, highly phasic stimuli and has several important mechanisms in place to maintain this selectivity. In 1997, Eric Vu et al. described a phasic mechanism that inhibits the LG immediately following the excitatory β component. This inhibition, labeled postexcitatory inhibition (PEI), was demonstrated through the use of a GABA_A channel blocker, picrotoxin (PTX), which preferentially enhanced the later EPSP (6 ms or later following the stimulus) compared to the α or β components. A normal EPSP decays following a narrow β peak, but preparations that were exposed to PTX showed a higher later component that maintains its amplitude for longer, which would likely increase the time where the LG may fire an action potential and trigger a tail-flip, reducing its selectivity for phasic stimuli. There was little effect to the β component, indicating that the inhibition was somewhat delayed. These results suggest the presence of inhibition occurring immediately following the primary excitatory component. Under normal circumstances, this inhibition reduces the width of excitatory input, ensuring the LG responds only to the most phasic stimuli. Since this PEI serves to make the LG more selective for phasic stimuli, it could be a major reason for the activation of non-giant (NG) tail-flips rather than LG tail-flips when the crayfish is presented with a gradual stimulus. It can be reasoned that the gradual stimulus will result in long-lasting excitatory inputs, inputs that given the presence of

PEI and the rectifying junctions would be shunted and would not lead to activation. It is believed that PEI originates from the same stimulus that activates the excitatory afferents and provides excitatory input to LG, simultaneously driving inhibition to arrive immediately following the β .

Tonic and Phasic GABAergic Inhibition

In addition to the postexcitatory inhibition (PEI) that occurs immediately following excitatory input to the LG (Vu et al., 1997), the LG is believed to be modulated through both a type of tonic inhibition and a different type of phasic inhibition that occurs following the activation of an LG or MG spike (Roberts, 1968). LG tonic inhibition is believed to be mediated by free-flowing GABA surrounding LG dendrites and is a possible mechanism by which the threshold for the LG can be manipulated, which can make the LG more or less likely to fire depending on whether this inhibition is decreased or increased. Because the LG is known as a command neuron, its activation directly generates behavioral output and regulating the neuron's excitability can be extremely important. Situations that have been shown to increase LG firing threshold include when the animal is feeding and when it is being restrained (Krasne & Wine, 1975; Krasne & Lee, 1988; Krasne & Edwards, 2002).

While tonic inhibition is thought to be located in the dendrites, phasic inhibition of the LG is believed to be mediated by GABAergic inhibition located proximal to the LG spike initiation zone (Vu & Krasne, 1992). Phasic inhibition takes the form of a long-lasting (~50 ms) depolarization activated recurrently by the LG or through MG activation and completely shunts the generation of any action potential in the LG while the inhibition is present. This prevention is accomplished by

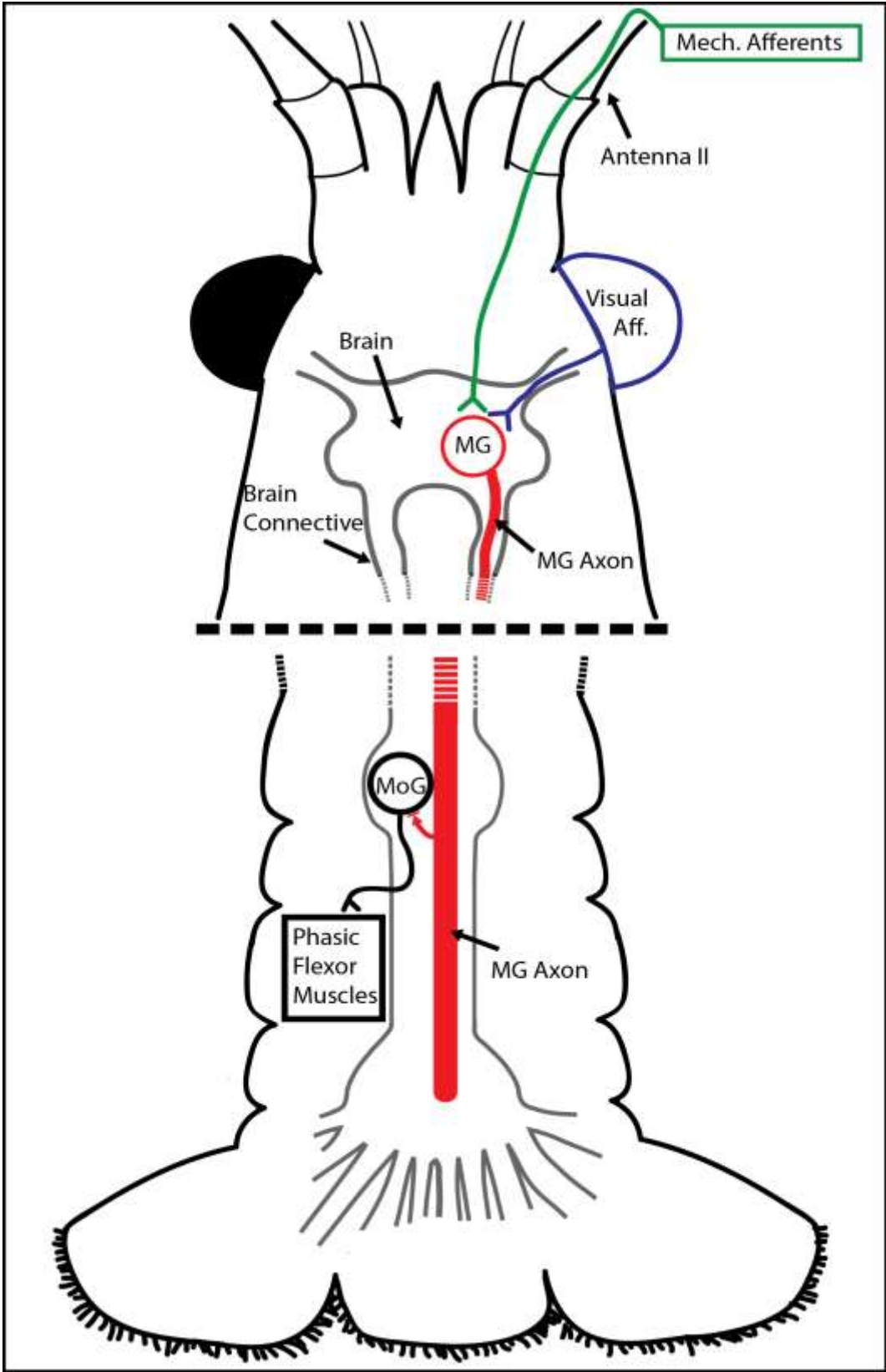
significantly reducing LG input resistance (Roberts, 1968). Hyperpolarizing inhibition would reduce Na⁺ channel inactivation leading to post-inhibitory rebound that is avoided due to the depolarizing nature of this phasic inhibition (Edwards, 1990). Recurrent inhibition is in place in the LG circuit to prevent the LG from continuous firing, as the barrage of sensory input following an LG tail-flip could be sufficient to drive subsequent activations, which would be behaviorally disadvantageous. Given the depth of knowledge regarding the LG circuit and its apparent GABAergic inhibitory mechanisms, the LG circuit makes an excellent model for the study of alcohol's cellular interaction with GABAergic systems and corresponding behavior.

Medial Giant Escape Tail-flips

In contrast to the expanse of knowledge surrounding the LG escape circuit, the MG is relatively poorly understood due to its reduced accessibility, and until recently, sensory inputs to the MG were still unknown. Behaviorally, MG tail-flips are used to escape from threatening mechanosensory or visual stimuli presented to the head or thorax (Liden & Herberholz, 2008; Liden et al., 2010). Additionally, MG tail-flips are important for crayfish agonistic behavior, often occurring when one animal subordinates itself to the dominant (Herberholz et al., 2001; Edwards et al., 2003). The MG has its cell body in the supraesophageal ganglion (crayfish brain) and transmits from the brain connectives to the abdomen in a single, unbroken axon that runs the length of the crayfish (Glantz & Viancour, 1983). In the brain connectives of an adult crayfish, the MG axon has been recorded to have a diameter of 200 μm . As described previously, it is known that the MG outputs to MoGs in all abdominal ganglia, causing the contraction of phasic flexor tail muscles in all abdominal segments (**Fig. 2.2**) (Wine, 1984). The MG responds to mechanosensory stimuli to the head or thorax as well as fast moving visual stimuli (Liden & Herberholz, 2008). The probability of initiating an MG tail-flip can be changed by the perceived value of a food odor stimulus when faced with a threatening shadow, implying that crayfish are capable of value-based decision making (Liden et al., 2010). A possible mechanism for this modulation is tonic GABAergic inhibition that adjusts the MG activation threshold similar to how the LG can be modulated when presented with a valuable food source (Krasne & Lee, 1988). Additionally, because the activation of the MG, like the LG, rarely leads to subsequent activations, phasic inhibitory

mechanisms are also likely to be present. These mechanisms of inhibition would prevent the MG from activating when it would be disadvantageous to do so, situations where tail-flipping may be the safer choice, but not the decision that is most likely to lead to the continued survival of the animal. The behavioral relevance of the MG makes it another obvious target for investigating the cellular mechanisms of alcohol exposure, but we know very little regarding the physiological properties of the MG excitatory post-synaptic potential (EPSP), or how it may be inhibited through GABA. For these reasons, it becomes the focus of a significant portion of my dissertation research in order to elucidate the inhibition that may be present in this circuit.

Figure 2.2: Simplified MG circuit diagram. Top) *The MG cell body, located in the supraesophageal ganglion (brain) receives excitatory visual afferents from the eye in addition to mechanosensory input from the head and crayfish antenna. Sufficient excitation of afferent input leads to an action potential of the MG that travels caudally through the brain connectives to the crayfish abdomen (Bottom) in a single, unbroken axon. In each of the six abdominal ganglion, the MG activates motor giant neurons (MoGs) that activate phasic flexor muscles of the entire crayfish tail. Size of crayfish nervous system relative to body and size of head relative to abdomen is exaggerated.*



Chapter 3: General Methods

Summary

The methods described in this chapter are used in most of the experiments throughout the course of my dissertation research. This section includes an overview of the research animals, housing conditions, electrophysiology equipment, superfusion system, data acquisition & analysis, and statistics that will be used throughout this dissertation. Specific methods for individual experiments will be discussed in their relevant chapter and references to general methods will be made throughout. Any modifications to these methods for individual experiments will be noted when describing the experiments in its respective chapter.

Animals

For all experiments, juvenile crayfish of the species *Procambarus clarkii* were used. Adult animals tend to prove more difficult for intracellular electrophysiology because maturation leads to the thickening of a sheath around the crayfish nervous system. Successful intracellular recording in adult crayfish typically requires the removal of this sheath in order to impale the neurons. Crayfish were purchased from a commercial biological supplier (Atchafalaya Biological) and housed in communal tanks (described below). Before use in any experiments, animals were checked for intactness and length. Crayfish that had molted within 48 hours or had significant damage to the body were not used for experiments. Body length was measured from

the rostrum to the tip of the animal's telson (tail fan) and an effort to use similarly-sized animals across experiments was made. As the animals were sexually immature, experimental groups consisted of crayfish of either sex. All animals were fed medium-sized shrimp pellets (Ocean Nutrition). Socially isolated animals (described below) were fed one pellet on the day of isolation and communal animals were routinely fed every Monday and Thursday with one pellet per crayfish placed into the communal tanks. Because of the nature of the communal tanks and competition between crayfish, the number of food pellets consumed by a communal animal over a given week cannot be controlled for.

Housing

Animals were housed communally in tanks with dimensions of 76 x 30 x 30 cm (L x W x H) typically holding between 30 and 50 other juvenile crayfish. All communal tanks contained approximately 2 cm of gravel and all animals were fed 1 medium-sized shrimp pellet every Monday and Thursday. Socially isolated animals that were used for the majority of the experiments conducted in this dissertation were removed from communal tanks 7-10 days prior to the experiment and placed in a small isolation tank (15 x 8 x 10 cm) with a 2 cm layer of gravel. Animals were fed one pellet on the day of their isolation. Both isolate and communal tanks were filled with room temperature tap water to several inches below the top of the tank. Isolation tanks were fitted with a lid to prevent tail-flipping out of the tanks during their 7-10 days of isolation and an air stone to maintain oxygenation of the housing water. Both

communal and isolate tanks were stored in an animal housing room with constant temperature ($22^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$) and on a 12hr light:dark cycle.

Electrophysiology Equipment

Intracellular electrodes were pulled using a Sutter micropipette puller and all electrodes had resistances measuring between 20-35 M Ω . Glass capillary tubes (World Precision Instruments) of diameter (outer diameter: 1mm, inner diameter: 0.58 mm) were used for all electrodes and were equipped with filaments to aid in filling with salt solution. 2M potassium acetate (CH_3COOK) solution was used for intracellular electrodes as the standard 3M Potassium Chloride that is typically used in these types of experiments could prime natural Cl^- currents (Herberholz et al., 2002; Liu & Herberholz, 2010). Excitatory post-synaptic potentials (EPSPs) and action potentials were elicited through extracellular stimulation via hook electrodes of sensory afferents to the giant interneurons. These hook electrodes were constructed using Teflon-coated silver wire with an uncoated diameter of 0.2 mm.

Impalement of the giant neuron of interest was confirmed by injecting sufficient current to activate the neuron and by analyzing the extracellular recording of the action potential. Because of the large diameter of the giant interneurons in relation to the other axons present in the crayfish CNS, the extracellularly recorded spike dwarfs any other action potentials and is easily identifiable as one of the giant interneurons. Additionally, the firing patterns of giant interneurons are very distinct and identifiable from other neurons in the crayfish nervous system. In any experiment

where sensory afferents were activated to produce EPSPs, a 90 second inter-stimulus interval was used to prevent habituation.

Electrophysiology data was collected using microelectrode amplifiers (Axoclamp900A: Molecular Devices), an A-M systems differential amplifier (Model 1700) and Grass stimulator (Model S88) for recording and stimulation using extracellular hook electrodes. A Digidata 1440A (Molecular Devices) was used to digitize analog data. Data were recorded and stored using pClamp 10.4 and Clampfit 10.4 software (Molecular Devices). All electrophysiological recordings were performed in a grounded faraday cage. Before amplitude measurements were taken, all EPSP recordings were filtered using a Gaussian filter. Measurements were performed to ensure that the application of the filter did not significantly alter the recorded amplitudes.

Crayfish Saline & Pharmacology Delivery

Following surgical preparation for experimentation, the preparations were bathed in 40 mL of crayfish saline. A modified van Harreveld's solution (van Harreveld, 1936) was used for all experiments, a standard crayfish saline consisting of the following salts in concentrations in mM: 202 NaCl, 5.37 KCl, 13.53 CaCl₂, 2.6 MgCl₂, and 2.4 HEPES (Antonsen & Edwards, 2002; Antonsen et al., 2005; Liu & Herberholz, 2010). In all experiments performed here, this saline served as the vehicle for drug delivery. Picrotoxin and muscimol (Sigma-Aldrich) solutions were created by measuring the desired quantity of picrotoxin or muscimol powder on a

scale (Mettler-Toledo AL204) and dissolved in crayfish saline to create the desired volume.

Fresh saline or pharmacological agent application was performed using a gravity flow superfusion system (**Fig. 3.1**). A fluid reservoir containing saline or a saline/drug combination was located on top of the faraday cage with an inflow tube running into the cage and into a Baxter flow control device that allowed for fine adjustment of the flow rate. Using this device, the flow rate was held consistent for all experiments at 5 ml/min. Drug application rate can have profound effects on the physiological response of crayfish circuitry (Teshiba et al., 2001) and therefore the rate was verified before each experiment was performed. To maintain the experimental dish volume at 40 mL of saline, a small outflow tube connected to a peristaltic pump (Thermo Scientific FH100 Series) was placed at the fluid surface before superfusion began. To reduce the influence of the peristaltic pump on electrical noise recorded in the preparation, the pump was placed below the faraday cage. For most pharmacology experiments, the experiments consisted of three distinct phases: 1) saline baseline, 2) drug exposure, and 3) saline washout to attempt to reverse the drug's affect. Between experimental phases, approximately 90 seconds were used to switch the reservoir of the flow system to the desired agent. The saline baseline was used to ensure that the superfusion itself was not responsible for any effects. The saline washout performed after drug superfusion was to ensure that the effects observed during the drug exposure were not the result of a decrease in the preparation's viability, evidenced by a measureable EPSP amplitude at the conclusion of the washout period. While this does not ensure that the preparation was not

“running down” by the time the washout was concluded, this would normally occur at times much later than drug exposure. Indeed, the typical response observed in preparations exposed to EtOH is not a “run down” effect, but rather a “runaway” effect where the observed facilitation that occurs during the drug exposure phase continues to increase during the saline washout.

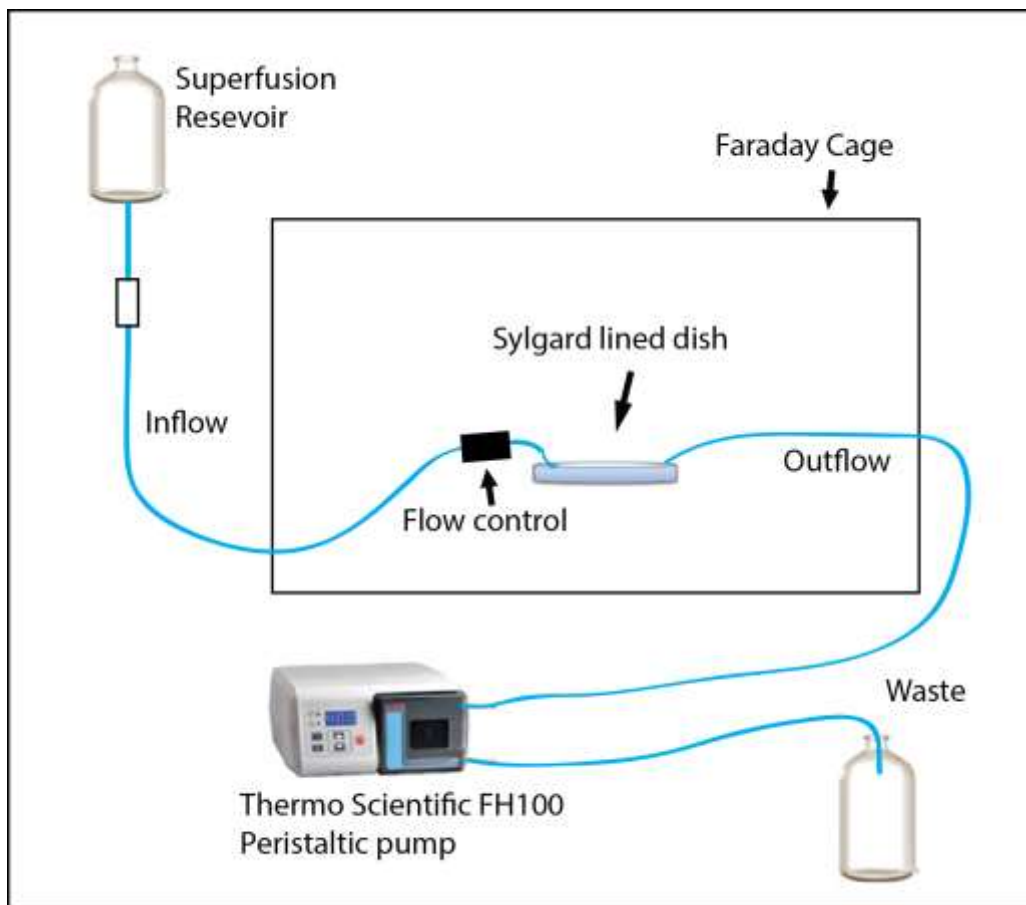


Figure 3.1: Gravity flow superfusion schematic. Reservoirs placed above the Faraday cage are used to superfuse saline or saline/drug mixtures to the preparation. Surgical dishes were filled with 40 ml of saline. Flow rate was controlled using a Baxter flow-meter with a small tube placed into the sylgard lined dish.

Simultaneously, an outflow tube placed at the surface of the saline in the dish pumps excess fluid out using a Thermo Scientific FH100 peristaltic pump and into a waste container. The peristaltic pump was set for 14 revolutions per minute which equates to a liquid removal rate slightly above the inflow rate. Before every experiment, reservoirs and tubing was flushed with de-ionized water. The flow rate was verified before every experiment using normal crayfish saline and maintained at 5 ml/min.

Selection of Drug Concentrations

EtOH

Initially, 100 mM EtOH was selected based on the work of Blundon & Bittner (1992). In this work, gas chromatograph showed that after 60 minutes of exposure to 434 mM EtOH, hemolymph EtOH concentrations were measured to be between 40-60 mM. Since behavioral experiments performed by the CNBL acutely exposed animals to 1M of EtOH for 60 minutes in its standard condition, we estimated a physiological exposure concentration should be approximately 10% of 1 M (Swierzbinski et al., in preparation). This estimation is in keeping with estimates of internal EtOH concentrations in crayfish over acute exposures (Blundon & Bittner, 1992). Reducing the concentration further allowed me to investigate the effects of lower EtOH concentrations on LG tail-flip circuitry as EtOH has previously been shown to have dose-dependent effects with lower concentrations inducing increased excitation and higher concentrations inducing greater incoordination in a variety of species.

Picrotoxin

The potent GABA_A channel blocker, picrotoxin (PTX), has a long history of use in crayfish electrophysiological work. A wide variety of concentrations were used over the past decades with concentrations largely ranging between 7 and 100 μ M (Krasne & Roberts, 1967; Roberts, 1968; Blundon & Bittner, 1992; Vu et al., 1997). The primary concentration used for the experiments described in this dissertation were based on the earlier work involving crayfish and EtOH, Blundon & Bittner (1992). In this study, a concentration of 50 μ M of PTX was applied to eliminate presynaptic inhibition in the claw opener excitator axon. Initially, this concentration was also used when exploring inhibition of the medial giant (MG) circuit, but led to significant convulsions that made intracellular recording of the MG difficult. This concentration was then reduced to 25 μ M, significantly improving the ability to record from inside the cell.

Muscimol

While PTX is a potent GABA_A antagonist, muscimol is a potent GABA_A agonist and is much more effective than GABA itself (Ticku & Olsen, 1977). Reports indicate that muscimol is between 10 and 20 times more potent than GABA (Ticku & Olsen, 1977; Hori et al 1978). In preliminary experiments not described here, 500 μ M GABA was shown to decrease EPSPs of the MG neuron. For the experiments reported here, a muscimol concentration of 25 μ M was used.

Quantification of Excitatory Post-Synaptic Potentials (EPSPs)

For experiments that measured the change in EPSP amplitude over the course of the experiment, specific methods were employed for consistency. EPSP amplitudes

were measured at preferred time points in relation to the stimulus artifact present in all afferent stimulations. Averages of three sweeps surrounding the desired time point of exposure were averaged to reduce the influence that outlier sweeps may have on the measured amplitude value. These averages were then normalized to the baseline amplitude at the same time point which is itself an average of the consistent baseline EPSP sweeps taken immediately prior to the drug exposure. Therefore, values greater than 100% indicate a facilitated EPSP amplitude at a given time point in comparison to that same time point in the saline baseline exposure. As expected, values lower than 100% indicate amplitudes below the baseline. This allows the quantification of the change in EPSP amplitudes over the entire experimental period of drug exposure as well as during washout. The normalization of EPSP amplitudes is standard practice in this line of research (Vu et al., 1997) and is advantageous for removing the weight that differently-sized EPSP amplitudes would have on an average amplitude. While necessary, this practice conveys one small negative in that very small baseline amplitudes that display modest shifts of voltage could in turn have large percentage changes.

In most experiments, the EPSP amplitude is quantified for 12 distinct time points in relation to the stimulus artifact. These time points were 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, and 25 ms following the stimulus artifact (**Fig. 3.2**). Values for these time points were recorded every 5 minutes of each experimental phase. Because of the reflexive nature of tail-flips, both lateral giants (LGs) and medial giants (MGs) would be expected to receive excitatory input rapidly following activation of sensory afferents. For the LG, it is known that the potential occurring before 3 ms following

the stimulus is mediated through excitatory ionic currents (Vu et al., 1997). The excitatory inputs for the LG are typically categorized as the α and the β component. The α typically occurs approximately 1 ms following the stimulus and is the result of direct electrical synapses of sensory afferents on the LG neuron. The β is the result of electrical synapses from sensory interneurons that are activated by afferents and this component typically occurs several ms after the α (~ 3 ms following the stimulus artifact) (**Fig. 3.2**) (Edwards et al., 1999). Later activity, for the LG at least, is believed to be largely inhibitory, increasing the bias of the giant interneurons for highly phasic inputs (Vu et al., 1997). Typically, select time points are represented to best convey changes to the different segments of the potential. While these components are well-understood components of the LG EPSP, the MG EPSP has never been investigated. The bulk of the analysis was focused around the peak of the EPSP and the time shortly thereafter, best represented by 3 and 6 ms following the stimulus. Throughout this dissertation, the 3 ms component will be referred to as the “early” component and the 6 ms component as the “late” component. These values also correspond to the β component and the postexcitatory inhibition (PEI) found in the LG, respectively.

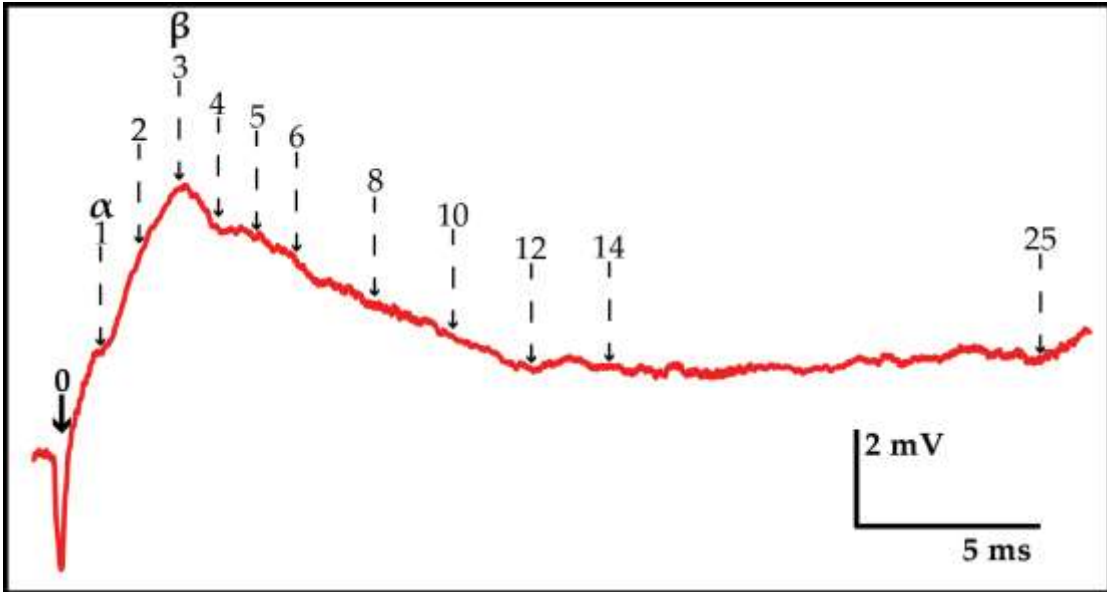


Fig 3.2: Quantification of the lateral giant (LG) excitatory post-synaptic potential (EPSP). An example trace of an excitatory post-synaptic potential from the lateral giant (LG) interneuron in the crayfish abdomen elicited through electrical stimulation of the sensory afferents of the terminal abdominal ganglion, A6. Numbers and arrows represent the time from the stimulus in milliseconds. α and β indicate the presence of the alpha and beta components of the LG EPSP. These components typically occur 1 ms and 3 ms following the stimulus artifact (0). LG action potentials rise from the β component. As shown in Vu et al. (1997), the component of the EPSP after the β is typically GABAergic inhibition deemed postexcitatory inhibition (PEI).

To record these different time points and to remove the human influence in measuring the amplitude at a given time, the amplitude recorded was the result of calculating the mean amplitude (mV) of the potential from the amplitude measured ± 0.05 ms around the time point of interest. In most cases, this would negate the influence of electrical noise in the intracellular recording, which could affect the

recorded value if this practice was not followed. This analysis was performed using Molecular Device's Clampfit 10.4 software. Normalization to baseline and averages were calculated using Microsoft Excel.

Statistics

To determine the presence of significance for all experiments certain statistical tests were used repeatedly throughout this dissertation. To compare data from independent groups a non-parametric Mann-Whitney U (MWU throughout text) test was used with a threshold value of $p \leq 0.05$ indicating the rejection of the null hypothesis with 95% certainty. For comparing data from dependent groups, a Wilcoxon signed-rank test was used, also with a threshold value of $p \leq 0.05$. Statistical tests were performed using SPSS (Version 21). For experiments focused on the differences between social groups or the effects of different drugs, MWU tests were used to determine the presence of statistical significance. Typically, experiments where values were compared to their baseline values, a Wilcoxon signed-rank test was used.

Chapter 4: Alcohol and the Lateral Giant Circuit

Summary

Behavioral and implanted electrode experiments have shown that crayfish are behaviorally sensitive to alcohol and that alcohol exposure decreases the activation threshold for lateral giant (LG) neurons in unrestrained animals. Importantly, this work has also shown that these effects are altered by changes to the crayfish's recent social experiences with communal animals (COM) being more sensitive to EtOH than animals that were socially isolated (ISO). In this chapter, I explore the effects of EtOH on the LG using single-cell electrophysiology. Similar to the behavioral and experiments with implanted electrodes that preceded this work, EtOH increases the excitability of the LG in a manner that can be observed on a single cell level; most interestingly, the different effects of social experience are paralleled as increased excitability in COM preparations when compared to ISO preparations. γ -Aminobutyric Acid (GABA) is a prime target for alcohol in the nervous system, and in this chapter I present data indicating that lower EtOH concentrations may facilitate LG excitability in COM preparations through interactions with known tonic GABAergic inhibitory mechanisms.

Background

The experiments performed here seek to establish the crayfish lateral giant circuit as a neurophysiological model system for the study of acute alcohol

intoxication. It consists of three experiments, the first two drawn from Swierzbinski et al. (in preparation). Previous experiments in the CNBL have shown behavioral intoxication in the presence of acute ethyl alcohol (EtOH) exposure by immersing freely behaving juvenile crayfish in different concentrations of EtOH solution (0.1, 0.5, and 1M) mixed in de-ionized water. Animals exposed to these EtOH concentrations displayed dose-dependent signs of intoxication with lower concentrations inducing intoxicated behaviors at a much slower rate. Intoxicated animals would experience an elevated posture, normally reserved for agonistic encounters in dominance hierarchy formation with other crayfish, followed by the onset of a period of spontaneous tail-flipping, an escape behavior that normally requires a significantly salient stimulus to elicit, and finally a period of incoordination, evidenced by an inability of the crayfish to remain upright. These behaviors are normally not produced by animals in the absence of EtOH, leading to their categorization as EtOH-induced behaviors. Of additional interest from these experiments is the fact that alterations to the recent social history of the crayfish resulted in different EtOH sensitivities, with animals socially isolated from other crayfish for 7 days prior to testing experiencing a slower onset of EtOH-induced behaviors than those taken from a communal housing environment.

Although the tail-flips observed during the period of EtOH-induced spontaneous tail-flipping are suggested to be mediated through non-giant circuitry (Herberholz, unpublished), the existing knowledge of the LG circuit makes it an effective target for testing the interaction of EtOH with GABAergic inhibition, which is expected to be involved in alcohol's behavioral effects. In addition, the experiments

performed here directly build on experiments where freely behaving juvenile crayfish were implanted with silver wire stimulation electrodes onto mechanosensory afferents that project to the LG neurons. This work showed that EtOH exposure reduced the voltage required to activate the LG. In these experiments, animals that were implanted and tested immediately following removal from a communal tank were found to be more sensitive to the threshold decreasing effects of EtOH. While the spontaneous tail-flips are believed to be non-giant tail-flips, the results from the implanted electrode data suggest that LG tail-flip circuitry is affected by EtOH exposure, which allows for the use of this circuit as a model to study the effects of EtOH on a single cell level. The experiments described in this chapter build on this behavioral and implanted electrode work, exploring the effect of EtOH on the spiking properties and excitatory postsynaptic potential (EPSP) of the LG.

A variety of literature in humans, vertebrates, and invertebrates indicate that higher EtOH concentrations lead to more inhibited behaviors while lower concentrations typically increase activity (Blundon & Bittner, 1992; Gerlai et al., 2000; Vonghia et al., 2008). Thus, I performed intracellular recordings of the LG using sharp micropipette electrodes while the preparation was superfused with EtOH. The experiment was designed with the goal of exploring both the excitatory (spontaneous tail-flipping) and inhibitory (incoordination) effects observed in the previous behavioral experiments through the use of both low (10 and 20 mM) and high (100 mM) concentrations of EtOH, respectively. Because of a higher than expected amount of fluctuation in control (i.e., saline) trials, I also repeated these experiments in preparations where the tail was isolated from the rest of the crayfish,

eliminating a possible source of the fluctuations originating in the crayfish brain. Next, I compared the changes in LG EPSPs between social groups during exposure to 100 mM EtOH as this concentration was effective for both COM and ISO preparations. Because of the results of the behavioral and implanted electrode experiments that showed accelerated EtOH-induced behaviors in communal animals I was interested in the effect that social experience might have on the ability of EtOH to bring LG EPSPs to threshold. I found that a higher proportion of communal semi-intact preparations (COM) exposed to lower concentrations of EtOH (10 and 20 mM) were brought to threshold during superfusion when compared to socially isolated preparations (ISO). The 20 mM EtOH concentration was most effective at eliciting a difference between the social groups in the percentage of preparations activated and in the average number of action potentials per preparation. As the EtOH concentration increased to the highest concentration, the difference between the social groups was largely eliminated. To explore additional social differences in the response to 100 mM EtOH, I measured changes to smaller LG EPSPs that were further removed from threshold. I found that socially isolated animals displayed larger EPSP facilitation compared to communal animals when exposed to 100 mM EtOH.

Experiment 4.1: Influences of Alcohol on LG Threshold

A defining property of the lateral giant (LG) interneurons is their role as a command neuron, meaning that activation of a single action potential in the LG is sufficient to drive a stereotyped escape tail-flip (Edwards et al., 1999). Additionally,

the circuit underlying the LG escape response is very well-understood, making it an excellent model for the study of acute alcohol effects. Implanted-electrode data showed that EtOH exposure reduces LG firing threshold and the degree of this effect is dependent on the animal's social history, but the cellular mechanisms cannot be thoroughly explored using this paradigm. For this reason, I measured the EtOH-induced effect using single-cell recording of the LG. The LGs receive excitatory input through sensory afferents located in nerve roots that can be easily stimulated using an extracellular hook electrode in a semi-intact preparation. Increasing the voltage of the stimulus increases the number of excitatory inputs recruited, and an increase beyond the LG's threshold for activation leads to the firing of an action potential in the LG ipsilateral to the stimulus. In a healthy preparation, this spike excites the contralateral LG as well, resulting in an easily identifiable pair of giant spikes in the extracellular recording of the ventral nerve cord (VNC). By placing the stimulating voltage just below threshold and superfusing EtOH, a number of different factors can determine whether EtOH increases LG excitability; what proportion of preparations were brought above threshold during EtOH exposure compared to the expectation that none of the preparations would normally be brought super-threshold during normal saline superfusion of similar duration; how many action potentials were recorded during the hour of exposure; and the latency to spike onset. For the preparations that did not fire an action potential during EtOH exposure, any changes to the EPSP amplitude can be recorded using the EPSP quantification method described in the general methods. Additionally, because previous behavioral and implanted electrode experiments performed in the CNBL have indicated a difference in alcohol sensitivity

between animals with different social experiences, the intracellular experiments described here allow for exploration of this disparity on a single-cell basis.

Methods

Animals measuring between 3 and 4.0 cm were removed from either socially isolated (N = 17; 3.64 ± 0.20 cm) or communal (N = 23; 3.53 ± 0.25 cm) housing conditions (described in the general methods) and placed on ice for 15 minutes. Walking legs and chelae (claws) were removed and the animal was pinned dorsal side up in a sylgard lined petri dish using three minuten pins through the telson, two pins into the thorax, and two pins into the antennal scales to keep the animal stable during experiments. The dorsal cuticle and tail-muscle was removed to provide access to the ventral nerve cord (**Fig. 4.1**). A small, rectangular piece of sylgard was placed between the ventral cuticle of the tail and the abdominal nerve cord for stability. The dish was filled with 40 mL of crayfish saline. One LG was impaled immediately rostral to the terminal abdominal ganglion (A6) using an intracellular electrode filled with 2M potassium acetate (**Fig. 4.2**). Extracellular electrodes were placed on A6 nerve roots ipsilateral to the impaled LG and stimulated to produce excitatory input to the LG. A second extracellular hook was placed more rostral on the ventral nerve cord (VNC) to record general interneuronal activity and to verify impalement of the LG. Additionally, a number of COM (N = 6, 3.75 ± 0.14 cm) and ISO (N = 6, 3.63 ± 0.16 cm) animals were exposed to two hours of saline superfusion rather than EtOH exposure to determine the effect long duration saline superfusion in the absence of EtOH. There was no statistical significance between the sizes of any of the groups tested in the EtOH conditions or the controls (MWU $p > 0.05$).

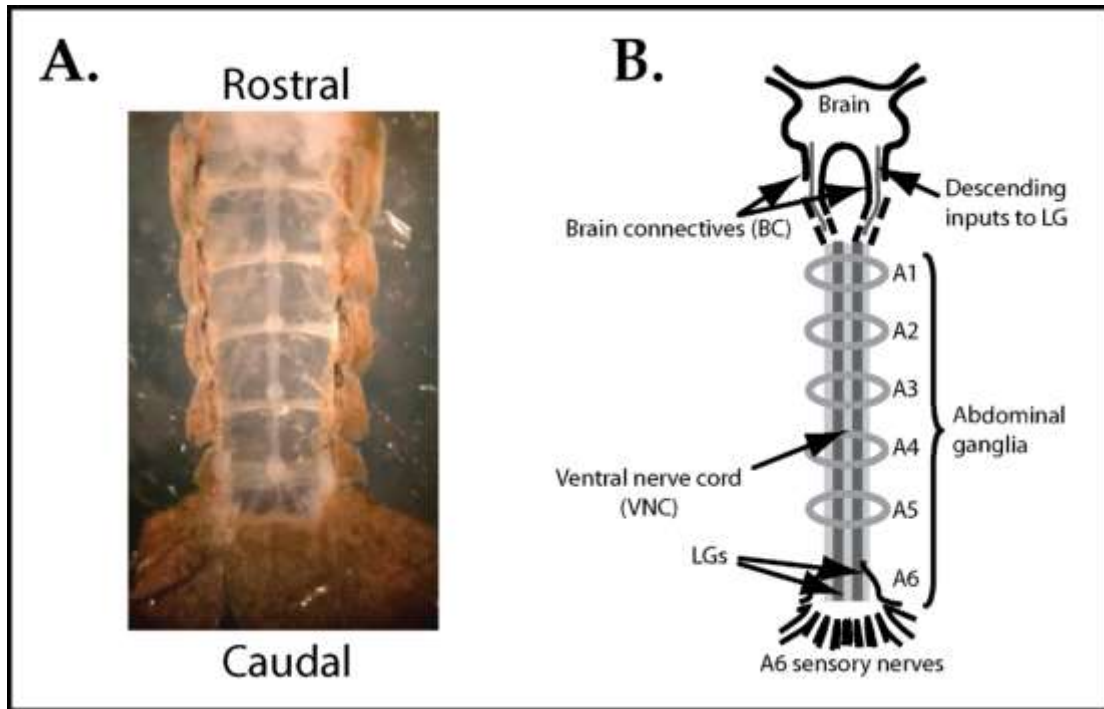


Fig 4.1: Semi-intact preparation to record from the LG of the abdomen. *A) Semi-intact crayfish preparations were largely intact when recording occurred. The animal was pinned dorsal side up in a sylgard lined dish. The dorsal cuticle of the abdomen was removed and the tail musculature was largely removed to expose the ventral nerve cord (VNC) of the crayfish central nervous system between abdominal ganglia 1-6. Care was taken to not damage the sensory nerve roots to the terminal abdominal ganglia (A6). To improve the stability of the VNC during intracellular recording, a rectangular piece of sylgard was inserted between the ventral cuticle and VNC. Nerve roots of A5 were typically pinned to this piece of sylgard to secure the VNC in place.* *B) Schematic of the crayfish VNC. In semi-intact preparations, nervous systems were intact. LG has cell bodies in each abdominal ganglia and its axon travels the length of the animal. It is known to receive descending inputs from the brain. (B modified from Liu & Herberholz 2010).*

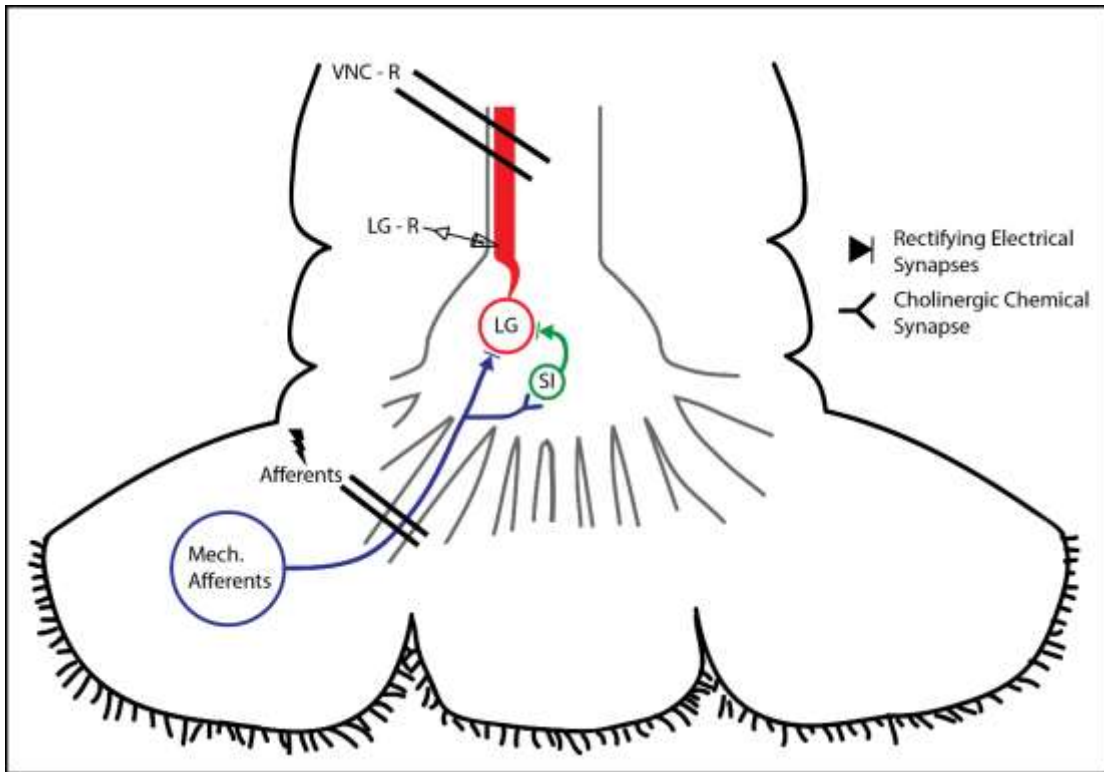


Figure 4.2: Electrophysiology recording of the LG in the crayfish abdomen.

Simplified circuit diagram of the LG. Extracellular hook electrodes were used to record general activity of the ventral nerve cord (VNC – R) and to stimulate mechanosensory afferents (Afferents), evoking EPSPs in the LG neuron. An intracellular sharp micropipette electrode was inserted into the LG axon proximal to its spike initiation zone (LG – R).

Semi-intact crayfish preparations of either social group were exposed to at least 10 minutes of a saline baseline and if the activation threshold was determined to be stable, the stimulus was set immediately below threshold (described below) and the preparation was then exposed to 60 minutes of one of the three EtOH concentrations, 10, 20, or 100 mM, dissolved in normal crayfish saline. At the

conclusion of this exposure, preparations were then washed out for 60 minutes using normal crayfish saline. The purpose of the saline washout was to ensure that the changes observed during any drug exposure period were the result of the drug and not the result of a decrease in the viability of the experimental preparation. Additionally, the washout can indicate how persistent the observed pharmacological effects are after the surrounding medium no longer contains the agent.

The voltage used to activate sensory afferent input to the LGs was placed directly below (0.2 V) a stimulus that would reliably elicit an LG action potential. To find the activation threshold for the LG, the sensory afferents were stimulated with an inter-stimulus interval (ISI) of 90 seconds to reduce the influence that habituation would have on the activation threshold. When the threshold had been found, the preparation experienced at least 5 stimulations at the sub-threshold level. To verify that this stimulus was still immediately below activation threshold, the stimulus was increased by 0.2V. If the LG then fired an action potential, the stimulus was then reduced back below threshold and the EtOH superfusion was initiated. To reduce the possibility that the superfusion itself could manipulate LG threshold, the threshold was found during superfusion of saline at a flow rate of 5 mL/min. EtOH was then superfused over the semi-intact preparations with the same flow rate as during the saline baseline. The flow rate was calibrated and verified before each experiment. Several communal and socially isolated preparations were exposed to normal crayfish saline for 120 minutes as controls.

In addition to the normal statistics described in the general methods, Fisher's exact tests were also performed to compare the number of action potentials in social

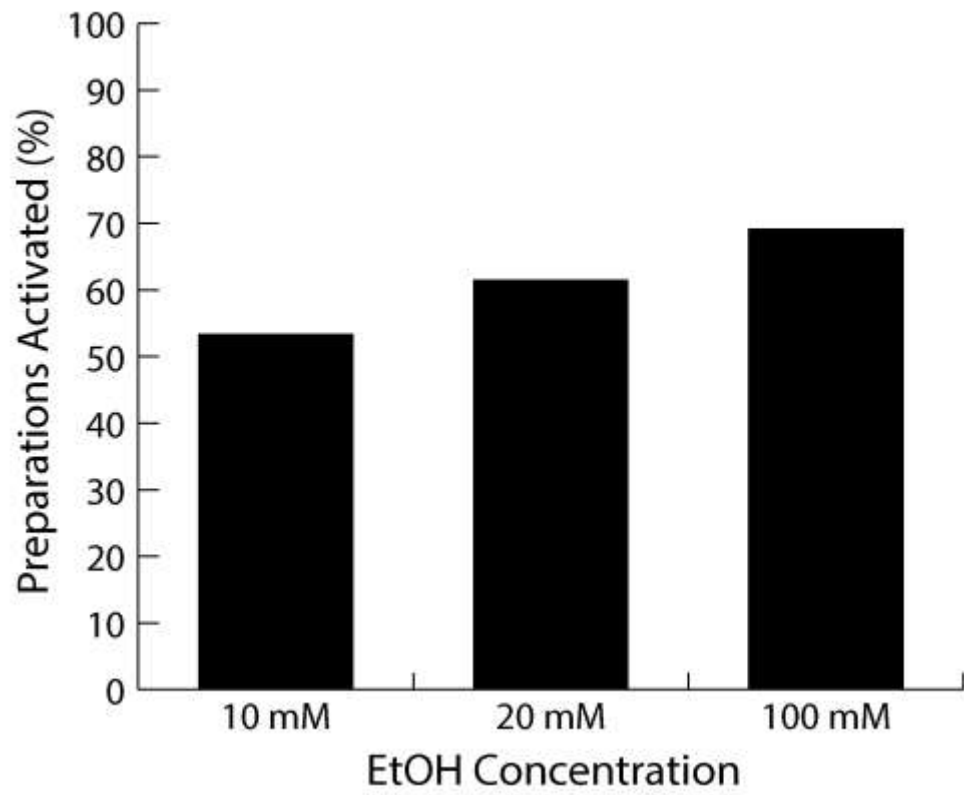
groups. To accomplish this, for every stimulus presented to a preparation (40), a 1 was assigned if the preparation fired an action potential and a 0 if the preparation did not. Fisher exact tests were performed to test the ratio of 1:0 between social groups. This method also allowed for increased scrutiny of smaller time bins so that the changes over time could be statistically compared.

Results

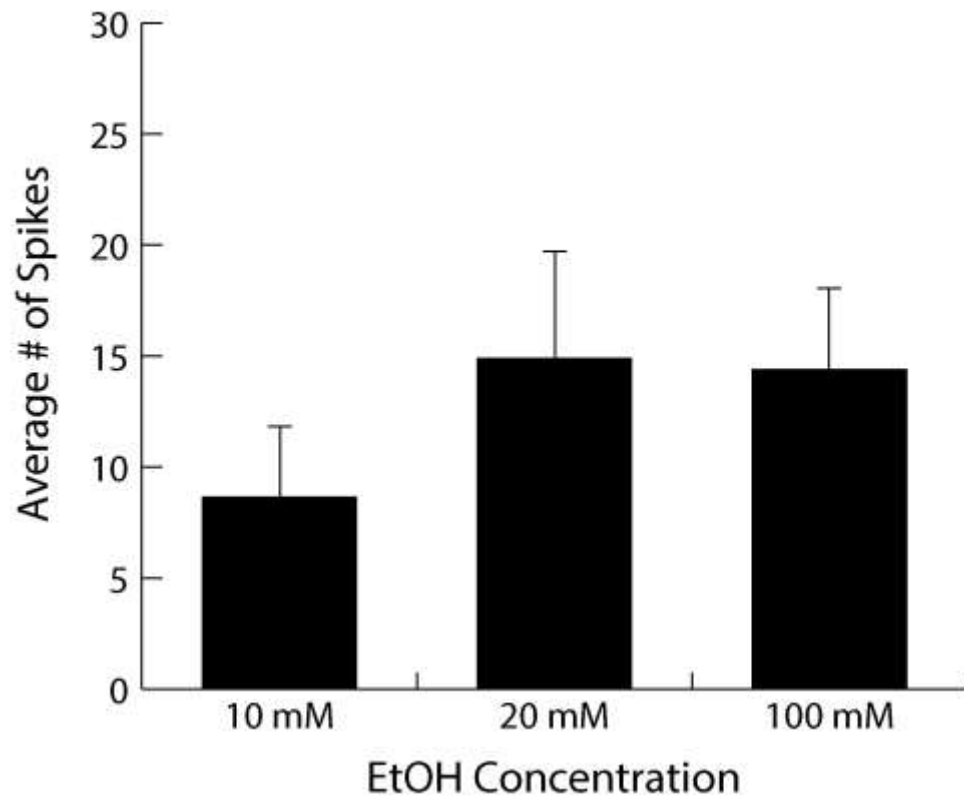
In previous behavioral experiments with juvenile crayfish, alcohol symptoms were dose-dependent with increasing alcohol concentrations inducing intoxication more quickly. Similar to these behavioral and implanted electrode experiments, intracellular recordings of the lateral giant (LG) neuron display a dose-dependent effect with a higher percentage of preparations brought above threshold when exposed to 100 mM EtOH (69.2%, N = 13) than 20 mM (61.5% , N = 13) or 10 mM (53.3%, N = 14). Increasing concentration also increased the average number of action potentials produced per preparation, but only when increasing the concentration from 10 (8.67 ± 3.16 spikes) to 20 mM (14.9 ± 4.79 spikes), although the increase was not significant (MWU $p = 0.33$) (**Fig. 4.3**). There was no difference between the number of spikes produced in 20 mM and 100 mM (14.4 ± 3.66 spikes) (MWU $p = 0.81$). The lack of difference between 20 mM and 100 mM EtOH might be related to the fact that these data pooled the COM and ISO groups and COM preparations were most sensitive to 20 mM EtOH while ISO preparations were most sensitive to 100 mM EtOH (**Table 4.1**).

Fig. 4.3: Increasing EtOH concentration increases the percentage of preparations brought above LG threshold during exposure (A) and increases the average number of spikes per preparation (B). Preparations were exposed to 10, 20, or 100 mM EtOH for one hour of exposure and stimulated every 90 seconds. A) Preparations activated (%) were recorded as the number of preparations that fired any action potentials divided by the total number of preparations for that concentration. B) Average number of spikes produced by all preparations (including those that did not fire).

A.



B.



	Isolates	Communals
10 mM	2/6	6/9
20 mM	2/6	6/7
100 mM	4/6	5/7

Table 4.1: Preparations that produced an action potential in relation to the total number of preparations included in that social group for different EtOH concentrations. A total of 40 preparations of either socially isolate or communal animals were exposed to one of three EtOH concentrations, 10, 20, or 100 mM with sub-threshold stimulation of LG sensory afferents. EtOH produced a greater number of action potentials in animals belonging to the communal group compared to socially isolated animals.

Previous work in the CNBL has shown that social experience modulates the behavioral sensitivity to EtOH and the LG activation threshold in electrode-implanted crayfish. To test the interplay between EtOH and social experience on lateral giant (LG) excitability using single cell electrophysiology, semi-intact surgically prepared juvenile crayfish were housed communally (N = 23, body length = 3.53 ± 0.25 cm) or in isolation (N = 17, 3.64 ± 0.2 cm) for 7-10 days prior to the experiment and exposed to different EtOH concentrations. An example of a preparation brought to threshold by EtOH superfusion can be seen in **Fig. 4.4**. I found that a greater proportion of communal preparations reached LG activation threshold when compared to animals that were socially isolated for 7-10 days prior to testing (**Fig. 4.5**). Pooling the different concentrations used and separating by social experience,

73.9% of communal preparations and 44% of socially isolated preparations were excited above LG activation during EtOH superfusion. For both 10 and 20 mM EtOH concentrations, only 33.33% of the ISO preparations (2 of 6 in each concentration) while 66.67% of COMs (6 of 9) exposed to 10 mM and 85.7% exposed to 20 mM (6 of 7) were brought to threshold. Interestingly, increasing the concentration to 100 mM doubles the number of activated socially isolate preparations (66.66%; 4 of 6) while the communal animals experience a small decline 71.42% (5 of 7). It is important to note that while the percentage of COM preparations firing to 100 mM EtOH reduces from the 20 mM value, this percentage is still higher than the proportion of ISO preparations.

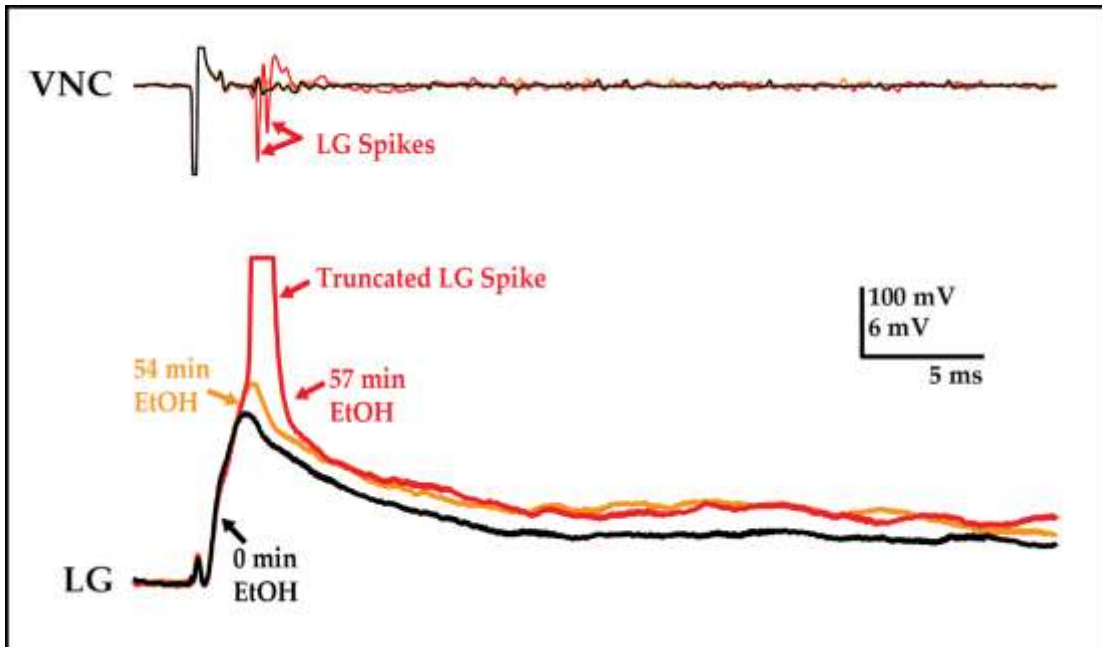
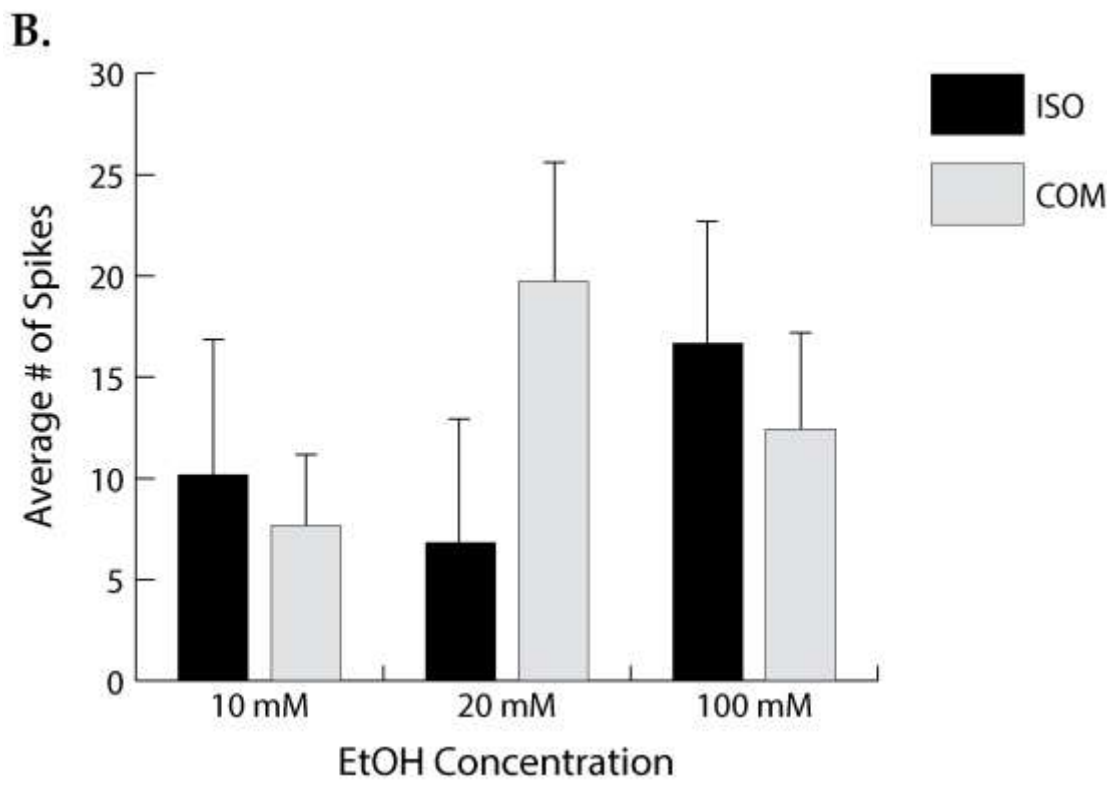
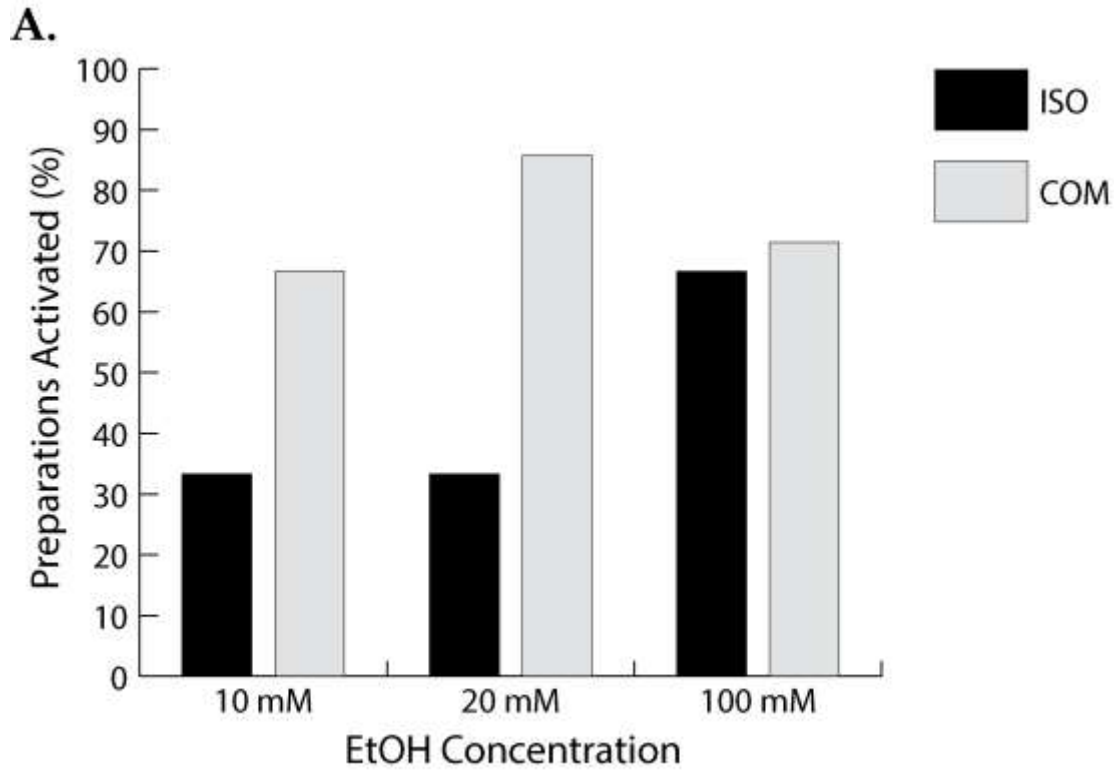


Fig. 4.4: Example of a communal animal LG preparation brought to threshold by superfusion of 100 mM EtOH. Sensory afferents were activated through an extracellular stimulating electrode providing a constant stimulus to the sensory afferents of the LG. Baseline EPSP recorded in normal crayfish saline at a flow rate of 5 mL/min superfusion (black sweep). Superfusion of 100 mM EtOH increases the β of the LG EPSP (orange sweep), bringing it above threshold after 57 minutes of exposure (red sweep). The LG action potential is easily distinguished from other interneuron activity in the extracellular recording electrode.

Fig. 4.5: Communal animals are more sensitive to lower EtOH concentrations than socially isolated animals. **A)** Percentage of preparations activated in each of the three EtOH concentrations used. **B)** Average number of action potentials produced per preparation exposed to EtOH. Bars indicate standard error. (MWU: $p = 0.133$).



Unexpectedly, ISO animals produced more spikes per preparation in 10 mM (10.17 ± 6.65) (mean \pm SE) EtOH than in 20 mM (8.2 ± 7.24). In COMs, raising the EtOH concentration increased the number of spikes from 7.67 ± 3.45 in 10 mM to 19.7 ± 5.83 spikes in 20 mM. Increasing the EtOH concentration from 20 to 100 mM more than doubled the average number of spikes per preparation in ISOs from 8.2 ± 7.24 to 16.67 ± 6.0 spikes (MWU $p = 0.443$) (**Fig. 4.5**). In contrast, COM animals displayed a decrease in spike number from 20 mM to 100 mM exposure (19.7 ± 5.83 spikes to 12.4 ± 4.75 spikes, MWU $p = 0.3$). When the EtOH concentration is removed and all preparations from a given social group are pooled together and the analysis focuses on only the number of action potentials produced by spiking preparations, MWU test reveals that there is no significant difference ($p = 0.28$) between the two social groups in the number of action potentials produced.

Comparing the total number of spikes in relation to the total number of stimuli -per social group (ISO or COM) for each concentration can also indicate LG excitability. There is no significant difference between ISO and COMs exposed to the 10 mM (Fisher's exact test: $p = 0.09$). However, a much larger percentage of stimuli resulted in spikes in COMs than in ISOs when exposed to 20 mM EtOH (49.29% of stimulations compared to 17.1%, $p < 0.01$) (**Fig. 4.6**). Interestingly, Fisher's exact test also revealed that more stimuli elicited action potentials in ISOs than in COMs exposed to 100 mM (41.7% of stimulations compared to 31.07%, $p = 0.012$). These results suggest that COM preparations are more sensitive to a lower concentration of EtOH, but higher EtOH concentration facilitates ISO preparations more than COM preparations. In fact, at this concentration, COMs are less responsive to EtOH than at

lower concentrations. This may be due to increasing EtOH-induced inhibitory effects countering the excitatory effects due to the elevated concentration.

A.

EtOH	Isolates		Communals	
	Spike	No -Spike	Spike	No -Spike
10 mM	61	179	69	291
20 mM**	41	159	138	142
100 mM*	100	140	87	193

B.

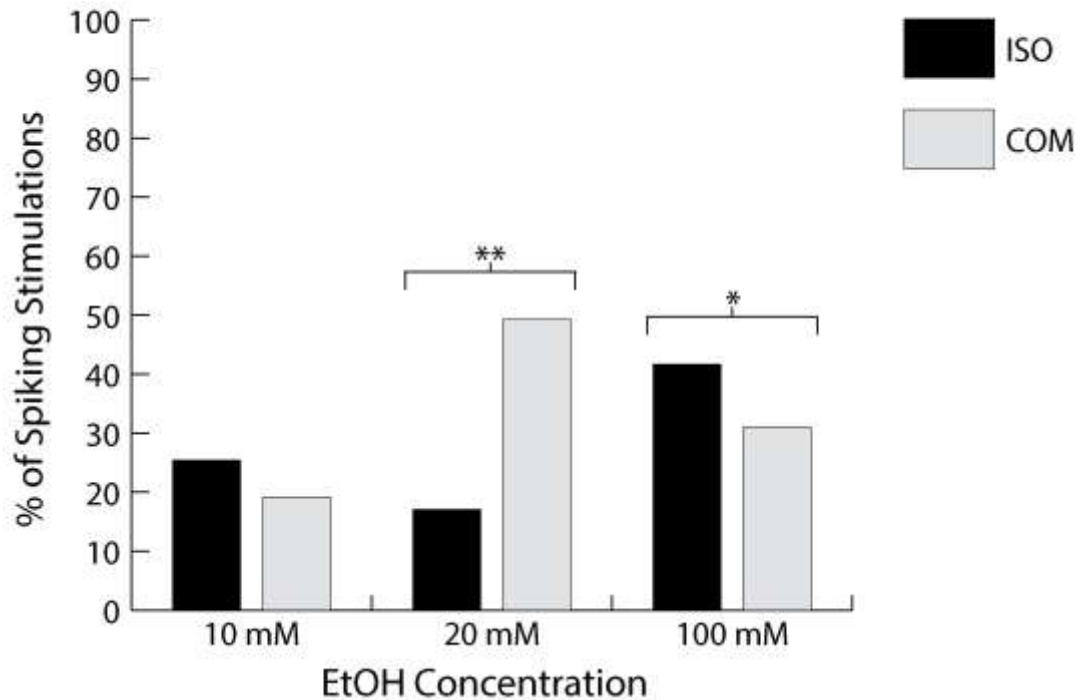


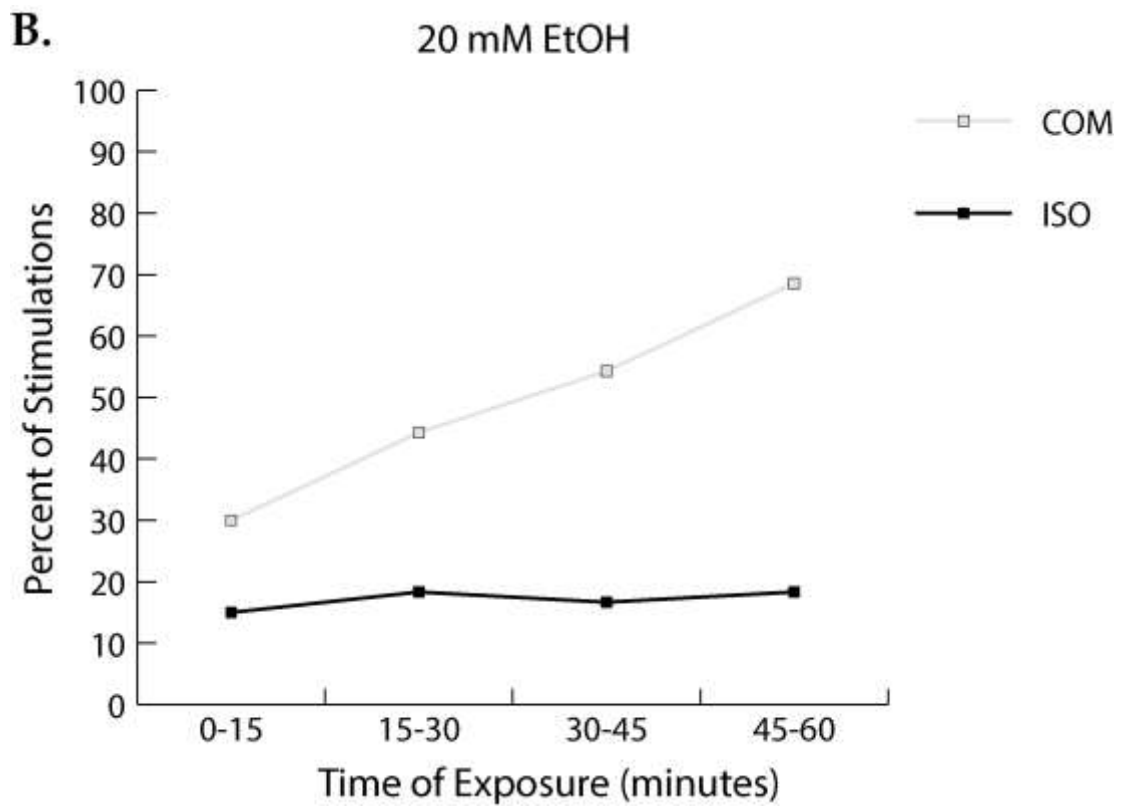
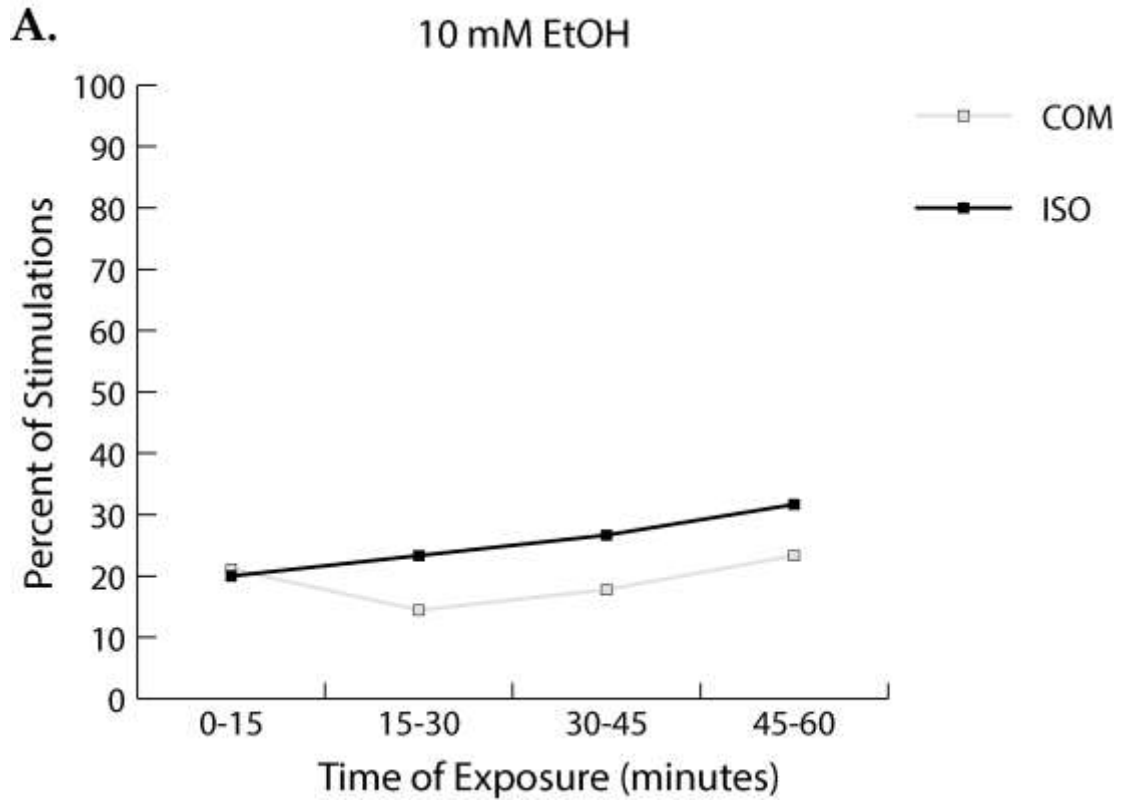
Figure 4.6: Total number of spikes produced across all preparations compared to stimuli that elicited no spike. A) Each preparation was stimulated 40 times during the hour of EtOH exposure. Each stimulus that resulted in an action potential was

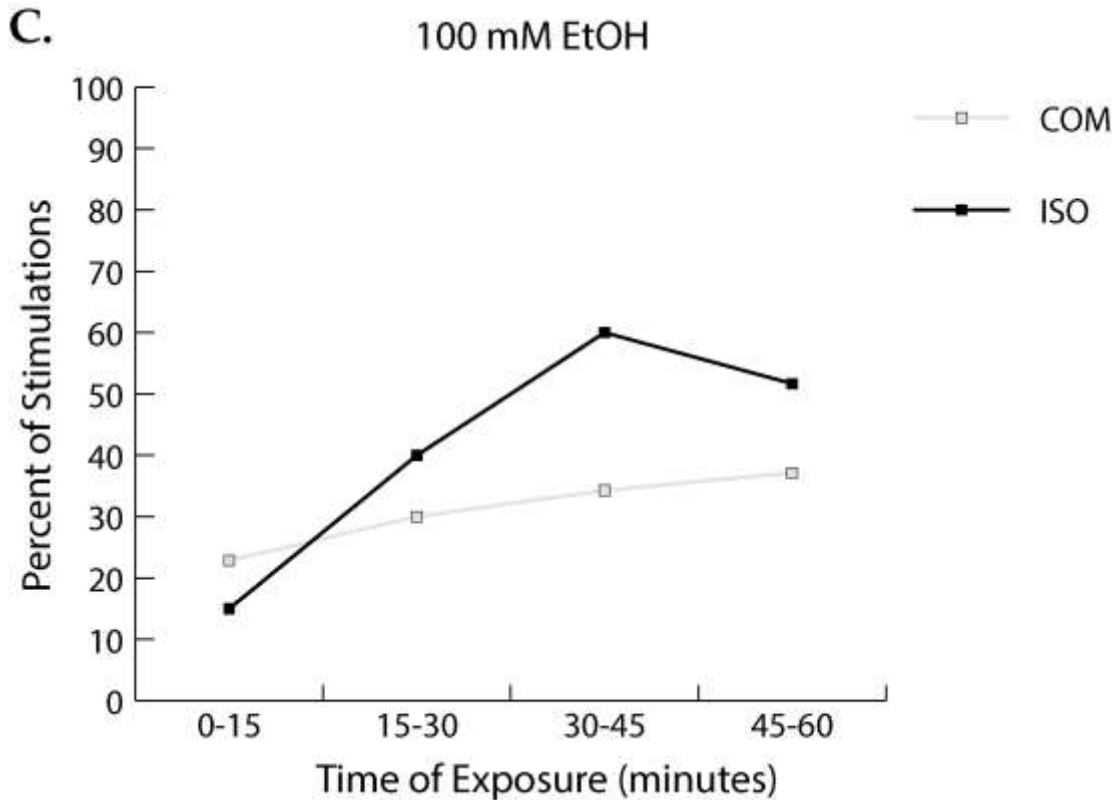
*recorded as a “Spike” and each stimulus that resulted in no action potential was recorded as a “No Spike” allowing for the comparison of the percentage of stimuli that produced action potentials in socially isolated and communal preparations across all three EtOH concentrations. B) Percent of stimulations that elicited spikes calculated from A. * indicate concentrations where significance was found using Fisher’s exact test ($p < 0.05$). ** indicates p values < 0.01 . Stimulations in the 20 mM EtOH were more likely to elicit spikes in COMs than in ISOs. In 100 mM EtOH, more stimuli elicited spikes in ISOs.*

Performing the same analysis for smaller time bins allows for more specific comparison of how EtOH superfusion affects LG activation threshold differently based on concentration and social experience. I did this by dividing the EtOH exposure period into four time bins, 0-15, 15-30, 30-45, and 45-60 minutes of EtOH exposure and totaled the number of spikes for each social group in each EtOH concentration. Performing a Fisher’s exact test using the total number of spikes per time bin in relation to the number of non-spike sweeps per time bin yields differences between ISO and COM groups primarily in the 20 mM EtOH concentration. COM animals exposed to 20 mM EtOH spiked significantly more than ISOs in the last three time bins (**Fig. 4.7**). In the 15-30 minute time bin 44.3 % of stimuli elicited action potentials in COM animals while only 18.3 % of stimuli generated spikes in the ISO group (Fisher’s exact test $p < 0.05$). In the next time bin (30-45 minutes) 54.3 % of COM stimulations were spikes in contrast to 16.7 % in ISO animals (Fisher’s exact test $p < 0.001$). In the final time point, the majority of stimulations in COM generated

action potentials (68.6 %) while ISO preparations remained less responsive (18.3 %) (Fisher's exact test $p < 0.001$). Interestingly, ISO animals spiked more than COMs when exposed to 100 mM EtOH concentration during the 30-45 minute bin with 60% of stimuli eliciting action potentials in ISO animals, but only 34.29% in COM animals (Fisher's exact test $p < 0.01$). This further suggests that this concentration has stronger facilitatory effects on ISOs than COMs. Overall, these results suggest a difference in sensitivity between social groups as lower EtOH concentrations evoke more action potentials in COMs and higher concentrations are needed to evoke more action potentials in ISOs.

Fig. 4.7: Percent of stimulations that result in a spike for 10, 20, and 100 mM EtOH concentrations in socially isolated (ISO) and communal (COM) groups. 20 mM EtOH concentration is most effective for animals with recent communal experience while 100 mM is most effective for socially isolated animals. Animals were exposed to either 10 mM (A), 20 mM (B), 100 mM (C) and the percent of stimuli that elicited action potentials was recorded and binned in 15 minute intervals.



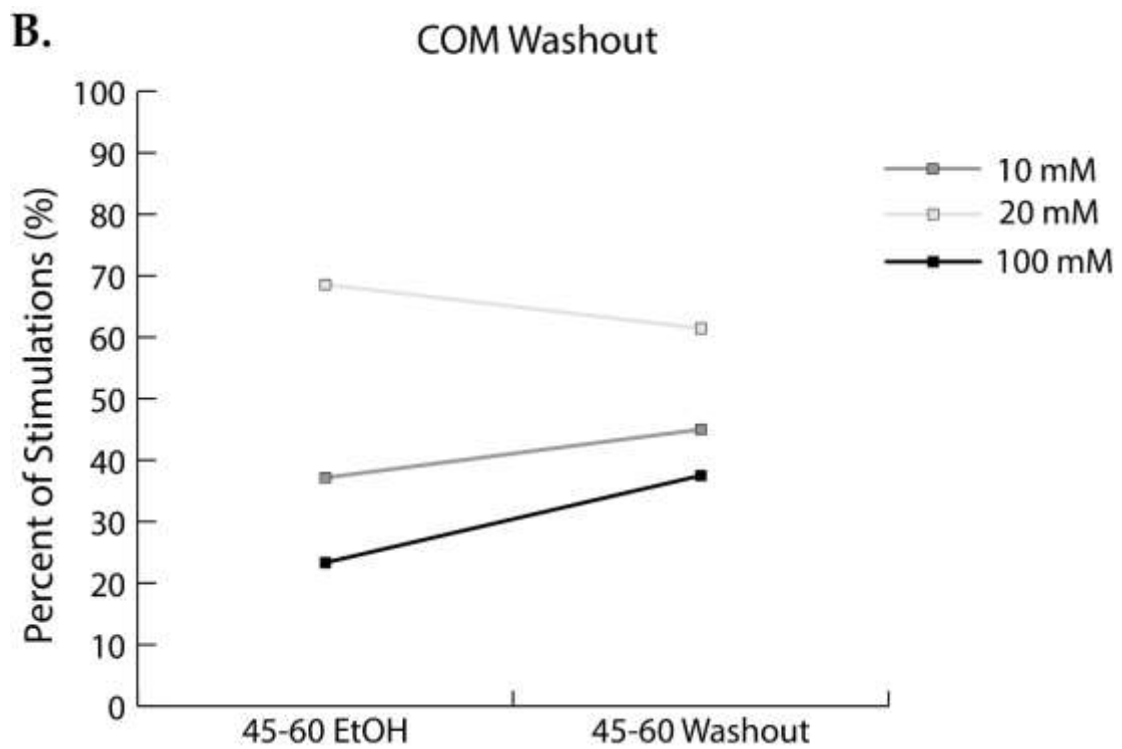
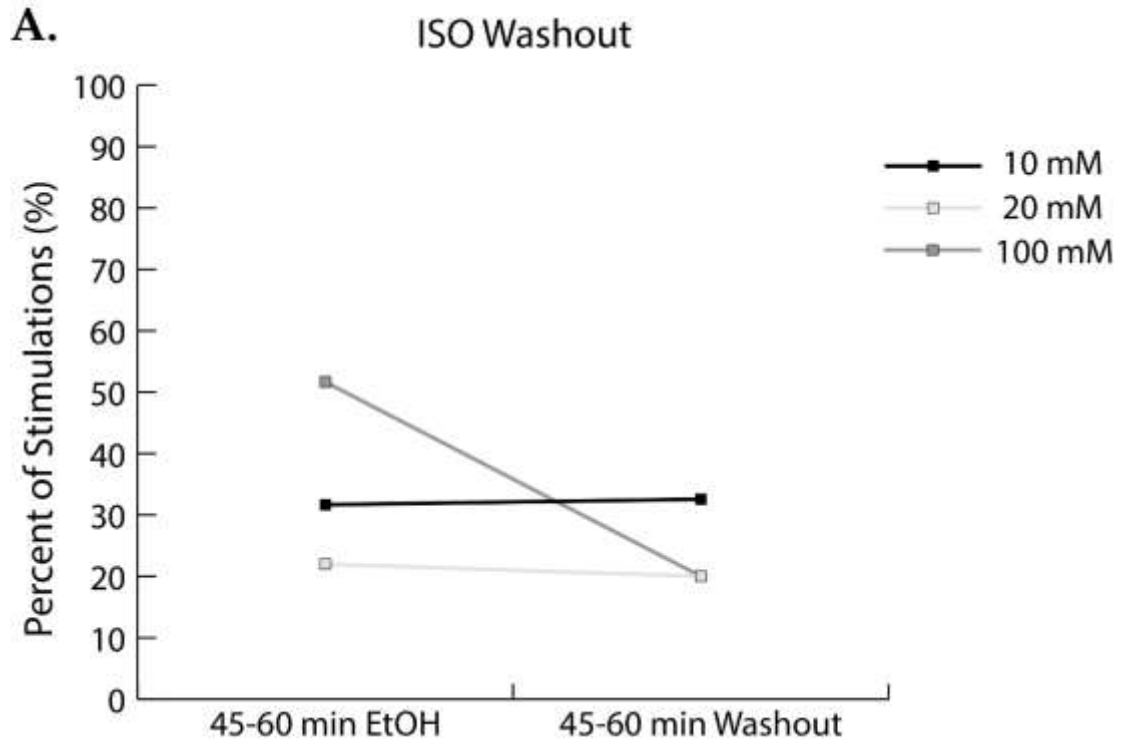


While most preparations received at least 60 minutes of saline washout, ISO preparations were highly persistent. Preparations that were brought above threshold during EtOH exposure generally continued spiking throughout the washout, but some changes did occur. During washout of each EtOH concentration, most ISO and COM preparations showed little change in the percent of stimuli that elicited spikes (**Fig. 4.8**). Only one group in the ISO showed a large decrease in percentage over the course of washout (100 mM: 51.67 to 20%), which was significant using a Fisher's exact test ($p < 0.01$). In COM preparations that were exposed to 10 (23.33 to 37.5%) and 100 mM (37.14 to 45%), the percent actually increased while those exposed to 20 mM decreased (68.57 to 61.43%). The increase in 10 mM was just above significance threshold (Fisher's exact test: $p = 0.06$) while the decrease observed in 20 mM was

significant ($p < 0.01$). The increase observed in the 100 mM in COMs was also significant ($p = 0.035$).

Fig. 4.8: Difference in the percent of stimuli that elicited spikes from the last time bin in EtOH to the last time bin in washout for both ISO (A) and COMs (B).

Following EtOH exposure, preparations were superfused with 60 minutes of normal crayfish saline. Percent of stimulations found by dividing the number of stimuli per time bin that elicited a spike by the total number of stimuli of the time bin.

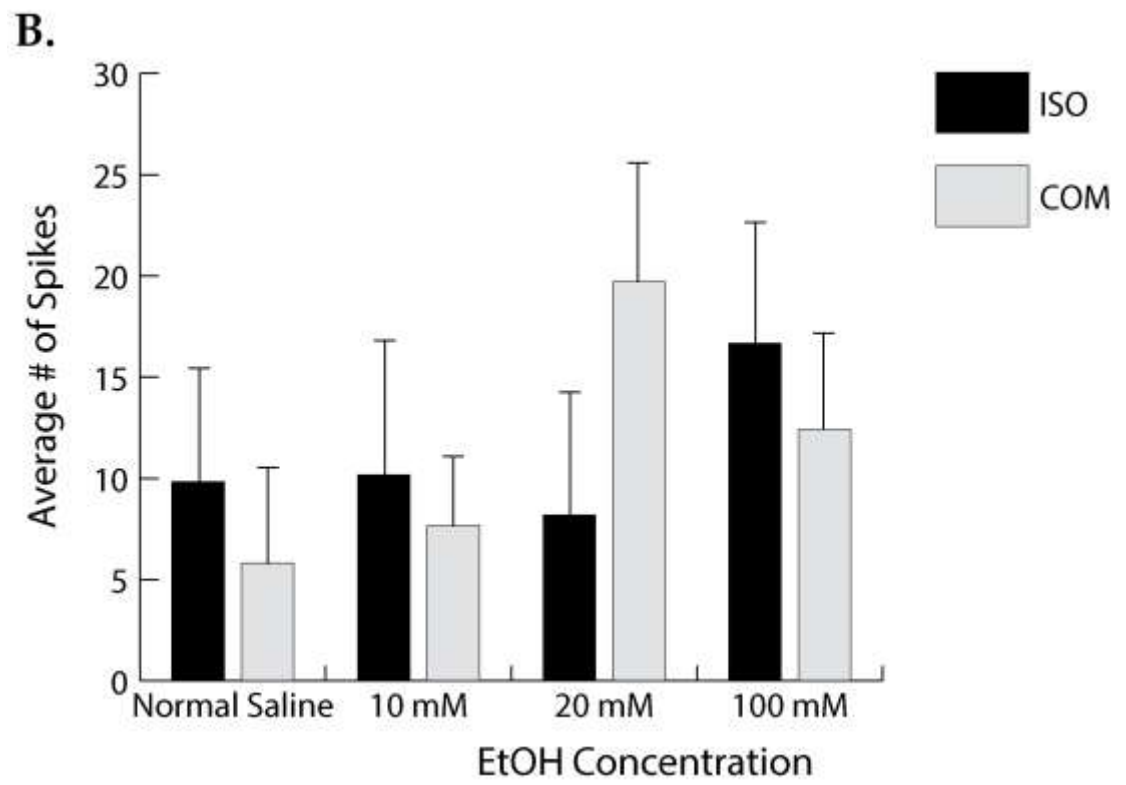
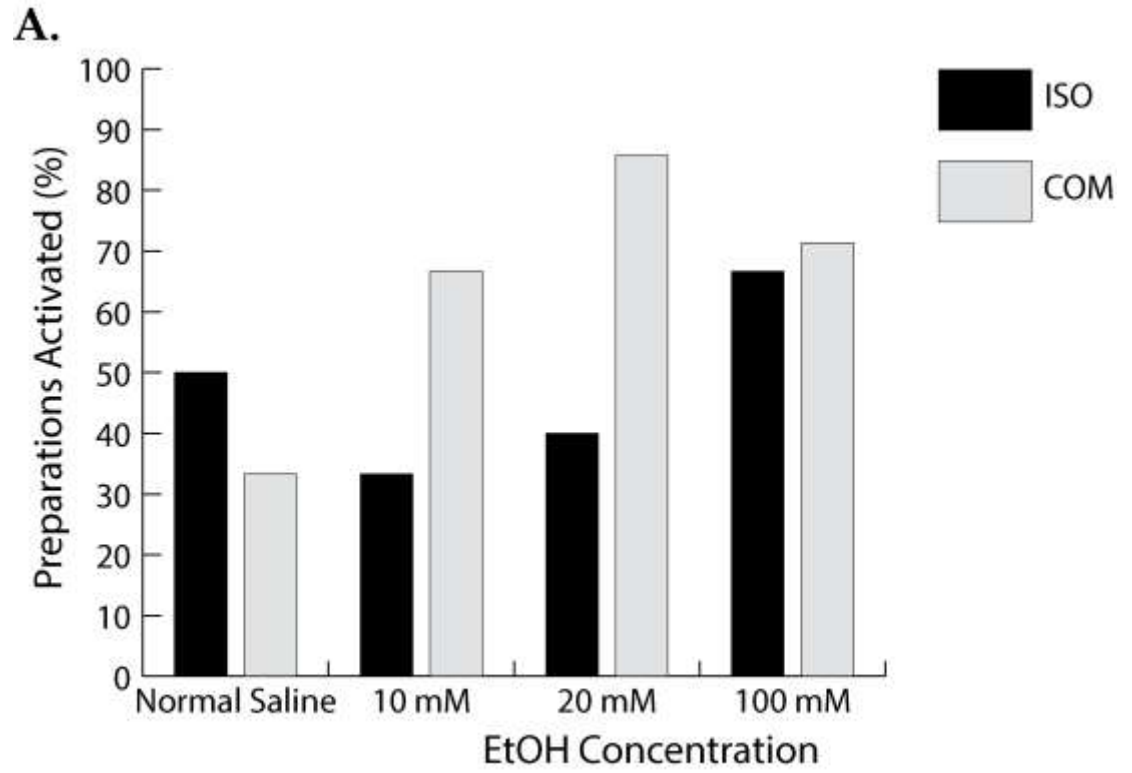


Up to this point, I have only discussed the results in regards to the preparations that produced action potentials, but the non-spiking preparations may still have been affected by EtOH. Interpreting results for each of the three EtOH concentrations separately (10, 20, and 100 mM) proves difficult as the prevalence of non-spiking preparations decreases as EtOH concentration increases. It can also be reasoned that these preparations, both ISO and COM, are the least likely to show enhancement of the EPSP during EtOH exposure, as any significant facilitation would be expected to bring the LG to threshold as the stimulus was already placed directly below threshold. Therefore, any enhancement that might be seen would likely produce action potentials, shifting the preparation to join the ranks of spiking preparations. While the behavioral relevance of a preparation activating an action potential is important, it can be reasoned that there is important information to be gained through observing changes to the EPSP when an action potential is unlikely. In the current experiment, it is also reasonable to suggest that this information cannot be gleaned from this experimental protocol and a new protocol must be implemented to study these sub-threshold responses. The effect of EtOH on sub-threshold EPSPs is the focus of experiment 4.3.

As described previously, LG activation threshold has been shown to decrease when crayfish are exposed to EtOH in the surrounding bath and stimulated with implanted electrodes. Because controls in these experiments displayed fluctuating activation thresholds when stimulated for an hour during water exposure only (no EtOH), similar controls were performed to determine the amount of fluctuation present in the intracellular semi-intact preparations. Six COM (3.75 ± 0.14 cm) and

six ISO (3.63 ± 0.16 cm) preparations were exposed to one hour superfusion of normal crayfish saline (no EtOH). Saline exposure alone caused 33.3% of COMs and 50% of ISOs to spike producing an average of 5.83 ± 4.74 and 9.83 ± 5.58 action potentials, respectively (**Fig. 4.9**). While I observed greater numbers of spiking preparations in the controls than expected, it is important to note that more COMs exposed to 20 mM EtOH were brought above threshold (85.7% vs. 33.3%), and they produced a higher number of average spikes (19.7 ± 5.83 vs 5.83 ± 4.74) than the controls. While not below the significance threshold of $P = 0.05$, both Fisher's exact test for number of spiking preparations ($p = 0.1$) and MWU test for number of spikes produced ($p = 0.07$) suggest a trend that COMs exposed to 20 mM EtOH were preferentially facilitated when compared to COMs that were exposed to saline alone. ISOs were most sensitive to 100 mM EtOH, but the average number of spikes (16.7 ± 6) and the proportion of spiking preparations (66.67%) does not suggest a clear difference to saline controls (MWU $p = 0.42$; Fisher's exact $p = 0.62$). These results suggest that activation thresholds of both ISOs and COMs change over time when exposed to normal saline with ISOs experiencing a greater degree of change. This was also observed in the experiments using implanted electrodes (Herberholz, unpublished). Together these results implicate a greater difference between COM and ISO groups; it is apparent that LGs of COMs exposed to EtOH are clearly facilitated while ISO preparations experience a much weaker effect.

Fig. 4.9: Comparison of saline-exposed controls to EtOH-exposed experimental groups. A) Percentage of preparations brought above threshold in controls, 10 mM, 20 mM EtOH, and 100 mM EtOH. B) Average number of spikes produced by each group. Error bars indicate standard error.



Experiment 4.2: LG EtOH Sensitivity is Modulated by Descending Brain Inputs

Because controls during the previous experiment indicate a high degree of variability in semi-intact preparations when exposed to normal crayfish saline, a means to reduce this variability was investigated. The presence of tonic inhibition, as described previously, is known to descend from the rostral portion of the animal and believed to originate in the brain. It is possible that this inhibition is the reason for the degree of activation during subthreshold stimulation when exposed to normal crayfish saline. To counter this, I was interested in how the removal of this tonic GABAergic inhibition influenced the effect of EtOH and normal crayfish saline on the LG activation circuit. I accomplished this by repeating experiment 4.1 with an isolated tail preparation. During surgery, the thorax and head were separated from the crayfish abdomen, eliminating any descending connections to the tail. The intracellular recording of the LG and stimulation of the LG sensory afferents were done in an identical manner to experiment 4.1. Interestingly, and unlike the semi-intact results, COM isolated tail preparations were most sensitive to 100 mM EtOH, displaying significantly lower activation thresholds following EtOH exposure compared to thresholds recorded in saline-exposed controls. COM preparations that were brought to threshold when exposed to 100 mM EtOH produced a higher number of spikes during EtOH exposure than ISOs, albeit only slightly. Additionally, the lack of effect seen in the 20 mM isolated tail COM group compared to the semi-intact preparations from experiment 4.1 suggests that this low concentration of EtOH might interact with the GABAergic tonic inhibition that is known to descend into the abdomen and modulate LG excitability. Much of the circuitry of the LG is located in the tail,

including sensory inputs and outputs to the MoGs and the SGs with the notable exception of the descending tonic inhibition (Edwards et al., 1999; Krasne et al., 2015). Therefore, it seems plausible that 20 mM EtOH is decreasing descending tonic inhibition in semi-intact animals to produce a higher degree of excitability in the LG.

Methods

All methods for experiment 4.2 were identical to those in the previous experiment, but animals were bisected between the thorax and abdomen. The isolated tail was pinned down to a sylgard-lined dish and the rest of the surgical preparation was performed in identical manner to experiment 4.1. Electrophysiological recording and stimuli were performed identically to the previous experiment and the threshold was recorded after the hour of EtOH (experimental groups) or normal crayfish saline (controls). COM and ISO experimental groups were exposed to 20 mM (COM N = 6, 3.65 ± 0.14 cm; ISO N = 6, 3.7 ± 0.2 cm) or 100 mM EtOH (COM N = 6, 3.65 ± 0.21 cm; ISO N = 6, 3.68 ± 0.18). COM (N = 4, 3.65 ± 0.1 cm) and ISO (N = 4, 3.68 ± 0.24 cm) controls were exposed to one hour of saline. There was no significant difference between any of the groups in terms of body length (Kruskall Wallace $p > 0.05$). Body lengths were recorded before the abdomen was separated from the thorax and head of the crayfish. Any preparation that produced any spikes during the hour of EtOH or saline were considered “spiking” and were included as such in the data. In addition to recording the threshold values before beginning exposure, all preparations had thresholds measured after one hour of EtOH or saline exposure.

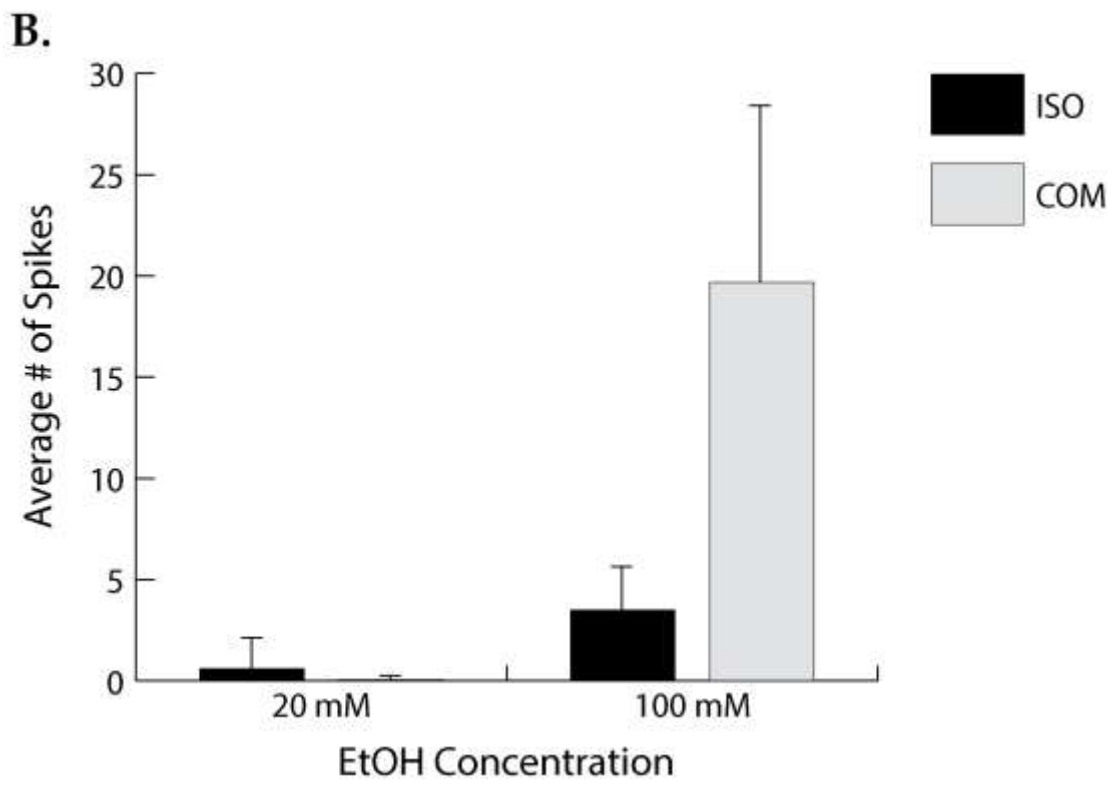
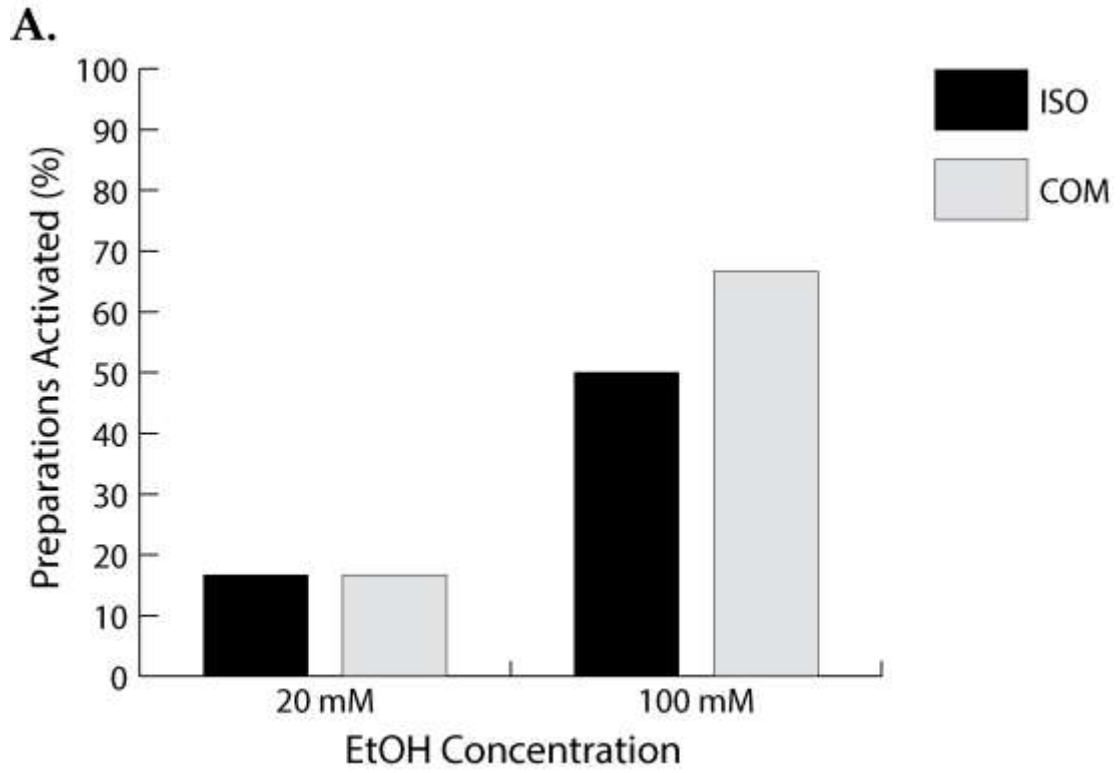
Results

Isolated tail preparations generally maintained more stable LG activation thresholds compared to semi-intact control preparations (experiment 4.1). While 33% of COM semi-intact controls (2 of 6) had spiked during the hour of saline, none of the isolated tail COM preparations (0 of 4) were brought to threshold. This trend is similar in the ISO controls as well where 50% of semi-intact preparations (3 of 6) fired action potentials while only 25% of isolated tail preparations (1 of 4) did. It is noteworthy that while the tail preparations showed lower activation than their semi-intact counterparts, ISO preparations were still more excitable than COM tail preparations. In the three semi-intact ISO crayfish that spiked during their hour of saline, 19.67 ± 7.68 (mean \pm SE) action potentials were produced. The one ISO tail preparation that spiked during saline produced only two action potentials at the end of the hour of saline exposure. The change in threshold after one hour of saline in isolated tail preparations was 103.45 ± 2 % and 98.87 ± 2.86 % of the starting threshold in COM and ISO groups, respectively. Control preparations from experiment 4.1 had thresholds of 102.98 ± 3.71 % in COMs and 103.6 ± 8.04 % in ISOs. When comparing controls between experiment 4.1 and 4.2, threshold values for neither COMs nor ISOs were significantly different (MWU: $p > 0.05$) Removal of the tail from the head and thorax of the animal before testing appears to have a larger effect on the ISO social group than on the COM group, possibly indicating an increase in the range of regulation of tonic inhibition in the ISO group.

While both 20 and 100 mM EtOH concentrations were tested, ISO and COM isolated tail preparations were most sensitive to 100 mM EtOH. During the hour of 100 mM EtOH exposure, 66.67 % of COM preparations were brought to threshold

producing on average 19.67 ± 8.51 spikes (mean \pm SE of all spiking and non-spiking preparations) (**Fig. 4.10**). While 50% of the ISO tail preparations were also brought above threshold during the hour, they produced fewer spikes on average (3.5 ± 2.08 spikes). While these values were not significantly different from their respective controls (COM MWU: $p = 0.06$, ISO MWU: $p = 0.31$), the recorded threshold after 1 hour of 100 mM EtOH was significantly lower (95.31 ± 3.58 % of starting threshold) compared to controls in the COM group (MWU $p = 0.02$). Thresholds recorded in ISO groups (96.1 ± 4.64 %) were not significantly different from saline-exposed controls (MWU $p > 0.05$). Directly comparing the ISO and COM experimental groups, however, yielded no significant difference. These data suggest that EtOH has a stronger effect in crayfish with recent communal experience compared to animals that had been socially isolated for 7-10 days prior to testing. As noted earlier, semi-intact preparations were resistant to washout, but COM tail preparations showed a decrease in firing compared to their hour of alcohol exposure (from 19.67 ± 8.51 to 7.83 ± 6.53 spikes). ISO tail preparations actually showed an increase in the average number of spikes during washout (3.5 ± 2.08 to 7.17 ± 6.58 spikes) due to one preparation brought above threshold at the end of EtOH exposure continuing to fire through all 40 stimuli in washout.

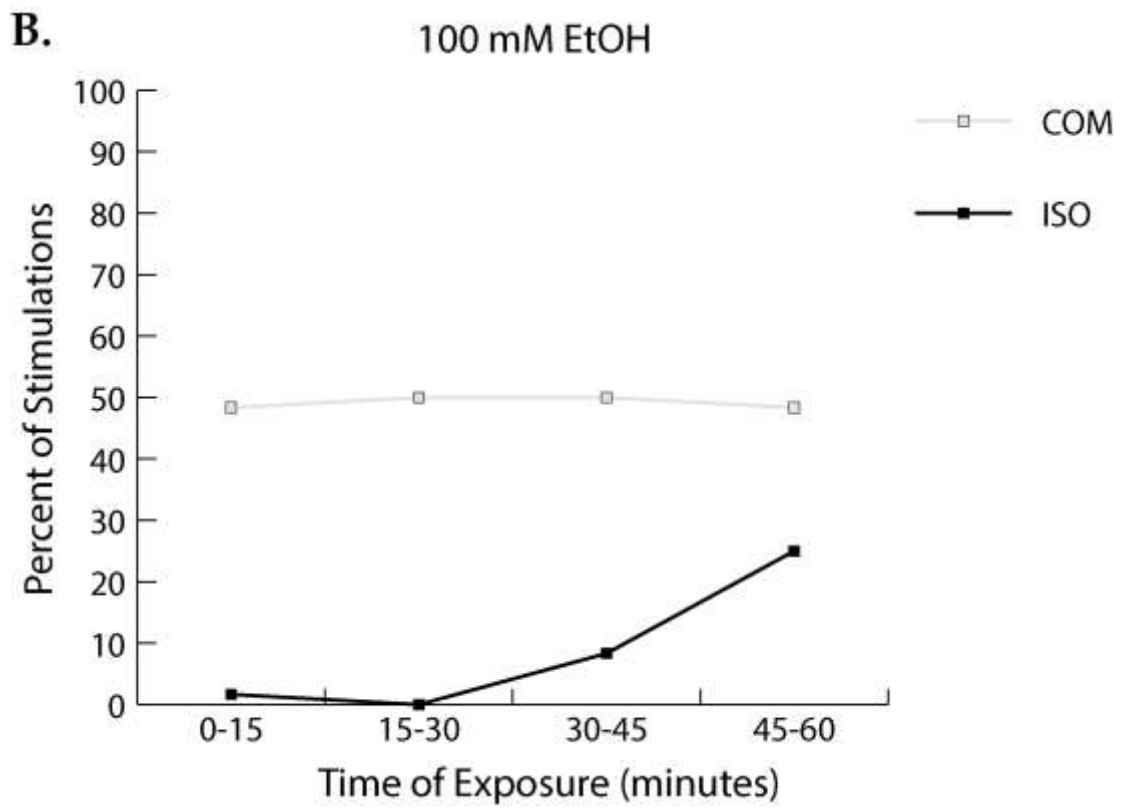
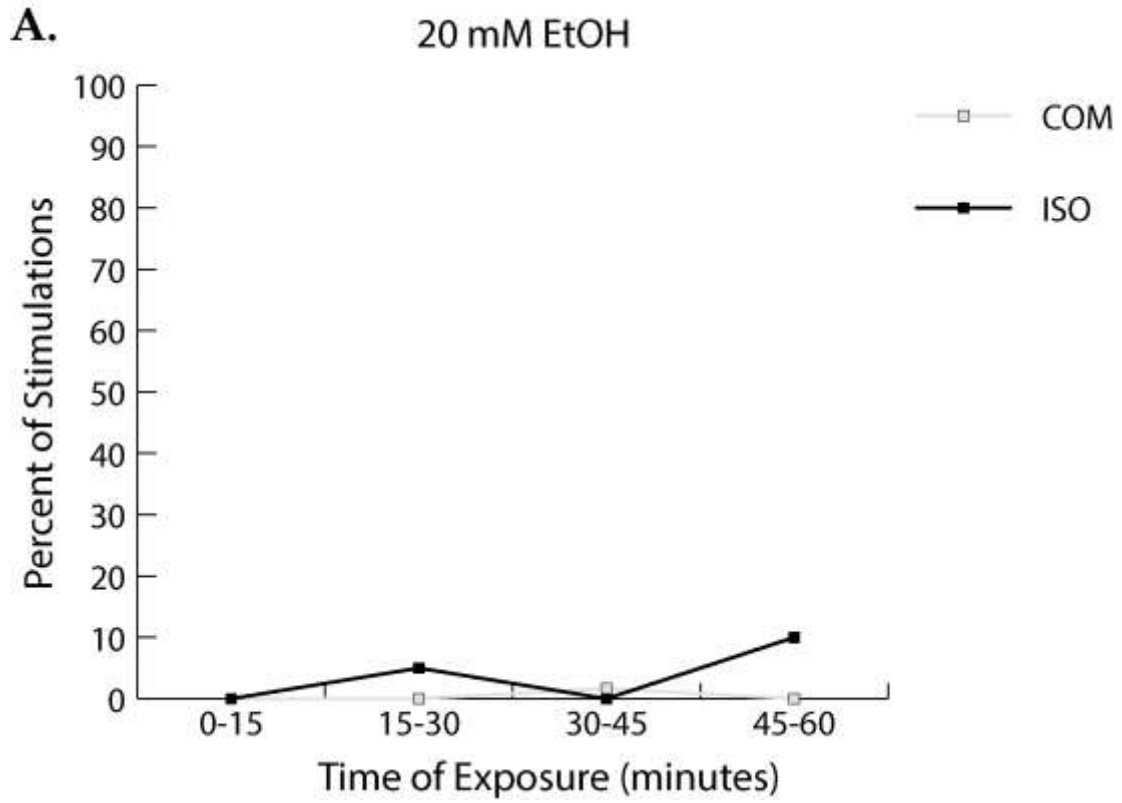
Fig. 4.10: Higher EtOH concentrations are necessary to produce an effect in isolated tail preparations. COM preparations are more sensitive to EtOH superfusion. A) Percentage of preparations activated in 20 and 100 mM EtOH. B) Average number of action potentials produced per preparation exposed to EtOH. Standard error is shown. (MWU $p = 0.133$).



Similar to control preparations, isolated tail preparations from either COM or ISO animals showed little change when exposed to 20 mM EtOH. This was unexpected as semi-intact COM preparations were most sensitive to 20 mM EtOH, producing the highest number of average spikes per preparation. Additionally, this concentration induced spikes in a higher proportion of preparations than any other concentration for either social group. For these reasons it is interesting that when the tail is removed from the thorax and head, eliminating tonic inhibition, EtOH no longer has an effect at this lower concentration suggesting that the sensitivity seen in semi-intact preparations may be due to interactions between EtOH and tonic inhibition. One COM preparation produced one spike during the 40 stimuli presented during EtOH exposure. Similarly, only one ISO preparation was brought to threshold, but produced 9 spikes over the course of exposure. Comparing spikes produced per preparation between semi-intact and isolated tail preparations indicates a significant difference in the 20 mM concentration for COMs (MWU, $p < 0.05$) but not ISOs (MWU, $p > 0.05$). Between surgical preparations, 100 mM did not have a different effect for either social group (MWU, $p > 0.05$). While the threshold values were shown to have shifted lower in the COM 100 mM group compared to controls, COM preparations exposed to 20 mM EtOH had threshold values of 102.85 ± 3.55 %. Following one hour of exposure, ISO threshold values were recorded as 98.69 ± 1.14 %. These threshold values were not significantly different from control thresholds (MWU $p > 0.05$).

Analyzing the number of stimuli that elicited spikes compared to the number of stimuli that did not for both ISO and COM groups in both concentrations shows that five times as many stimuli presented to COMs exposed to 100 mM EtOH produced spikes compared to ISOs exposed to the same concentration. Of 240 stimuli presented to COMs during EtOH exposure, 118 elicited spikes while only 21 were successful in ISOs (Fisher's exact test $p < 0.001$). For this analysis, both COM and ISO stimuli produced more spikes in 100 mM than in normal crayfish saline (Fisher's exact test $p < 0.01$). Unsurprisingly, in each 15 minute time bin, COM stimuli elicited more spikes than in ISOs with p values below 0.01 for the first three time bins (0-15, 15-30, 30-45 minutes) (**Fig. 4.11**). In the final time bin, p values were slightly increased, but still significant ($p = 0.013$). Neither the total stimuli nor the 15 minute time bins for the 20 mM concentration showed significance between the social groups, or their respective controls. The late increase in the number of spikes produced by ISOs in the 100 mM concentration implies that it might require a longer period of time for 100 mM EtOH to exert its effects on ISO tail preparations than COM tail preparations.

Fig. 4.11: Percent of stimulations of isolated tail preparations that result in a spike for 20 and 100 mM EtOH concentrations in socially isolated (ISO) and communal (COM) groups. 20 mM EtOH is insufficient to excite either social group. COMs exposed to 100 mM EtOH are brought above threshold earlier than ISO counterparts. Animals were exposed to either 20 mM (A) or 100 mM (B) and the percent of stimuli that elicited action potentials was recorded and binned in 15 minute intervals.



Experiment 4.3: Influences of Alcohol on LG EPSPs

In the previous experiment, I found that increasing concentrations of EtOH elicit a greater proportion of immediately sub-threshold LG preparations to produce action potentials with 100 mM effectively driving cells above threshold in both socially isolated (ISO) and communal (COM) preparations. For this experiment I was interested in the effect of EtOH on sub-threshold EPSPs. Because of the nature of the previous experiment, the stimulating voltage was placed directly below activation threshold, and very little changes to the EPSP were observed before a preparation made the transition from “non-spiking” to “spiking”. While the action potentials themselves would make measuring EPSP amplitudes impossible, the LG also produces recurrent inputs following its action potential that prevents further activation (Roberts, 1968; Edwards, 1990). For these reasons, animals with a recent history of social isolation or communal housing were surgically prepared in identical fashion to Experiment 4.1 except the stimulating voltage was placed well-below activation threshold. This significantly reduced the possibility of the preparation being brought to threshold through EtOH-induced facilitation. In fact, only one preparation produced action potentials at the conclusion of EtOH exposure. This preparation was excluded from the present analysis. Animals were exposed to the higher EtOH concentration, 100 mM, with the same exposure protocol as the previous experiment.

Methods

The effect of EtOH on sub-threshold EPSPs of crayfish escape circuitry were explored by measuring the effects of 100 mM EtOH on electrically stimulated LG EPSPs. Animals were tested after removal from a communal housing tank (N = 8) or

after being socially isolated for 7-10 days ($N = 7$) in accordance with the general methods for housing. Communal animals (COM) had average body lengths of 3.58 ± 0.17 cm (mean \pm SD) and animals belonging to the socially isolated group (ISO) had average lengths of 3.73 ± 0.21 cm. These sizes were found to be statistically insignificant using a MWU test ($p > 0.05$), indicating that any difference between these social groups is likely not the result of any difference in size of the animals.

Semi-intact crayfish preparations of either social group were exposed to at least 10 minutes of a saline baseline and if the measured excitatory post-synaptic potential (EPSP) was stable, the preparations were then exposed to 60 minutes of 100 mM EtOH dissolved in normal crayfish saline. At the conclusion of this exposure, all preparations were then washed out for at least 60 minutes using normal crayfish saline. The purpose of the saline washout was to ensure that the changes observed during any drug exposure period were the result of the drug and not the result of a decrease in the viability of the experimental preparation. Additionally, the washout can indicate how persistent the observed pharmacological effect may be after the surrounding medium no longer contains the agent.

EPSP amplitudes were measured as a percentage of the EPSP baseline for each individual experiment and then averaged together for graphical representation. This was performed in accordance with the previously described EPSP Quantification methods described in detail in the General Methods. Statistically, EPSP amplitudes were compared between social groups using a MWU test.

Results

Acute exposure to 100 mM EtOH increased LG EPSP amplitude over the course of one hour of exposure. ISO EPSPs continued to increase in amplitude during the washout, suggesting the continued facilitation even when the dish EtOH concentration should have been decreasing. These results are consistent with those from Yeh et al. (1996), which showed that ISO preparations are more resistant to washout compared to animals with recent social experience as subordinates or dominants. This effect was not seen in the COM preparations as the modest increases in EPSP amplitude did not continue into the washout (**Fig. 4.12**). By analyzing the EPSP and comparing each component of it to the amplitude of that component during the saline baseline, I can analyze alcohol's facilitating effect as an increase of each samples' amplitude. The amplitude was measured at the β component of the LG EPSP (3 ms after the stimulus artifact) and during postexcitatory inhibition (6 ms) (Vu et al., 1997). By plotting the amplitude as a percentage of the baseline average over the course of the 60 minute EtOH exposure, it is obvious that the 100 mM concentration has a significant effect on both the β component and the component governed by postexcitatory inhibition (PEI).

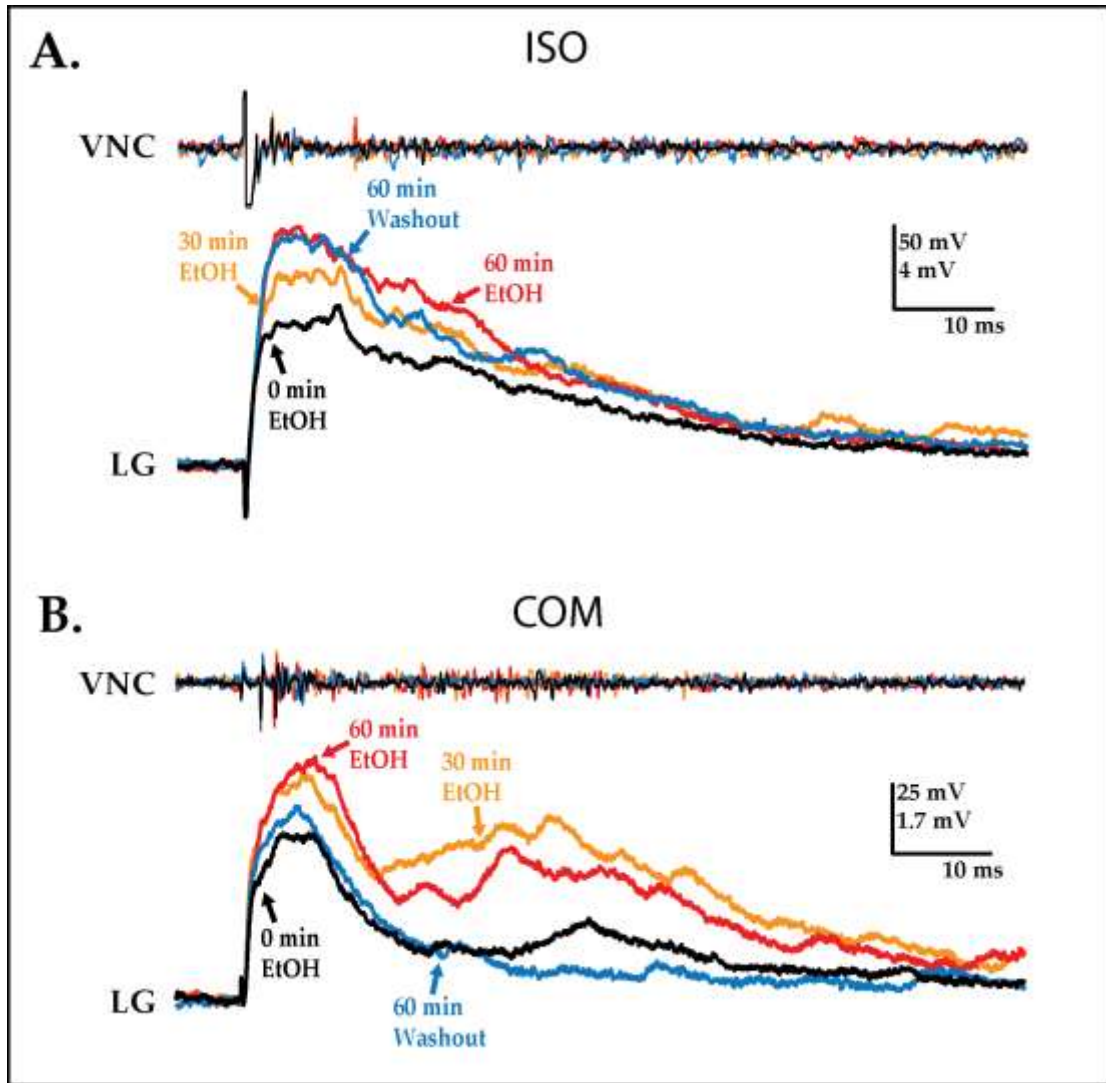


Fig. 4.12: Sample EPSPs from ISO (A) and COM (B) preparations. Intracellular recording from the lateral giant (LG) neuron from an animal that was socially isolated for 7-10 days prior to testing (A) and an animal that was housed in a communal tank with other crayfish before testing (B). Baseline EPSP amplitudes in response to electrical activation of LG sensory afferents were recorded in normal crayfish saline (black sweep). The EPSPs recorded from the same voltage stimulation of afferents after 30 min (orange sweep) and 60 min (red sweep) of 100 mM EtOH

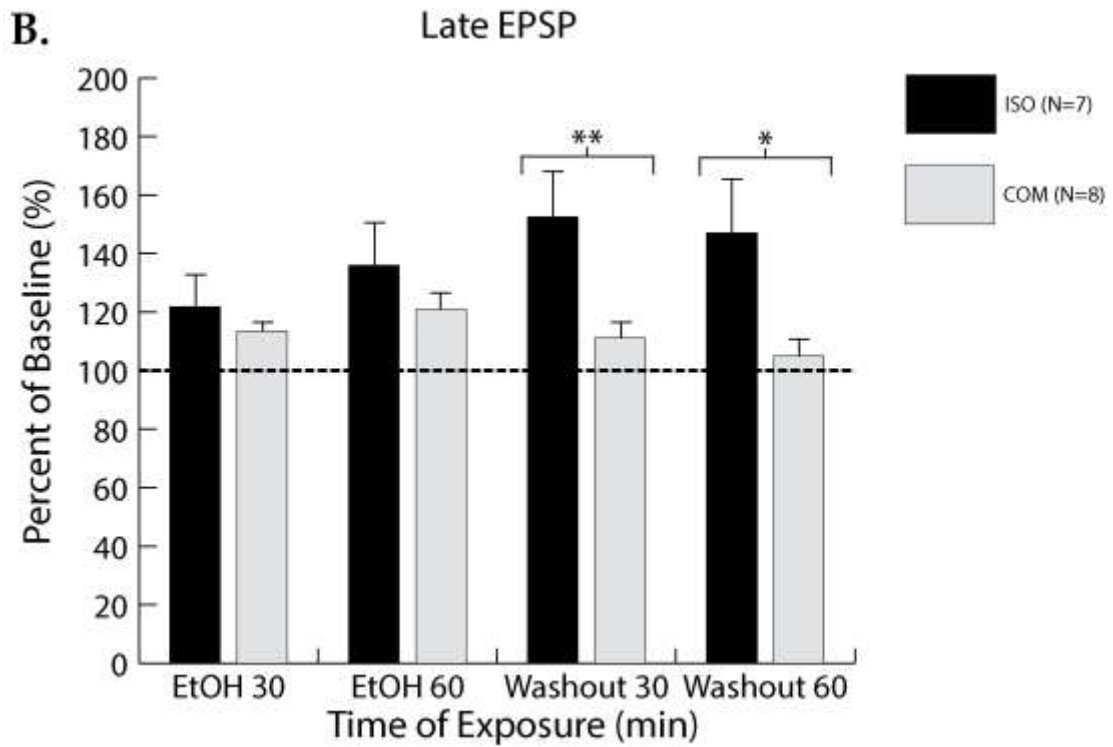
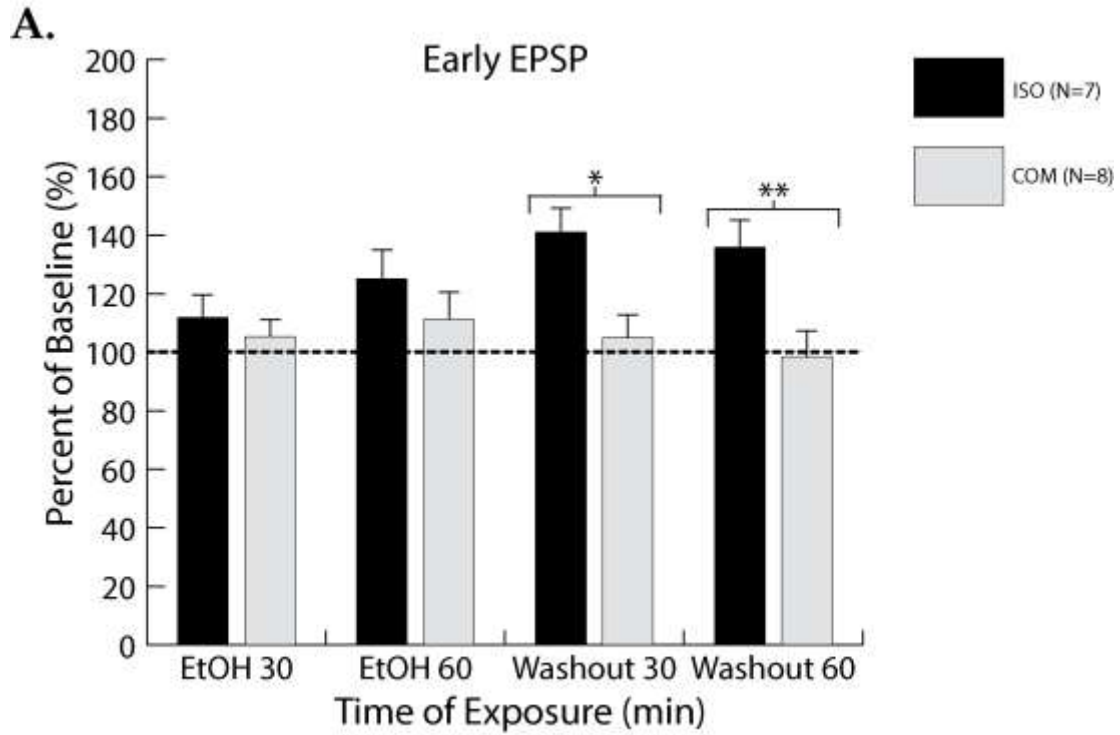
exposure. The resultant amplitude after 60 minutes of washout following EtOH exposure is indicated by the blue sweep.

COMs displayed an average facilitation of 105.38 ± 5.63 % (mean \pm SE) in the early component and an average of 113.45 ± 3.1 % in the late component after 30 minutes of 100 mM EtOH exposure. After 60 minutes of exposure, early components were 111.21 ± 9.28 % of baseline and late EPSP components were 120.94 ± 5.66 % of baseline. ISOs experienced similar trends of facilitation: After 30 minutes of 100 mM EtOH exposure the early EPSP showed minor facilitation of 111.91 ± 7.54 % of baseline while the later EPSP component showed an increased average facilitation of 121.86 ± 10.73 % (Fig. 2B). After 60 minutes of EtOH exposure, ISOs recorded early component amplitudes of 125.06 ± 9.63 % of baseline and late components were 135.91 ± 14.59 % of baseline (**Fig. 4.13**). During EtOH exposure, the early EPSP component of COMs was not significantly different from baseline (Wilcoxon, $p > 0.05$) after 30 or 60 minutes of exposure. However, the late component was significantly enhanced over baseline values after 60 minutes of exposure (Wilcoxon, $p < 0.05$). In contrast, 60 minutes of EtOH exposure increased both ISO early and late components over saline baseline values (Wilcoxon, $p < 0.05$). Comparing the difference of enhancement between the early and late component by subtracting the early value from the late value showed that the EPSP components of COMs and ISOs were similarly effected by EtOH exposure (MWU, $p > 0.05$).

Testing for significance between ISO and COM samples using a MWU test yielded no significant difference during all EtOH exposure time points. However,

during the washout, socially isolated preparations maintained their facilitation in comparison to communal animals. Following 30 minutes of saline washout, isolate early and late components were significantly higher than communal preparations (ISO early = 141 ± 8.04 %, COM early = 104.97 ± 7.89 %, $p < 0.05$), (ISO late = 152.46 ± 15.37 %, COM late = 111.24 ± 5.15 %, $p < 0.01$) (**Fig. 4.13**). This difference continues through the end of the 60 minutes of washout for both components (ISO early = 135.81 ± 9.07 %, COM early = 98.45 ± 8.9 %, $p < 0.01$), (ISO late = 147.13 ± 18.14 %, COM late = 104.99 ± 5.76 %, $p < 0.05$). Additionally, during washout, both ISO early and late components were increased over baseline values for all time points (Wilcoxon, $p < 0.05$) while COM EPSP components were not (Wilcoxon, $p > 0.05$). These data suggest a difference between socially isolated and communal animals, where the enhancement seen in isolates continues through washout, while changes that occur in the communal animals, are generally reversed over the course of the washout. This result is consistent with other crayfish work where socially isolated animals were more resistant to washout after serotonin exposure than animals with recent agonistic encounters (Yeh et al., 1996). It may seem contradictory that the previous experiments displayed increased excitability in COM preparations over ISO preparations, but it is important to note that the ISO preparations were most sensitive to this EtOH concentration (100 mM) while COMs were more affected by a lower EtOH concentration (4.1.).

Figure 4.13: Percent enhancement (mean \pm SE) over baseline of LG EPSPs between communal and isolate preparations between early (A) and late (B) EPSP components. Preparations were exposed to 5 mL/min of 100 mM EtOH following a saline baseline. All preparations were then washed out for 60 minutes (Washout). * indicates values that were significantly different ($p < 0.05$) through MWU non-parametric test. ** indicates significant difference with a $p < 0.01$ between social groups.



Chapter Discussion

The data collected from these experiments leads me to believe that the existence or absence of social exposure plays a key role in alcohol sensitivity in *Procambarus clarkii*. It is known that LG circuitry in crayfish with different social experiences respond differently to serotonin exposure (Yeh et al., 1996). In this study, Yeh et al. observed socially isolated animals displaying significant and prolonged enhancement when exposed to serotonin. In socially dominant animals, some enhancement was observed as well, but socially subordinate animals were inhibited. It is believed that these social differences are based on varied distribution of serotonin receptors in the LG circuit (Yeh et al., 1996). The interaction between social experience and alcohol has also been explored in other model systems. Humans have been shown to increase alcohol consumption based on the social environment in which they are imbibing (Collins & Marlatt, 1981; Collins et al., 1985). Additionally, social anxiety itself has been shown to increase consumption. In mice, subordinate mice that had recently experienced a defeat to a dominant cage-mate were shown to consume more alcohol than dominant animals (Hilikivi-Clarke & Lister, 1992). Because crayfish are believed to form linear dominance hierarchies (Bovbjerg, 1953), crayfish in groups are likely to have experienced being both dominant and subordinate while socially isolated animals may lose this communality. While it is still unclear exactly what changes are occurring between socially experienced and inexperienced animals, altered receptor distribution is a likely candidate for the differences observed in our current data. The simple nervous system of this model also allows a more detailed investigation of these differences in the future.

I found, when repeating implanted electrode experiments using intracellular electrophysiology, that superfused EtOH decreases the activation threshold of the LG and that this effect is concentration dependent with higher EtOH concentrations typically inducing more preparations to activate. Additionally, when altering the recent social experience of the animal, I found that animals removed from a communal environment and placed in social isolation showed a decreased sensitivity to the effects of EtOH when compared to communal animals. This is evidenced by communal preparations spiking significantly more in lower concentrations compared to isolates. This difference was negated with higher doses of EtOH superfusion. This suggests a saturation of disinhibition (and possibly inhibition) in communal animals at the high dose, and a higher concentration needed to evoke similar changes of excitability in socially isolated preparations.

Variables that have been shown in the past to positively influence sensitivity to alcohol include increased alcohol synthesis of the neuro-steroid $3\alpha,5\alpha$ -TH PROG (Morrow et al., 2001) and disruption and antagonism of CB1 receptor systems (Naassila et al., 2004). Existing evidence suggests that stress to mammalian systems brought on by social isolation and isolated rearing can cause changes to these variables that could potentially effect an organism's sensitivity to some of the pharmacological effects of alcohol (Serra et al., 2003; Malone et al., 2008). While the results in these past experiments are the opposite of what I found in *Procambarus clarkii*, it is possible to attribute this contradictory result to the differing characteristics of the social behaviors in the model organisms. Social isolation would prove incredibly stressful to a species that has evolved to require communal

environments for normal behavioral development. However, in a model such as *Procambarus clarkii*, it is possible that removal from the constant struggle for dominance reduces stress compared to communal animals. Little to no data exists that would suggest that crayfish exhibit any social behaviors other than those of an agonistic nature in order to compete for resources and mating. Developing a method to measure stress in crayfish and a behavioral paradigm to simulate communal stress could be used to further investigate the possibility that communal animals express increased alcohol sensitivity as a result of increased stress by way of increased agonistic interaction compared to isolated individuals. Further investigation into these factors and their relationship between the inhibitory and dis-inhibitory behavioral effects of alcohol on crayfish may prove insightful.

While the EtOH concentration used in experiment 4.3 was selected to observe both the excitatory and inhibitory effects seen in the previously performed behavioral experiments, the use of a lower concentration may have shown a larger difference between the socially isolated and communal groups. In the 100 mM EtOH exposure, socially isolated and communal animals showed no significant difference between the two groups during the EtOH exposure, ISO preparations were more significantly enhanced throughout the washout as the late component of COM preparations was shown to wash out more readily than enhanced ISO preparations. Interestingly, when comparing levels of enhancement to their pre-EtOH baselines, ISO preparations were shown to be facilitated in both the early and late components while the COM preparations only showed significance in the late component. In ISO preparations, it took longer for preparations to display this significance in the early component than

in the late component. These results indicate a strong effect of EtOH on the GABAergic inhibition occurring after the β component. Additionally, the differences observed between the two social groups may be the result of different representation of GABA receptors in ISO preparations when compared to COM preparations.

Control trials in the semi-intact threshold experiments yielded a larger degree of fluctuation than expected with several preparations in both ISO and COM groups producing a high number of action potentials during the saline exposure. This certainly calls into question the notion that EtOH exposure was having significant effects on the LG activation threshold. While the use of a semi-intact preparation is more relevant to the previously described results from behavioral experiments, the presence of descending tonic inhibition from the rostral portion of the animal could be the reason for the fluctuation observed. As the experimental time increased, the level of tonic inhibition may have been changed from when activation threshold was first found, leading to changes in the threshold possibly independent of the effect of any superfused EtOH. For this reason, the threshold experiments were repeated in preparations where the tail and abdominal nervous system were isolated from the rest of the animal. Interestingly, while 20 mM EtOH was sufficient to drive excitation in the COM semi-intact group, this concentration had little effect in either the ISO or COM tail preparations. Instead, 100 mM was much more effective for both social groups implying that EtOH has a local effect on the LG circuit, which does not require the crayfish brain. While the number of preparations brought to threshold during EtOH was not different between COM and ISO groups, thresholds following EtOH were significantly reduced in COM preparations compared to saline-exposed

controls while ISO preparations were not different from their controls. Perhaps a concentration between 20 mM and 100 mM would display the same social differences observed in the semi-intact preparations. Because the isolated tail preparations eliminated descending tonic inhibition and this is the main difference between semi-intact preparations and isolated tail preparations, it stands to reason that tonic inhibition is a probable site of action for low EtOH concentrations in COMs. While not represented by statistical significance, COM preparations that were brought to threshold produced more action potentials on average than ISO preparations exposed to the same concentration. In summary, COMs were found to be more sensitive to EtOH than ISOs and descending inputs from the brain to the local LG circuit increases the sensitivity to EtOH.

Chapter 5: Alcohol and the Medial Giant Circuit

Summary

The previous chapter established the lateral giant (LG) as an effective model circuit for studying the physiological effects of EtOH exposure and how these effects can be modulated through changes in the recent social history of the animal. In this chapter, I begin exploring the other escape tail-flip circuit, the medial giant (MG). While the LG circuit is one of the best understood neural circuits in the animal kingdom, comparatively little is known regarding the MG. Because of its important role in crayfish intra-specific agonistic encounters and economic decision making, I was interested in the effects of alcohol on the MG circuit. Moreover, I wanted to know whether the EtOH-induced effects observed in the LG circuit would generalize to the other giant escape circuit in the crayfish. Thus, I measured EPSPs in the MG during 100 mM EtOH exposure in socially isolated preparations. I found that, like the LG, EtOH facilitates early and late excitatory post-synaptic potential (EPSP) components in the MG. Facilitation of the early component plateaus rapidly and remains elevated throughout the saline washout. Changes to the later EPSP component also occur rapidly, but the enhancement decreases as EtOH exposure and washout continue, which may indicate a biphasic effect with increasing EtOH concentrations.

Background

Unlike the lateral giant, the medial giant receives input from both mechanosensory neurons and visual neurons (Liden & Herberholz, 2008). Through the use of bath electrodes that can detect the activation of giant interneurons without touching the animal, and a setup that presents a threatening shadow stimulus to the crayfish during foraging behavior, it is known that the MG can be activated visually (Liden & Herberholz, 2008). In this experimental paradigm, the juvenile crayfish makes its way along a submerged narrow passage towards a food odor flow port. At a pre-determined location along the corridor, a moving shadow is passed over the crayfish, resulting in the animal either freezing in place or MG tail-flipping away from the food port back towards the starting compartment. Because of the incredibly large diameter of the MG neurons, action potentials in these cells generate enormous field potentials that electrodes in the bath can record without touching the animal (Herberholz et al., 2001). The circuitry controlling the freezing behavior is not currently known. Later work showed that varying the concentration of the food odor stimulus, thereby changing the perceived value of the food to the animal, changes the number of freezes in relation to tail-flips (Liden et al., 2010). Higher food values reduced the number of animals that tail-flipped in response to the shadow stimulus, suggesting that the animals make an economic decision based on the perceived value of the food stimuli. The more valuable the food source, the more advantageous it would be to stay closer to the food and so the animal freezes rather than tail-flips. It is believed that the tail-flip is the less risky option of the two, but takes the animal further from the food source so that the degree of risk vs. reward is weighed in order

to select the behavior performed. Additionally, holding the amount of the food constant and elevating the hunger-state of the animal also changes the value of the food, and it leads to a decrease in the probability of initiating a tail-flip (Herberholz et al., unpublished). Taken together, the MG circuit is a well-suited model for testing the effects of EtOH as the established behavioral experiments offer an opportunity to investigate the interaction of EtOH and risk-taking behavior.

Behavioral Characteristics

The MG circuit is activated by a threatening visual stimulus or a phasic mechanosensory stimulus to the head or thorax. This tail-flip sends the crayfish directly backwards away from this stimulus. As described in chapter 2 of this dissertation, the behavioral output of the MG tail-flip is due to MoG activation in each abdominal ganglia, leading to phasic flexor muscle contraction throughout the entire tail.

Medial Giant Anatomy and Physiology

While the physiological workings of the MG are not as well-understood as the LG, some structural comparisons between the two giant interneurons can be made. The MGs, which are electrically coupled to each other, have only a single pair of cell bodies located in the supraesophageal ganglion (brain) (Wiersma, 1947; Wine & Krasne, 1972). Activation of one MG in the brain connective through intracellular current injection typically activates the other MG, resulting in a pair of spikes that descend from the brain. The MG axons consist of two caudally projecting axons, unbroken by the presence of septate junctions (Wiersma, 1947; Wine & Krasne,

1972). The two axons project to the terminal abdominal ganglion (A6), and activate motor giants (MoGs) in all six abdominal ganglia (Wine, 1984). In contrast, the LGs have their cell bodies in each of the six abdominal ganglia of the crayfish ventral nerve cord (VNC) (Edwards et al., 1999). Structurally, each LG is comprised of a series of electrically coupled axons, connected through specialized gap junctions, called septate junctions. Each cell body receives excitatory mechanosensory input from sensory nerves at each ganglion. In each of these abdominal ganglia, LGs are also electrically coupled and pass electrical current between the ipsilateral and contralateral LG (Antonsen & Edwards, 2003).

While the location of the MG cell bodies and their projections are known, much of the physiology remains unexplored. The specifics of the MG sensory inputs are still unclear, but it has been shown that the MG receives excitatory input from the antenna II (Glantz & Viancour, 1983). Electrical and chemical synapses are an important part of the LG EPSP through primary afferent input and interneurons, but the types of inputs that make up the MG EPSP are unknown, but are believed to be more chemical than electrical (Glantz & Viancour, 1983). Additionally, the GABAergic inhibition of the LG circuit is fairly well-understood, but inhibition of the MG has not previously been studied. This dearth of information includes whether GABAergic inhibition is even present in the MG circuit. Because of the behavioral and functional similarities, the MG likely contains similar physiological and neurochemical properties as the LG, but this has not been investigated previously and will be presented in my dissertation for the first time.

Role in Economic Choice in Crayfish

The MG circuit has significant behavioral relevance for both social (Edwards et al., 2003) and value-based decision-making (Liden et al., 2010) and could provide an avenue to study the effects of alcohol on these two types of behaviors. In a behavioral paradigm where *Procambarus clarkii* are exposed to a threatening visual stimuli while walking towards a food odor at the opposite end of the tank, animals typically perform one of two behavioral outputs, freezing in place or activating a MG tail-flip away from the shadow (Liden & Herberholz, 2008; Liden et al., 2010). In this situation it is believed that tail-flipping is the most risk-averse behavior, but it adds critical distance between the animal and the food source. When the concentration of the food odor is increased, and therefore its perceived value, more crayfish froze in response to the shadow stimulus, implying the ability of these animals to make value-based decisions (Liden et al., 2010). Because inhibition is a significant component of the other giant-mediated tail-flip, the lateral giant (Roberts, 1968; Vu and Krasne, 1993; Vu et al., 1993), inhibition is a prime suspect for regulating MG activation threshold, which could be the underlying mechanism for selecting between tail-flip or freezing behaviors. The research performed here will inform future studies of the role of EtOH in decision making.

Role in Crayfish Agonistic Behavior

At the conclusion of an agonistic encounter, one animal will activate an escape, sometimes an MG tail-flip, subordinating itself to the other crayfish (Herberholz et al., 2001; Edwards et al., 2003). In Herberholz et al. (2001), agonistic and tail-flip behaviors were recorded using video and bath electrode

electrophysiology recording, respectively. Because of the size of the giant neurons involved in LG and MG tail-flips, the electrical field potential can be recorded through electrodes placed in the water without actually touching the animals. In this study, it was found that animals that had subordinated themselves after engaging in an agonistic encounter with another paired animal showed a significant increase in the occurrence of MG tail-flips while LG tail-flips were unaffected (Herberholz et al., 2001). Importantly, the authors indicated that many of these MG tail-flips occurring in the new subordinate were performed to the mere presence of the dominant and that subordinates had lower MG thresholds than dominants. These results indicate that the MG can play an important role in crayfish aggressive behavior. Perhaps altering patterns of MG inhibition could affect MG threshold and therefore the outcome of agonistic encounters and formation of dominance relationships in crayfish. Because GABA is a target for EtOH and may be implicated in modulating MG activity, this circuit may serve as a model to study the mechanisms underlying the interaction of EtOH and crayfish social behavior.

Experiment 5.1: Influence of Alcohol on MG EPSPs

In the previous chapter, I have shown that the well-understood LG tail-flip circuit is physiologically sensitive to superfusion of EtOH (Ch. 4). While the LG was exposed to three different concentrations, 100 mM was selected for use on the MG as it had the largest amplitude change on LG EPSPs in socially isolated preparations. All animals were socially isolated for 7-10 days prior to testing. Because it is difficult to bring the MG to activation threshold through the stimulation of the antenna II nerve,

experiments performed in the MG focused on the effect of EtOH on MG excitatory post-synaptic potentials (EPSPs). While action potentials in these command neurons may have more behavioral significance as one spike would lead to a discrete behavior, facilitation or inhibition of the EPSP in these neurons should change the probability of producing an action potential. The effect of EtOH on the MG EPSP will be measured in this chapter and the mechanisms will be further studied in the next chapters (chapter 6 & 7) through investigation of the physiological properties and GABAergic inputs to the circuit and by manipulating EtOH's effect through the use of a GABA agonist.

Methods

Socially isolated animals ($N = 8$, 3.48 ± 0.23 cm body length) were surgically prepared for intracellular recording of the MG neurons. Crayfish were socially isolated for 7-10 days prior to surgical preparation. Animals were chilled on ice for 15 minutes and pinned ventral side up in a sylgard lined dish with a rectangular trough down the center of the dish for the animal's head, thorax, and abdomen to be placed. Pins were inserted into the telson (tail-fan), thorax, and antennal scales to secure the animal in place. Ventral cuticle was removed from the abdomen in order to expose the ventral nerve cord (VNC) of the abdomen and sever motor nerves to the abdominal ganglia in order to reduce the amount of twitch induced by activation of the giant interneurons. Cuticle rostral to the mandibles was removed and the green glands were extracted to expose the brain connectives (BC) where the impalement of the MG is performed (**Fig. 5.1**). While the MG can be accessed in the abdomen, it receives excitatory sensory input from the thorax and head, which necessitates

recording near the spike initiation zone of the MG to measure changes to the EPSPs. The antennal II nerve was exposed by removing a rectangular piece of cuticle from the basal segment of the 2nd antenna. An extracellular electrode was then placed on the ipsilateral antenna II nerve through this opening. Contact with the nerve was verified through observation of spontaneous action potentials and mechanosensory-sensitive spikes.

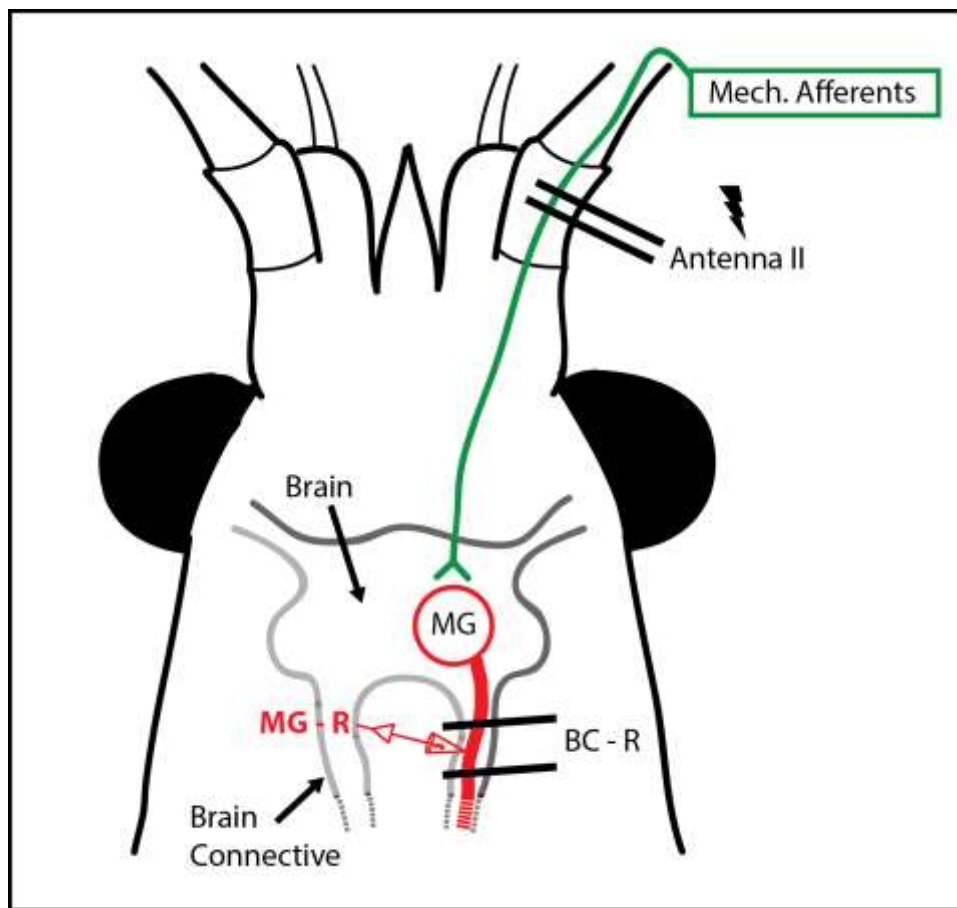


Figure 5.1: *Circuit diagram and recording paradigm.* Crayfish supraesophageal ganglion (brain) was exposed from the ventral surface of the head. Extracellular hook electrodes (2 parallel black lines) were used to record activity of the brain

connective (BC- R) and to stimulate the antenna II nerve (lightning bolt) to evoke an EPSP in the MG neuron. The MG neuron was recorded using sharp intracellular micropipette electrodes (MG – R) placed into the brain connective ipsilateral to the stimulated antenna II nerve. The size of the crayfish nervous system is exaggerated compared to the crayfish head.

Excitatory post-synaptic potentials (EPSPs) were elicited through electrical stimulation of the antenna II nerve ipsilateral to the impaled MG interneuron. 100% Ethyl alcohol (EtOH) was dissolved in normal crayfish saline to create a 100 mM (4.6 g/L) EtOH solution to be superfused for 60 minutes. All preparations were exposed to a baseline with superfusion of normal crayfish saline, followed by 60 minutes of 100 mM EtOH superfusion. Finally, all preparations received saline washout for at least 60 minutes. EPSP amplitudes were quantified and normalized in accordance with the methods described in Chapter 3 (**Fig. 5.2**). Averages of EPSPs in response to three stimuli were recorded every 5 minutes during drug exposure resulting in 12 data points over the course of a 60 minute period. However, the bulk of the analysis was focused on key points during exposure. While the amplitude for each of these averaged EPSP sweeps was recorded at 12 different times after the stimulus, analysis was focused on the EPSP component occurring 3 ms after the stimulus (referred to as early) and 6 ms after the stimulus (referred to as late). The early component is generally the first peak of the EPSP and the late component is located well after the peak. In the LG, the early EPSP component is associated with the peak of the β component, which is excitatory, while the late EPSP component is associated with

postexcitatory inhibition (PEI). In some MG preparations a second peak can be observed after the late EPSP component that can reach amplitudes almost as high as the early EPSP. Additionally, a second group of animals ($N = 5$, 3.6 ± 0.16 cm) was exposed to 90 minutes of normal crayfish saline to observe the EPSP fluctuations that might occur over an extended period of saline exposure. These controls were designed to ensure that physiological changes observed in the EtOH-exposed preparations were due to the EtOH and not a natural response to superfusion. The two groups are not statistically different in size when compared using a MWU test ($p = 0.27$).

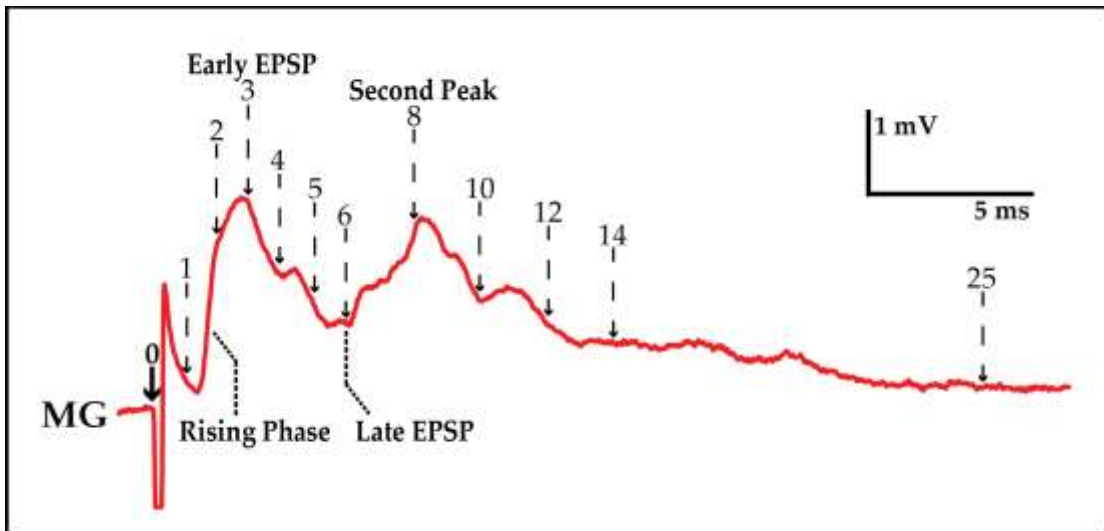


Fig. 5.2: Sample MG excitatory post-synaptic potential (EPSP) with labeled time points. MG EPSP elicited through electrical stimulation of the antenna II nerve. Numbers above EPSP waveform represent latency (in ms) from the stimulus (0 timepoint). Early (3 ms after the stimulus) and late (6 ms after the stimulus) EPSP components were used for analysis in all MG chapters.

All animals used were isolated for 7-10 days before surgical preparation in accordance with the isolated housing condition described in Chapter 3. Additionally, 100 mM EtOH concentration was selected as it had the strongest effect on socially isolated LG preparations. Numerous comparisons were made in this chapter comparing the effect of EtOH on the MG with those results on LG discussed in Chapter 4. Additionally, the effects of EtOH can be compared to the effects of normal crayfish saline. When possible, these comparisons were done using statistical tests such as the MWU non-parametric test for statistical significance between independent groups when comparing to other data sets, and a Wilcoxon signed-rank test for dependent groups. As indicated in the general methods, these statistical tests were performed using SPSS (Version 21).

Results

To investigate the effect of EtOH on the MG interneuron, I first repeated experiment 4.3, exposing the MG EPSP to 100 mM EtOH. Similar to the LG circuit, EtOH exposure dramatically increased EPSP amplitudes during the 60 minutes of exposure (**Fig. 5.3**). By measuring the averaged EPSP amplitude of three electrophysiology sweeps every 5 minutes, a progression of the observed facilitation can be seen. By the 30th minute, the amplitude of the early MG EPSP component has plateaued in most preparations with average facilitations of 159.62 ± 15.52 % of baseline (mean \pm SE) (**Fig. 5.4**). Late EPSP amplitudes also plateau quickly with an average amplitude of 183.22 ± 32.87 % of baseline after 30 minutes of exposure. Amplitudes recorded 60 minutes into EtOH exposure were 155.63 ± 16.77 % and

166.86 ± 38.16 % in early and late components, respectively. While the later component typically shows higher amounts of facilitation, this effect appears to be much more variable across the different preparations. Although the variability of the late component of the MG EPSP is high, statistical comparison of the EPSP amplitude during EtOH exposure is significantly different from baseline values (Wilcoxon signed-rank test: $p < 0.05$), but only in 6 of 12 time points during EtOH exposure. In contrast, because of lower variability in the early EPSP component the enhancement is significantly different from baseline in all 12 recorded EtOH time points (Wilcoxon signed-rank test: $p < 0.05$).

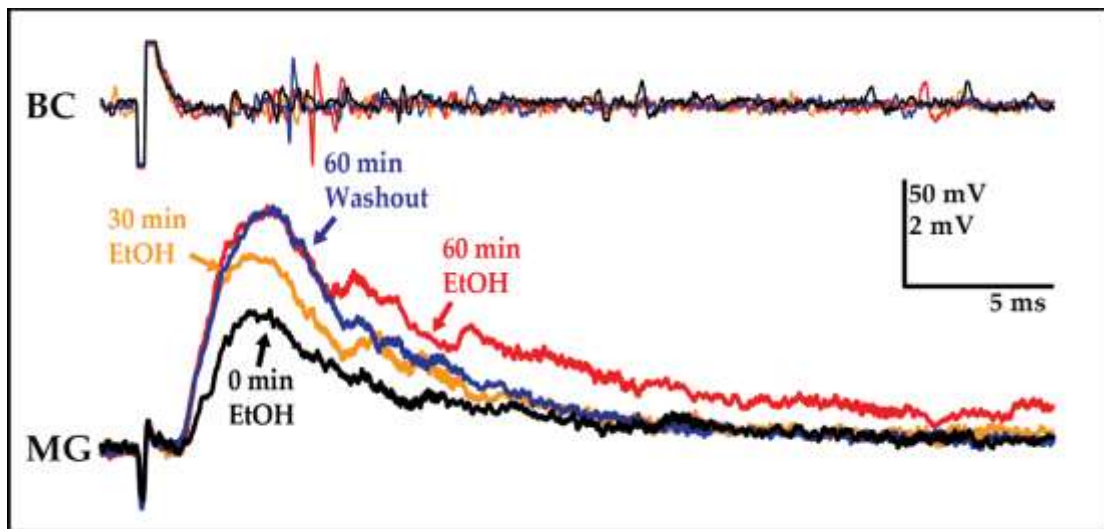
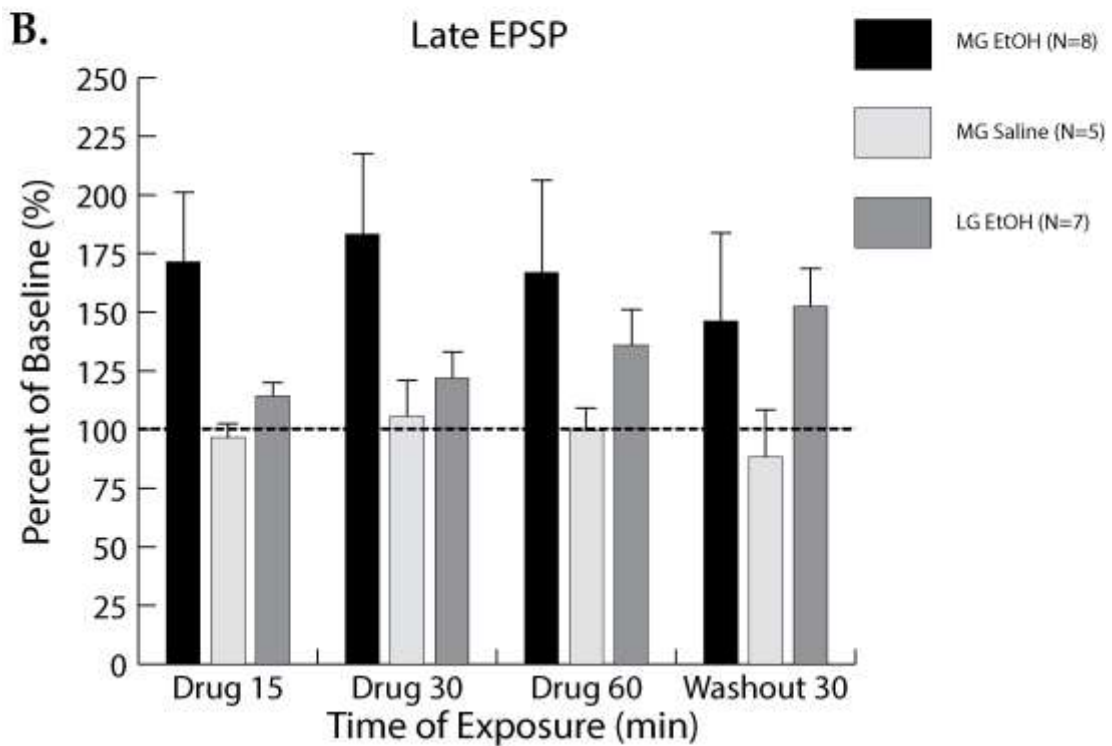
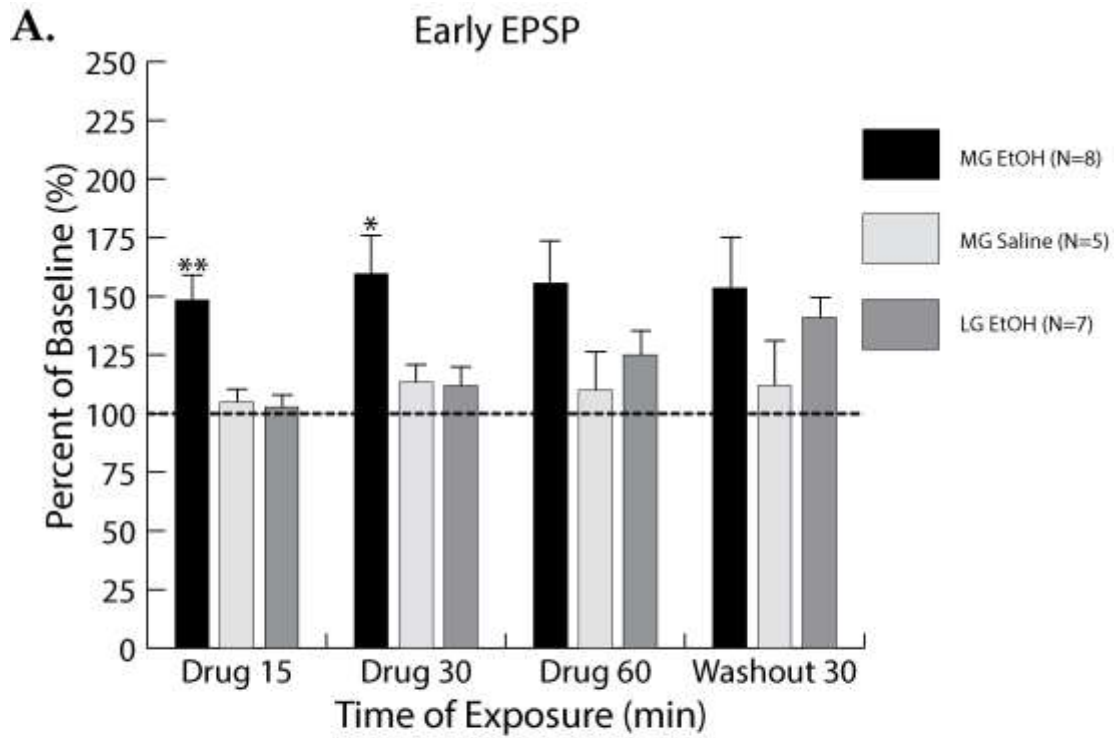


Fig. 5.3: 100 mM EtOH produces enhancement of both early and late EPSP components that persist through washout. Example trace of various averaged EPSPs recorded after exposure to 100 mM EtOH for 30 minutes (orange sweep) and 60 minutes (red sweep). All preparations received saline washout (blue sweep) for 60 minutes. Brain connective (BC) recording was placed on the ipsilateral brain connective to the stimulated and recorded MG neuron. EPSPs were generated using

a consistent stimulating voltage applied to the ipsilateral antenna II nerve. The facilitation of 100 mM EtOH is persistent throughout EtOH exposure and through washout in some preparations.

Fig. 5.4: Comparison of average enhancements (mean \pm SE) of the LG and MG to EtOH as well as the effect of normal crayfish superfusion on the MG. Changes in EPSP amplitude over time during exposure of 100 mM EtOH on MG and LG preparations. Additionally, the effect of normal crayfish saline superfused for 90 minutes is indicated by the MG Saline group. The MG saline group was not exposed to EtOH during the experiment. Graphs represent changes in EPSP amplitude of early (A) and late (B) EPSP components. Statistical significance of the MG EtOH group is indicated by * to indicate $p < 0.05$ using a Kruskal-Wallis non-parametric test. ** indicates $p < 0.01$.



Superfusion of normal crayfish saline (N = 5) produces no significant enhancement of the early or late component of the MG EPSP over its preceding baseline values even when that superfusion lasts for 90 minutes (Wilcoxon signed-rank test: $p > 0.05$). While EtOH produces large increases to both components, normal crayfish saline shows little effect by comparison. Thirty minutes of saline superfusion resulted in average amplitudes of 113.51 ± 6.82 % for the early component and 105.65 ± 14.76 % for the late component. Nothing changes after 60 minutes of saline exposure (early = 110.05 ± 15.41 %, late = 99.5 ± 9.42 %). When compared to saline exposure on the MG using a MWU test, EtOH exposure is significantly different in 7 of 12 time points during EtOH exposure ($p < 0.05$) for the early EPSP component. The late component, while more variable, is significantly different from late EPSP components measured during saline exposure in 4 of 12 time points (MWU: $p < 0.05$).

As can be seen in figure 5.4, the EPSP remains facilitated throughout the duration of the saline washout. This enhancement can sometimes even be higher than the final EtOH amplitudes. This was not the case with all preparations as some were brought back closer to baseline. Some even returned below their starting values. After 30 minutes of saline washout, the average amplitude of the early component was 153.44 ± 20.8 % of baseline. The effect of EtOH on the late EPSP component in these preparations was also difficult to wash out, with average amplitudes after 30 minutes of 146.22 ± 35.93 % of baseline. Even after 60 minutes of washout, both EPSP components were largely unchanged with average amplitudes of 154.86 ± 16.9 % and 130.33 ± 27.56 % of baseline, respectively. This persistence was not present in all

preparations, with some over 200 % of baseline while others near 50 % of their starting values (**Fig. 5.4**). These results indicate varying effects of EtOH on MG preparations similar to semi-intact LG preparations, and this occurred despite controlling for social experience.

It may be most informative to interpret the comparison between MG and LG response more in relation to the pattern of changes in the preparations rather than the difference in the numerical enhancement itself. Comparing the patterns of EtOH-induced enhancement between the two escape circuits indicates that for both early and late components of the EPSP, the MG experiences facilitation early on that quickly plateaus while the LG undergoes a more gradual increase in both 3 ms (β) and 6 ms (PEI) components with the region governed by PEI experiencing the largest increase relative to baseline.

Chapter Discussion

The comparison between the effect of EtOH on the MG and the LG yields some interesting differences. While the LG is facilitated gradually through the EtOH superfusion in both early and late components of socially isolated animals, the MG is more quickly facilitated to a certain level of enhancement where it remains for the duration of EtOH exposure. MG EPSPs showed no significant increase between the EPSP amplitudes at 60 minutes and 30 minutes, indicating the saturation of the EtOH effect for these evoked EPSPs halfway through the exposure period. The two preparations have similar amplitudes during washout, however, as the EtOH-induced MG facilitation appears to decrease in both early and late components while LG

preparations continue to be facilitated through a steady increase while washout continues. When comparing the effect of EtOH on the MG with the effect of normal crayfish saline, it is clear that EtOH produces a significant enhancing effect compared to normal fluctuations that occur during saline superfusion. It may be expected that saline superfusion should result in no changes to the EPSP, but the fluctuations observed may be the result of adjustments to the level of inhibition present in the MG. Although MG inhibition has not been explored before, similar inhibition to that which is present in the LG may change over time. In experiment 4.2, I found that semi-intact LG preparations were more sensitive to EtOH than preparations where the abdomen was isolated from the head and thorax. This isolation removed descending inputs from the brain. It is important to note that the MG preparations are semi-intact and inputs from the brain could also regulate MG excitability as they do in the LG.

Because of the superfusion flow rate used, it is believed that the EtOH concentration in the dish reaches approximately 95% of the 100 mM EtOH concentration after 30 minutes. The concentration then experiences little change for the rest of the exposure and this appears to be reflected in the lack of change after 30 minutes of EtOH exposure. However, why would this plateau effect not be present in the LG circuit? There are a number of possible explanations for the difference in effect between the LG and MG in this EtOH superfusion paradigm. Fundamentally, these differences are likely to be due to differences in receptor distributions and physiological mechanisms between the two circuits. While I believe the effect observed in the LG is likely due to EtOH-induced alterations of GABAergic mechanisms, it is premature to make the same assertions for the MG circuit.

GABAergic inhibition of the LG circuit is well-described through evidence indicating the presence of descending tonic inhibition that alters firing threshold (Vu and Krasne, 1993; Vu et al., 1993), postexcitatory inhibition that narrows the peak of the β EPSP component increasing the preference of the LG for extremely phasic stimuli (Vu et al., 1997), and recurrent inhibition that prevents subsequent LG activations (Roberts, 1968). The MG mediated tail-flip shares many behavioral similarities with the LG tail-flip in that they are both reflexive, respond only to phasic stimuli, and activate only once in order to generate a motor response. It therefore seems likely that the MG also needs some mechanism to alter firing threshold to prevent unwanted escape behavior. Thus, it is reasonable to suggest that the MG may have similar inhibitory mechanisms in place. This may account for the similarities in the response to EtOH, but the observed differences suggest that these inhibitory characteristics may be mediated through other processes in the MG. For these reasons, it becomes necessary to further investigate the presence and characteristics of GABAergic inhibition of the MG.

Chapter 6: GABAergic Inhibition of the Medial Giant Escape Circuit

Summary

The two previous chapters have established both lateral giant (LG) and medial giant (MG) tail-flip circuits as being physiologically sensitive to EtOH exposure. Because GABA is considered a major target of alcohol's effect on the nervous system and is a major component of LG tail-flip circuitry, it also becomes a primary target for investigating its effects on the MG circuit. While the LG circuit is thoroughly understood and much is known regarding GABAergic inhibition, the inhibitory properties of the MG are mostly unexplored. The experiments performed in this chapter describe in detail, for the first time, the physiological and inhibitory properties of the MG neuron. This detailed understanding will play a major role in understanding the mechanisms by which EtOH exerts its effects on the MG (as described in the previous chapter) and will be guiding the experiment performed in the next chapter. Physiologically, it is known that the MG can be activated through both threatening tactile stimuli as well as visual stimuli (Liden & Herberholz, 2008; Liden et al., 2010, Liu & Herberholz 2010). Excitatory inputs can be generated through electrical stimulation of optic nerves and the antenna II nerve (Liu & Herberholz, 2010). While the antenna II nerve is thought to contain mostly mechanosensory afferents, a portion of the cells activated by an electrical stimulus are believed to be chemosensory. However, it is thought that the bulk of chemosensory inputs that may play an important role in value-based decision making originate in the

antennules, the smaller, more medial antennae (Liden et al., 2010). As discussed in the background to chapter 5, crayfish are capable of modulating the probability of initiating an MG escape response based on the external situation and the internal state of the animal, and this has been implicated in value-based decision making (Liden et al., 2010). The modulation of the MG escape response may be the result of adjustments to levels of tonic inhibition similar to mechanisms that are known to be present in the LG circuit (Vu & Krasne, 1993). Alterations of this inhibition, either through increases or decreases, would change the probability of activation of a tail-flip by changing the MG threshold, and thus biasing the animal towards different behavioral decisions.

In this chapter, I show for the first time that the MG EPSP appears to consist of both electrical and chemical EPSP components. This is evidenced by the habituation of one specific part of the EPSP while other parts appear resilient following high-frequency stimulation. I also show through experiments altering the MG resting membrane potential and the external concentration of chloride (Cl^-) ions that the region of the MG EPSP that follows the early excitatory component is mediated by Cl^- currents. These Cl^- currents are likely involved in GABAergic inhibition and may be indicative of a postexcitatory inhibitory (PEI) -like mechanism, a form of inhibition in the LG that occurs after the early EPSP component, sharpening the EPSP peak. In addition, I performed neuropharmacological experiments to further explore the GABAergic inhibitory properties of the MG circuit. Antenna II sensory inputs to MG were exposed to both a powerful GABA_A agonist and antagonist, muscimol and picrotoxin (PTX), respectively. I found that in many PTX preparations

and in some muscimol preparations, exposure increased early EPSP components. However, similar to the LG, PTX had stronger effects on the later MG EPSP components, but decreased both early and late components during saline washout. Muscimol was also found to decrease both early and late EPSP components during saline washout. Because these experiments raise the question of whether the locus of GABAergic action is pre-synaptic or post-synaptic, I performed additional experiments measuring input resistance during muscimol exposure. I found an increase in input resistance coinciding with preparations that displayed increased MG EPSP amplitudes and when the EPSP subsequently decreased during washout, the input resistance decreased slightly. Changes in the MG input resistance during increases and decreases to the EPSP amplitude indicate muscimol-induced effects directly on the MG. All of these experiments taken together indicate a significant GABAergic effect on the MG, mediated by chloride current, which primarily affects the later part of the MG EPSP, and suggests the presence of a similar postexcitatory inhibitory mechanism as seen in the LG circuit (Vu et al., 1997).

Background

Crayfish are able to produce rapid escape tail-flips that make use of one of two giant interneurons, the lateral giants (LGs) or the medial giants (MGs). The LGs have been the focus of behavioral and physiological research for decades, but the MGs are still poorly understood, despite an increasing understanding of their behavioral relevance in decision making paradigms (Liden et al., 2010). Inhibition plays an important role in regulating activation of the LGs through both phasic and

tonic GABAergic mechanisms. A better understanding of the mechanisms that regulate MG activation is an important step in determining the means by which decisions can be made on the neural circuit level to create one behavioral choice over another. For the first time, I show here the inhibitory mechanisms that regulate the MG escape circuit using intracellular physiology and neuropharmacology, and I am able to make comparisons to the existing literature on the inhibitory properties of the well-understood LG circuit.

GABAergic Inhibition in the crayfish

Because GABA_A channels in crayfish, much like GABA_A channels in vertebrates and mammals, are mediated by the flow of negatively-charged chloride (Cl⁻), manipulation of Cl⁻ concentrations would have large effects on any portions of the MG EPSP that are mediated by this type of inhibition. Because the internal concentration of Cl⁻ in crayfish neurons is much higher than the external concentration, activation of Cl⁻ currents produces a depolarization of the cell membrane (Wallin, 1966; Roberts, 1968). While the presence of GABA-mediated depolarizations is not very common (other than in the developing nervous system), in the crayfish it inhibits the cell through a number of different mechanisms, and it is believed to hold certain advantages over the more common hyperpolarizing inhibition. While hyperpolarizing inhibition may lead to post-inhibitory rebound, biasing the cell towards activation following inhibition, depolarizing inhibition is believed to lead to the inactivation of voltage-sensitive sodium currents, inhibiting the cell without the possibility of an excitatory rebound (Edwards, 1990).

Much of the work in crayfish investigating inhibition of tail-flip circuitry has been performed on the LG circuit. As described in chapter 2, the LG is known to have GABAergic depolarizing inhibition that immediately follows the earlier excitatory α and β EPSP components, and this has been called postexcitatory inhibition, PEI (Vu et al., 1997). As first shown in Vu et al., (1997), superfusion of the GABA_A channel blocker picrotoxin (PTX) preferentially enhances the EPSP component following the β component where this PEI occurs (**Fig. 6.1**). This inhibition biases the LG towards responding to highly phasic stimuli (Vu et al., 1997).

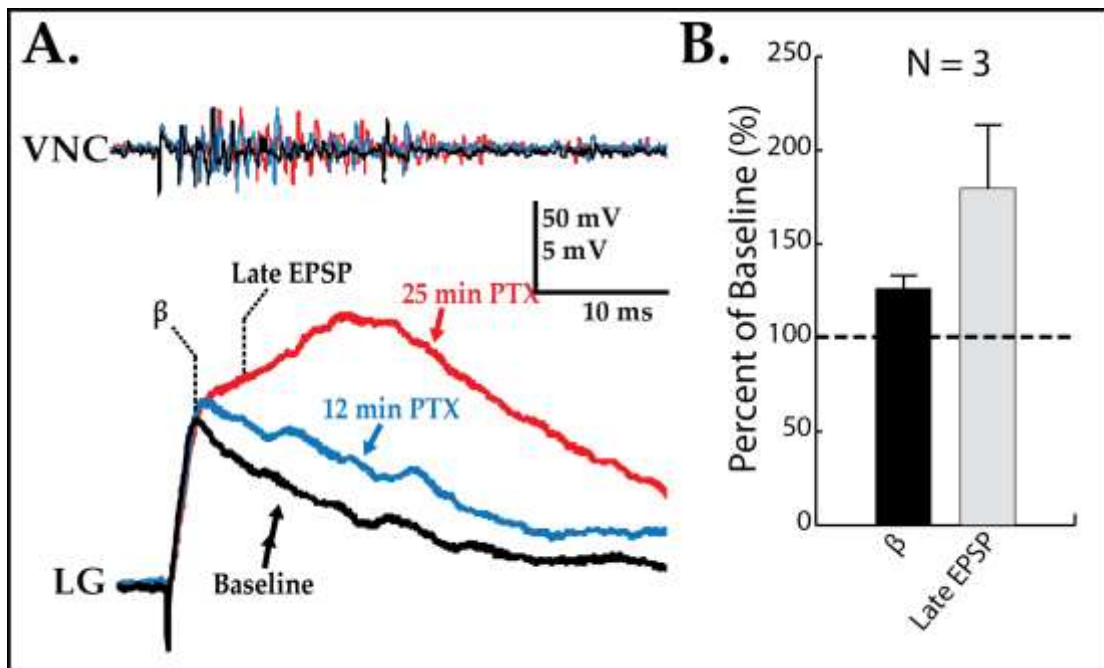


Fig. 6.1: Effect of 50 μ M picrotoxin on lateral giant (LG) EPSP. 50 μ M PTX superfusion facilitates electrically-evoked sensory afferent EPSPs of the LG. This facilitation is largely biased towards late EPSP components occurring after the α and β components that are known to be excitatory. This type of experiment was first

performed by Vu et al. (1997) and the repeated trials that I performed show similar effects.

Experiment 6.1: Habituation of Sensory Input of the MG

As discussed in the background for the crayfish lateral giant (LG) circuit, LG EPSPs consist of both electrical (α) and chemical (β) components that typically occur approximately 1 and 3 ms following an electrical stimulus to the sensory afferents of the last abdominal ganglion (A6). As shown in previous work, rapid stimulation reduces the β component of the EPSP due to a decrease of synaptic neurotransmitter at the chemical synapse between afferents and interneurons that connect to LG (Zucker, 1972). To test whether the MG EPSP experiences similar habituation, I exposed the antenna II nerve of two different MG preparations to 60 seconds of 10 Hz stimulation while recording MG EPSPs. It has previously been shown that an inter-stimulus interval (ISI) of 60 seconds is low enough to produce habituation of the LG, evidenced by decreased EPSP amplitudes (Araki & Nagayama, 2005; Nagayama & Araki, 2015). For this reason, an ISI of 0.1 seconds (10 Hz) should be highly effective in habituating the MG. Indeed, I found that the early EPSP component (occurring 3 ms after the stimulus) reduced in amplitude following 10 Hz stimulation suggesting the presence of habituation similar to the LG. Also, the rising phase of the EPSP that immediately precedes the early component was resistant to this stimulation and remained a constant amplitude throughout.

Methods

Juvenile crayfish ($N = 2$, 3.6 ± 0.14 cm body length) were socially isolated and surgically prepared in an identical fashion to those used in the previous chapter. All animals used were socially isolated for one week and fed one medium-sized shrimp pellet on the day of their isolation. Electrophysiological recordings were also performed in an identical manner with an intracellular electrode placed into the MG inside the brain connectives (BC). The BC is the descending decussating nerve cord from the supraesophageal ganglion (brain) to the subesophageal ganglion. The BC was also recorded extracellularly on the ipsilateral side of the MG intracellular electrode using a silver-wire hook electrode. EPSPs were generated in the MG through electrical stimulation of the ipsilateral antennal II nerve. In all cases, the intracellular electrode was confirmed to be located in the MG neuron by activating it through intracellular current injection. The MG action potential can be easily distinguished from other cells in the extracellular recording of the brain connectives due to it receiving antennal input and because of the amplitude of its extracellularly recorded action potential. Due to the immense diameter of the MGs in the brain connectives, the action potential amplitude measured extracellularly dwarfs all other interneuron potentials.

While the inter-stimulus interval (ISI) in previous experiments was 90 seconds, in order to measure habituation in this experiment, an ISI of 0.1 seconds was used. While the 90 second ISI is specifically used to avoid the possibility of habituation, this much decreased ISI is specifically designed to elicit habituation, indicating the presence of chemical synapses present in the MG EPSP. Experiments

were performed in normal crayfish saline and preparations were given at least 10 minutes to recover between bouts of stimulation.

Results

Before high frequency stimulation, some observations of the MG EPSP and comparisons to the LG EPSP can be made. Stimulation of the antenna II nerve produces EPSPs of considerably lower amplitude compared to those EPSPs in the LG. This is not surprising as the recording site in the brain connectives is further from the MG dendrites than in the LG recording paradigm, leading to smaller amplitude EPSPs. This may also indicate the presence of multiple synapses before sensory inputs make their way to the MG. The MG EPSP is characterized by a rising phase, occurring between 1 and 2 ms following the stimulus artifact (**Fig. 6.2**). This is in contrast to the LG, where the EPSP rises from baseline very rapidly (< 1 ms following the stimulus). This first rising phase is the α component and is mediated through electrical synapses from primary sensory afferents. In most MG preparations, a second rising phase occurs following the first, peaking in amplitude at 3 ms and maintains a stable amplitude for approximately 1 ms before beginning to decline. The LG also has a second rising phase which ends as the EPSP reaches its peak amplitude, termed the β , which is the result of chemical synapses from sensory interneurons. The amplitude of this early EPSP can be increased through increasing the magnitude of the stimulus. In many preparations, after the increase in voltage fails to evoke further increases in the early component, a second later peak sometimes appears. This peak typically occurs more than 6 ms after the stimulus artifact, yet is, at times, as large as the initial EPSP component (**Fig. 6.2**). In most of these preparations the electrical

stimulation of the antennal II nerve is generally insufficient to generate an MG action potential.

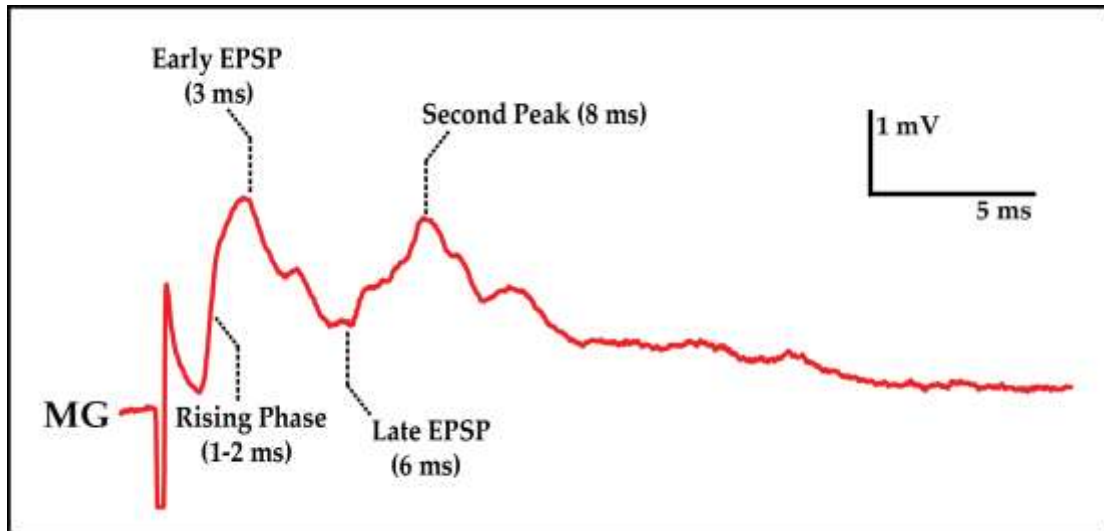


Fig. 6.2: Sample MG excitatory post-synaptic potential (EPSP) with labeled components. MG EPSP recorded in the MG axon located in the brain connectives. EPSP was elicited through electrical stimulation of the ipsilateral antenna II nerve. Rising phase occurs between 1-2 ms following the stimulus, Early (3 ms after the stimulus) and late (6 ms after the stimulus) EPSP components were used for analysis in all MG chapters.

Prolonged, frequent stimulation of the antennal sensory inputs to the MG indicates the presence of habituation-resistant components of the MG EPSP that occur approximately 2 ms following the stimulus. The early EPSP (3 ms) is reduced, which indicates that this EPSP component is likely evoked by chemical transmission in the input circuit. These results mirror those described in the LG in previous research where repetitive stimulation of the sensory afferents to the LG result in

decreased amplitude of the β component, implicating the involvement of habituation and chemical synapses in the LG circuit (Krasne, 1969; Wine et al., 1975). In preparations where the change between the baseline EPSP and the habituated EPSP were measured, the rising phase of the EPSP was reduced to approximately 78.27 ± 2.32 % of baseline (mean \pm SE) while the early component was reduced to 52.87 ± 7.18 % of baseline (**Fig. 6.3**). While these percent changes are measured in two different preparations only (each exhibiting three different bouts of stimulation), the low standard error values are indicative of the consistency of the response.

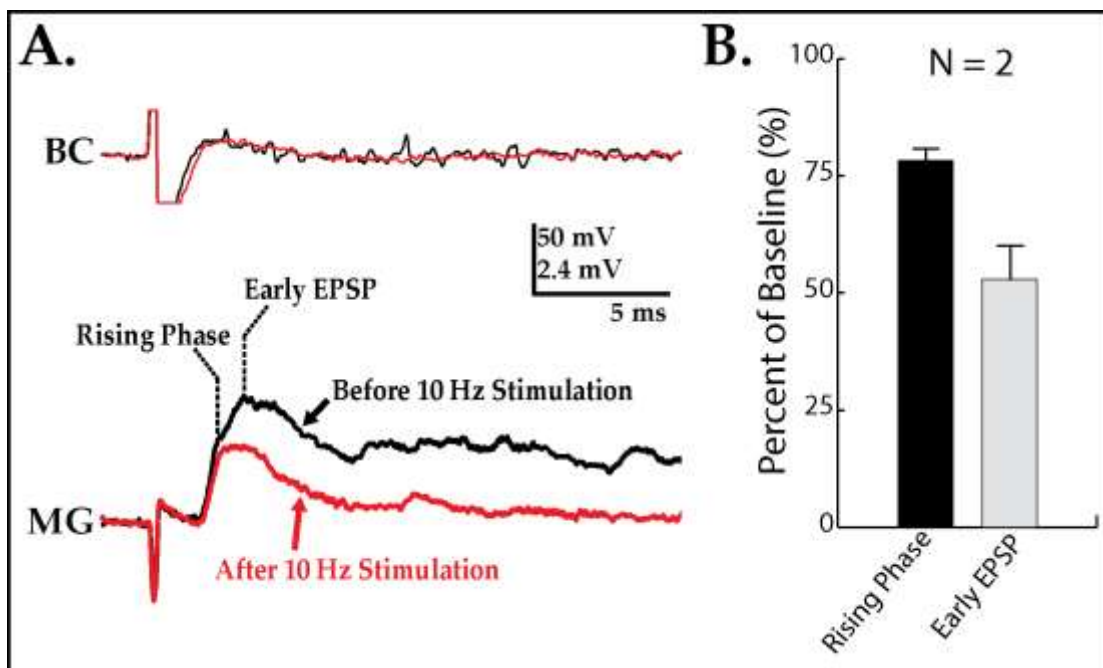


Fig. 6.3: Repeated stimulations reduce part of the MG EPSP while other portions remain unaffected. A) Electrical stimulation of the medial giant (MG) EPSP inputs with prolonged (60 sec. of 10 Hz) stimulation. Black sweep indicates the normal MG EPSP before the barrage of stimulations. Red sweep indicates the EPSP recorded following the 10 Hz stimulation. Extracellular recording of the ipsilateral brain

connective (BC) indicates a significant decrease in interneuron activity following after 10 Hz stimulations. B) Average amplitude as a percent of baseline of the rising phase and the early EPSP component following 60 seconds of 10 Hz stimulation.

Interestingly, the habituation-resistant EPSP component of the MG occurs later than might be predicted given that the excitatory component of the LG occurs approximately 1 ms following the stimulus. This is likely due to the increased distance of the recording electrode from the excitatory inputs received from the antennal stimulation. This distance results in a small delay of the observed rising phase of the MG EPSP.

Experiment 6.2: Modulation of MG Resting Membrane Potential

Experiment 6.1 from this chapter suggests the presence of both chemical and electrical synapses involved in the excitatory inputs from the antenna II nerve to the MG neuron. The following experiment continues the analysis of the MG EPSP through the use of two intracellular electrodes to depolarize the resting membrane potential (RMP) of the MG. This allows me to determine the presence of chloride (Cl^-)-mediated currents that indicate the presence of GABAergic inhibition of the MG. Because Cl^- is more highly concentrated inside the neurons of the crayfish nervous system, GABAergic inhibition working through Cl^- currents depolarizes the cell rather than hyperpolarizing it, which would be expected of “regular” types of GABAergic inhibition. The depolarization in crayfish is caused by the outward flow of negatively-charged Cl^- ions because the reversal potential of chloride is

depolarized from the resting membrane potentials of the MG and other crayfish neurons (Wallin, 1966; Roberts, 1968). When the membrane potential is depolarized from its normal value of approximately -70 - -80 mV closer to the reversal potential of Cl^- (approx. -55 mV), the outward flow of Cl^- ions when channels open is reduced as the electrostatic pressure has decreased. Depolarizing the membrane potential will also affect sodium (Na^+) currents, but to a much lesser degree as the Na^+ reversal potential is above 0 mV. I depolarized the MG RMP, which is typically around -80 mV, by at least 15 mV and compared the EPSP measured during this depolarization to an EPSP measured at RMP. I found that depolarization significantly affected regions of the MG EPSP that follow the early excitatory phase. This suggests the presence of Cl^- mediated currents after the early EPSP component, which may indicate a mechanism very similar to the postexcitatory inhibition (PEI) that has been described in the LG circuit (Vu et al., 1997).

Methods

Juvenile crayfish ($N = 3$, 3.2 ± 0.1 cm) were surgically prepared to expose the brain connectives near the crayfish brain in order to insert two intracellular electrodes into the MG neuron. Extracellular stimulating hook electrodes were placed on the antenna II nerve ipsilateral to the impaled MG and electrically stimulated to generate EPSPs. Electrophysiological recording were largely unchanged except for one important difference. Rather than one intracellular electrode inserted into the MG neuron, for this experiment I inserted two intracellular electrodes into the same MG in close vicinity. By inserting two electrodes into the same cell, I was able to depolarize the cell through injection of positive, sub-threshold current through one electrode.

With the other, I would observe the voltage change in the resting membrane potential and stimulate the sensory afferents of the antenna II nerve to elicit an EPSP while the MG is depolarized. Positive current required to shift the membrane potential ranged between 100 and 300 nA. The amount of current injection required was largely dependent on the distance between the two intracellular electrodes with greater distances requiring more current to cause the same membrane potential shift. I measured EPSP amplitudes of the early (3 ms after the stimulus) and late (6 ms after the stimulus) components before and after the membrane depolarization through current injection. Average changes in the EPSP amplitude for each animal was calculated from 1-3 stimuli with the EPSP at normal RMP and at RMP +15 mV. If a preparation received more than one stimuli, all measurements for that preparation were averaged together. Each preparation contributed one value to the average shown in the results below.

Results

Shifting the resting membrane potential of the MG (normally between -70 to -80 mV) by injecting positive current through one of the electrodes caused a reduction of both early (3 ms after the stimulus) and late (6 ms after the stimulus) EPSP components when the membrane was depolarized by at least 15 mV (**Fig. 6.4**). This depolarization resulted in membrane potentials of approximately -55 to -65 mV. The early component was measured as 58.9 ± 6.26 % (mean \pm SE) of the early component measured when the RMP was unaltered. Compared to the early component, membrane depolarization had a greater effect on the late component, decreasing it to 35.28 ± 18.89 % of the non-depolarized MG RMP. This result is expected if the late

component was mediated by larger amounts of Cl⁻ current when compared to other EPSP components. However, this result was not consistent across all preparations. One preparation showed reductions of the late component of only 30% while the other two preparations were more significantly reduced with average reductions over multiple measurements of more than 75% and 90%. These differences may be the result of different levels of inhibition present depending on the varying internal states between the preparations. Overall, these results suggest the importance of Cl⁻-mediated currents in the later component of the EPSP.

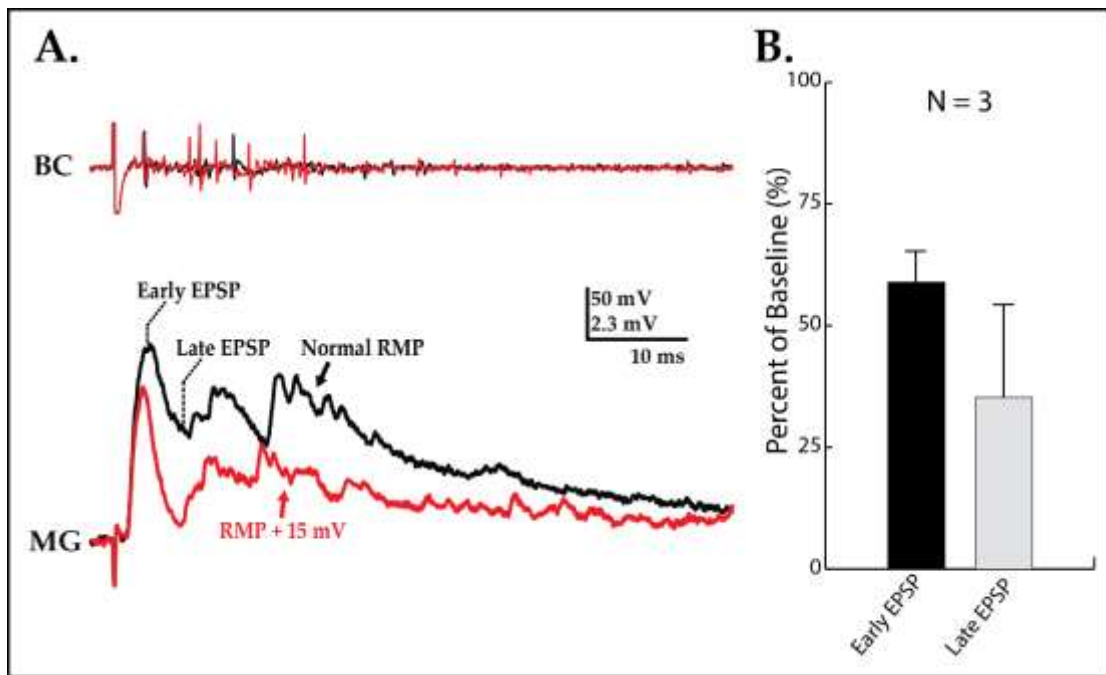


Fig. 6.4: Depolarization of MG resting membrane potential preferentially reduces late EPSP components. Injected 120 nA and depolarized by 15 mV near Cl⁻ reversal potential. A) Impalement of the MG with two intracellular electrodes allowed for the membrane depolarization of the MG by injecting 120 nA of current through one intracellular electrode and recording voltage changes with the other (MG).

Antennally-evoked EPSPs in the MG when the membrane is not depolarized (Black Sweep) compared to when the MG resting membrane potential is depolarized by 15 mV (Red Sweep). A 15 mV depolarization brings the MG resting membrane potential (RMP) close to chloride's reversal potential in the crayfish (-55 mV). Membrane depolarization decreases early and late components of the MG EPSP, but the effect on the late component is significantly higher indicating that the late MG EPSP component is mediated by Cl⁻ currents. B) Average amplitude of early and late EPSP in percent of baseline after depolarizing the MG resting membrane potential by 15 mV. Error bars represent standard error.

Experiment 6.3: Medial Giant and Low Chloride Saline

The previous experiment strongly suggest the presence of Cl⁻ currents that underlie the late (6 ms after the stimulus) component of the MG EPSP. Manipulating the concentration of chloride ions of the extracellular medium can also indicate the presence of Cl⁻ mediated currents (Le Bon-Jego & Cattaert, 2002) and in this case will serve to verify their presence before exploring whether these currents are created through GABAergic mechanisms in following experiments. Because movement of Cl⁻ across the membrane is most commonly associated with the opening of GABA_A channels, it can be reasoned that superfusion of a low Cl⁻ saline would cause significant changes to the MG EPSP if it undergoes GABAergic inhibition. Mechanistically, while depolarizing the membrane from the previous experiment reduces the electrostatic pressure for Cl⁻ ions to leave the cell when channels are opened, reducing the concentration of Cl⁻ in the extracellular medium would have the

opposite effect. Because there is a greater concentration of Cl^- ions inside the cell (Roberts, 1968), opening channels causes an efflux of ions. Reducing the extracellular concentration of Cl^- further increases Cl^- conductance. The result of this manipulation is that EPSP components that are partially mediated by Cl^- currents will be increased

Methods

Socially isolated animals ($N = 3$, 3.47 ± 0.47 cm) were surgically prepared to expose the brain connectives in the head of the crayfish. MG neurons were impaled using an intracellular electrode and verified by activating the MG through current injection. EPSPs were electrically evoked using extracellular hook electrodes placed on the antenna II nerve ipsilateral to the MG being recorded.

Normal crayfish saline was superfused for 10 minutes to record baseline amplitudes, then low Cl^- saline was added. For the drug exposure phase of the experiment, low- Cl^- saline was superfused into the dish for 15 minutes. Finally saline washout was performed for 60 minutes. Normal crayfish saline consists of concentrations in mM: 202 NaCl, 5.37 KCl, 13.53 CaCl_2 , 2.6 MgCl_2 , and 2.4 HEPES. To drastically reduce the chloride concentration in the saline, sodium propionate was used in substitution for NaCl, (Le Bon-Jego & Cattaert, 2002).

Because of the volatility of the low Cl^- saline, exposure times were often much lower than the usual 30 minutes used for other experiments. I found that because the effects were very rapid, most preparations could be switched from low- Cl^- saline to saline washout after 15 minutes while still displaying significant changes to the MG EPSP. In some cases after superfusion of low Cl^- saline, electrical

stimulation of the antenna II nerve failed to elicit any EPSP in the MG. At this point, saline washout was started and a normal EPSP returned during washout.

Results

To measure the contribution of chloride mediated currents to the MG EPSP, a low Cl^- saline solution was superfused into the dish with the semi-intact preparation following a baseline of normal saline. Consistently in each tested preparation ($N = 3$), the low Cl^- saline facilitated the MG EPSP. While both early (3 ms after the stimulus) and late (6 ms) components of the EPSP showed increases, the change to the later component is much greater than changes to the early component (**Fig. 6.5**). In all preparations, this change occurred rapidly within the first minutes after low Cl^- saline was added and suggests a large component of Cl^- mediated currents that contribute to both early and late EPSP components. In all preparations, washout with normal crayfish saline rapidly reversed the effects as low Cl^- saline and EPSPs returned to their normal pre-exposure values. Prolonged exposure to low Cl^- produced a complete lack of depolarization in one preparation when the antenna II nerve was stimulated. Cessation of the low Cl^- saline superfusion and returning to normal crayfish saline quickly reversed this effect. Because the crayfish is known to have a higher concentration of Cl^- inside the cell than outside, in a low Cl^- saline environment, activation of GABA_A channels is followed by a high outward flow of Cl^- , which depolarizes the cell. In cells where the internal Cl^- concentration is generally lower than the external concentration and activation of Cl^- channels leads to an influx, low Cl^- saline would have the opposite effect as seen here.

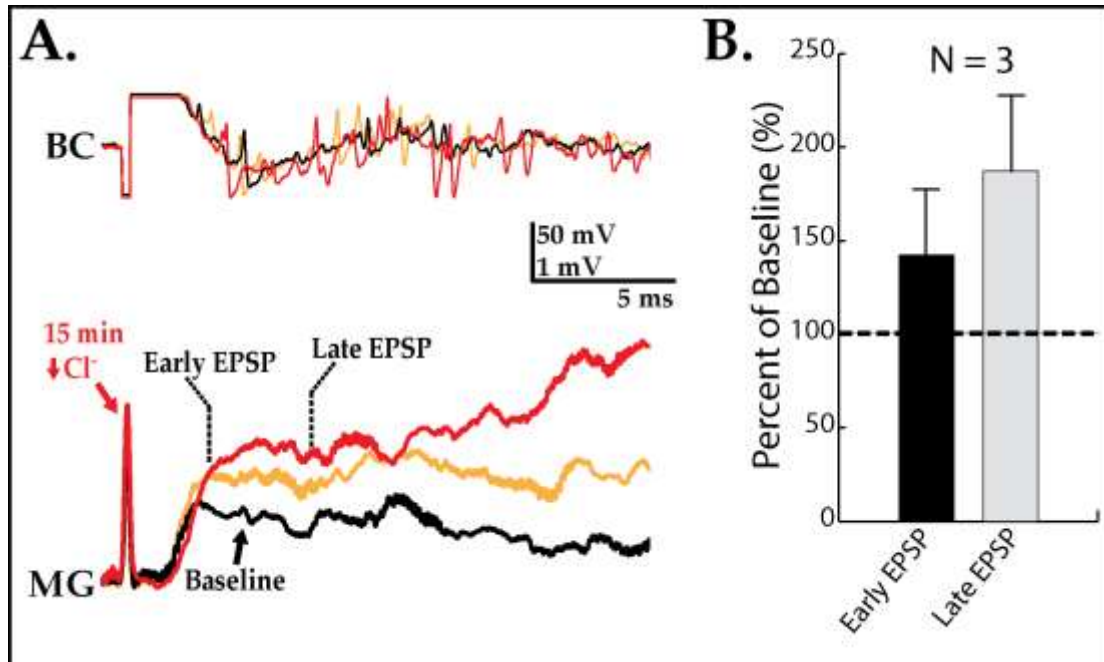


Fig. 6.5: Low Cl^- saline greatly increases late MG EPSP components. Exposing the MG EPSP to a saline solution with a low concentration of Cl^- significantly enhances the MG EPSP. NaCl present in normal saline in high quantities was substituted with sodium propionate ($\text{C}_3\text{H}_5\text{NaO}_2$). Much higher internal Cl^- concentration exacerbates outward Cl^- currents that are present during the later EPSP components. This efflux of Cl^- produces large depolarizations located after the early component. The effect of low Cl^- saline had a rapid onset and was quickly reversed (not pictured) during normal saline washout. A) Example electrophysiology trace of EPSP amplitude in normal crayfish saline (black sweep). Effects of low Cl^- saline can be seen after 7.5 minutes of exposure (orange sweep) and after 15 minutes of exposure (red sweep). B) Average amplitude of early (3 ms) and late (6 ms) EPSP as a percent of the baseline value after 15 minutes of low Cl^- saline.

While only three preparations were used for this experiment, the results were highly consistent and showed relatively little variation. The early MG EPSP component after 15 minutes were 142.27 ± 34.49 % of baseline (mean \pm SE). The late component displayed amplitudes of 187.19 ± 39.84 %. As can be seen in **Fig. 6.5**, the largest effects due to the low Cl⁻ saline superfusion appears to be on even later components of the EPSP. For example, the EPSP amplitude measured 14 ms after the stimulus had an average amplitude of 429.3 ± 80.7 % of baseline. The large effect observed may indicate that this component is primarily mediated through Cl⁻ currents.

Experiment 6.4: Medial Giant Responsivity to GABA_A Channel Blocker, Picrotoxin and GABA_A receptor agonist, Muscimol

So far in this chapter I have shown that the MG EPSP has a significant Cl⁻ mediated current following the early EPSP component. It is likely that this current is created by GABAergic inhibition that produces depolarizing inhibition after the early excitatory EPSP component. The next step in investigating the presence of GABAergic inhibition in the MG EPSP is to repeat previous experiments performed first in the LG by Vu et al., (1997), exposing the preparation to the GABA_A channel blocker, picrotoxin. Naturally, a significant effect of picrotoxin (PTX) on the MG EPSP would indicate the presence of the GABAergic inhibition in the MG circuit. PTX was shown to facilitate the LG EPSPs in the segment of the potential following the β component, indicating the presence of postexcitatory inhibition (PEI). PEI is believed to ensure the LG's responsivity to only the most phasic stimuli (Vu et al., 1997), excluding more "tonic" stimuli that is more likely to activate non-giant tail-flip

circuitry (Wine & Krasne, 1972). Before I exposed the MG to PTX, I repeated the Vu et al. (1997) experiments in the LG and found the same PEI (**Fig. 6.1**). I was also interested in the effect that a GABA agonist, muscimol would have on the MG circuit. This experiment, when combined with the PTX experiment could also indicate the presence of GABAergic inhibition in the MG.

Methods

Individual *Procambarus clarkii* were socially isolated in accordance with the general methods (see above) and exposed to either 25 μ M PTX dissolved in saline (N = 5, 3.38 ± 0.31 cm), 25 μ M muscimol (N = 6, 3.62 ± 0.16 cm), or normal crayfish saline (N = 5, 3.6 ± 0.16 cm). The MG in one of the brain connectives was impaled using micropipette electrodes as described in experiment 6.1 of this chapter and exposed to one of the three experiment conditions after establishing a baseline during saline superfusion. EPSPs were elicited through electrical stimulation of the antenna II nerve ipsilateral to the intracellular MG recording. Preparations that were exposed to either muscimol or picrotoxin received 30 minutes of drug exposure followed by 60 minutes of saline washout. The LG has been exposed to PTX in previous work by Eric Vu et al (1997) where it was shown to significantly increase late EPSP components, suggesting the presence of later inhibition arriving after primarily excitatory inputs. Vu et al. theorized the involvement of this postexcitatory inhibition (PEI) in aiding the LG's preference for highly phasic mechanosensory stimuli and to play a role in coincidence detection of sensory inputs. Efforts were initially made to expose the MG to the same concentration of PTX, but 50 μ M concentrations produced significant convulsions in MG preparations, which include the brain and are

more intact than tail preparations used in Vu et al.. Because of this, the concentration was reduced and all MG preparations were exposed to 25 μ M PTX. All samples included in the data received at least 60 minutes of normal crayfish saline washout following the PTX exposure. Only animals that still had measureable EPSP amplitudes at the end of the 60 minutes of washout were used in the data analysis. While picrotoxin (PTX) is known to be a powerful GABA_A channel blocker and has been used in the past to investigate inhibition in the crayfish (Roberts, 1968; Wine and Krasne, 1972; Vu et al., 1997), it is also known to bind to less common glutamatergic-mediated chloride channels (Lingle & Marder, 1981). For these reasons, the GABA_A receptor agonist, muscimol, was also used to further explore the influence of GABAergic inhibition in the MG neuron.

While most of the analysis focused on four specific time points of exposure (15 min drug exposure, 30 min drug exposure, 30 min washout, 60 min washout), average amplitudes were recorded for every 5 minutes of drug exposure and washout.

Results

As shown previously (Fig. 6.1, page 112), I repeated the experiment first performed by Vu et al. (1997), and measured the amplitude of the LG excitatory post-synaptic potential (EPSP) when it was exposed to PTX. As expected, PTX facilitated the amplitude of LG EPSPs with a particularly strong effect on EPSP components following the well-described α and β components that are known to result from stimulating sensory afferents to the LG. In the three LG semi-intact preparations, enhancement of the β component after 25 min of PTX application resulted in mean amplitudes of 126.28 ± 6.85 % (mean \pm SE) of baseline. Similar to results from Vu et

al. (1997), the amplitude of the late potential underwent larger facilitation with amplitudes of 179.73 ± 33.54 % of those recorded in baseline (**Fig. 6.1**). As described in Vu et al. (1997), these results suggest the presence of a postexcitatory inhibition mediated through ionotropic GABA_A channels that are activated following the primary components of the EPSP.

While the concentration used for the LG experiments was 50 μ M, this concentration led to significant convulsions that prevented stable intracellular recordings of the MG for the duration of the drug exposure. Therefore, the PTX concentration was halved, but results are compared to Vu et al. and my own LG experiments. The early and the late component of the MG EPSP activated through antennal II nerve stimulation underwent facilitation, with both components showing similar levels of enhancement (**Fig. 6.6**). Amplitudes measured at the early component after 15 minutes of exposure had average values of 115.73 ± 8.29 % of baseline (mean \pm SE) and late EPSP amplitudes were measured as 105.2 ± 9.24 % of baseline at the same time point of exposure (**Fig. 6.7**). In saline control groups, 15 minutes of exposure led to early EPSP amplitudes of 104.87 ± 5.12 % of baseline and late EPSP amplitudes of 96.57 ± 5.98 %. Using a MWU to test for significance indicates no elevation of PTX preparations over saline-exposed preparations ($p > 0.05$). PTX values recorded after 30 minutes of exposure (early: 118.79 ± 7.27 %, late: 122.48 ± 16.1 %) were also not different ($p > 0.05$) from saline controls (early: 113.51 ± 6.82 %, late: 105.65 ± 14.62 %). As can be seen in figure 6.7, values during washout decrease dramatically and are significantly different from saline controls. After 30 minutes of washout, PTX amplitudes (early: 73.83 ± 10.5 %, late: $58.18 \pm$

17.51 %), while decreased, were not significantly lower than saline controls (early: 110.05 ± 15.41 %, late: 99.5 ± 9.42 %) (MWU early: $p = 0.1$, late: $p = 0.15$). After 60 minutes of saline washout following PTX, early EPSP amplitudes were 47.4 ± 13.87 % of baseline and late EPSP amplitudes were 24.1 ± 10.34 % of baseline while values of saline control preparations remained largely unchanged (early = 111.86 ± 18.59 %, MWU $p < 0.05$; late = 88.32 ± 19.45 %, MWU $p < 0.05$). Typically, saline-exposed preparations ($N = 5$, 3.6 ± 0.16 cm) showed a slight elevation over baseline of the early EPSP component during the course of the superfusion. When tested for statistical significance between dependent groups using a Wilcoxon signed-rank test, none of the saline exposed controls were significantly different from baseline during any of the measured time points ($p > 0.05$).

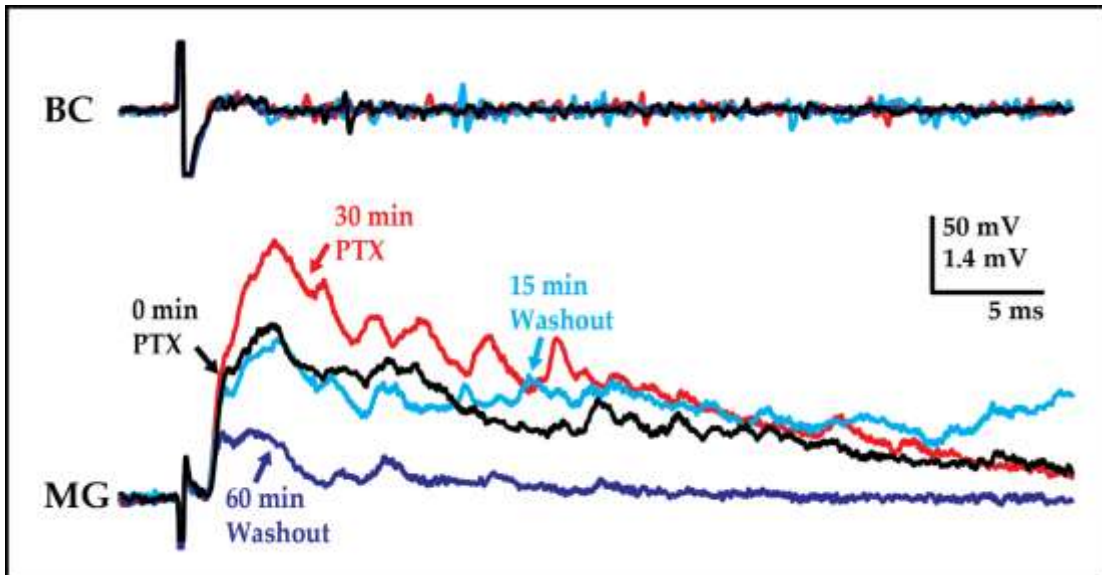
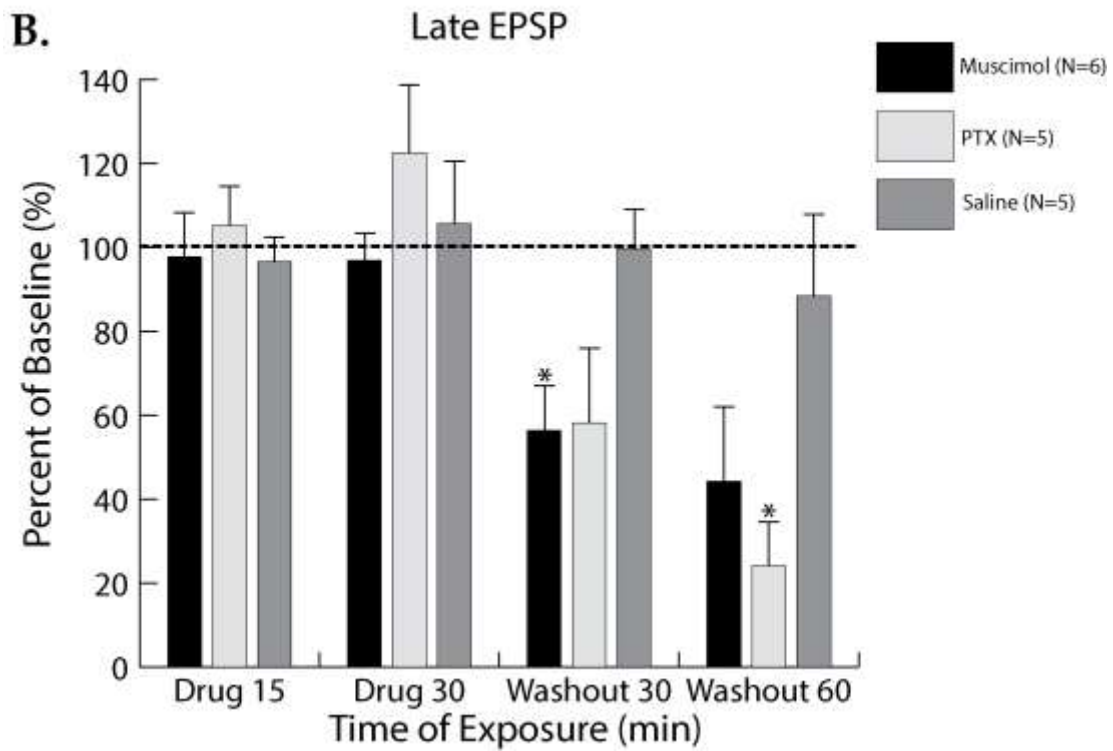
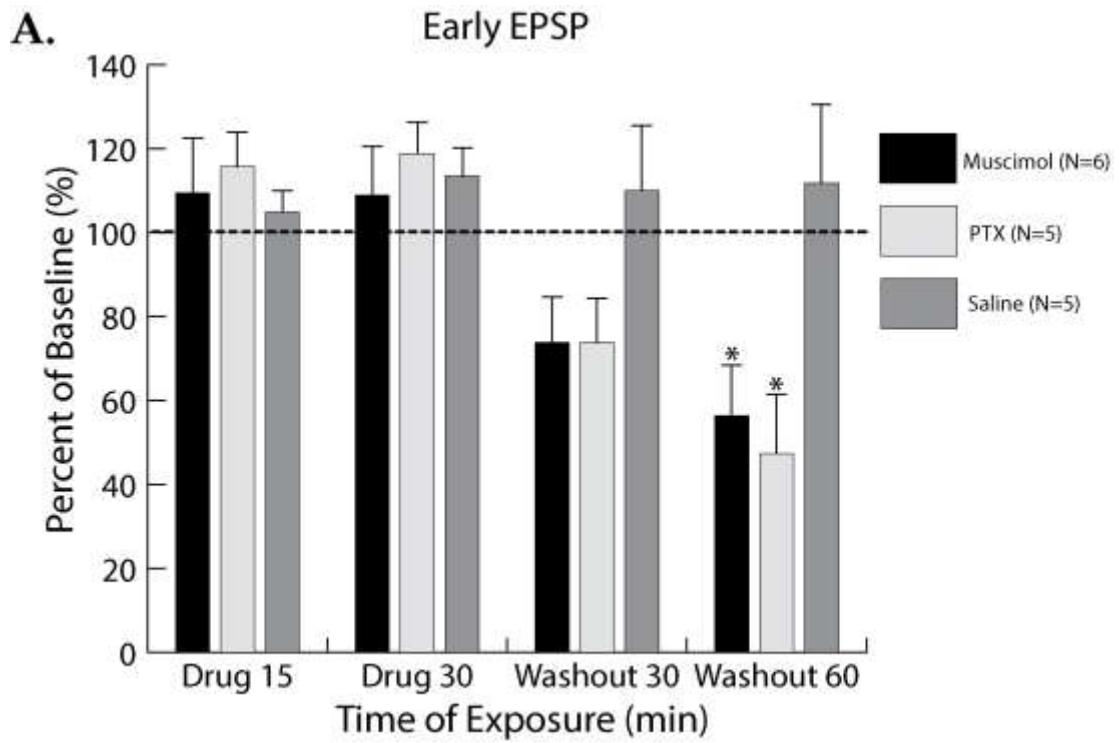


Fig. 6.6: Response of MG neuron to picrotoxin (PTX) superfusion. Superfusion of $25 \mu\text{M}$ PTX facilitates MG EPSPs in some preparations. Both early and late EPSP components of the MG appear to be facilitated. EPSPs were evoked using electrical stimulation of the antenna II nerve ipsilateral to an impaled MG neuron in the brain

connective. Preparations were exposed to a saline baseline (black sweep), 30 minutes of 25 μ M PTX (red sweep), and finally 60 minutes of saline washout (blue sweeps). The change during saline washout is shown in the difference between 15 minutes of washout (light blue) and 60 minutes of washout (dark blue).

Fig. 6.7: Effects of different pharmacological agents on the early (A) and late (B) MG EPSP components. Animals were socially isolated for 7-10 days and surgically prepared for intracellular recording of the medial giant (MG) neuron in the crayfish brain connectives. Excitatory post-synaptic potentials (EPSPs) were elicited through extracellular electrical stimulation of sensory afferents of the ipsilateral antennal II nerve of the impaled MG neuron. All preparations were exposed to at least 10 minutes of baseline where a stable EPSP was recorded before being exposed to one of three agents; normal crayfish saline (Saline), 25 μ M muscimol dissolved in crayfish saline (Muscimol), or 25 μ M picrotoxin dissolved in crayfish saline (PTX). The change in EPSP amplitude was measured as its % of the amplitude recorded during baseline. Muscimol and PTX groups were exposed to 30 minutes of drug exposure (Drug 15 and Drug 30) and then were washed with normal crayfish saline for 60 minutes. Saline groups were exposed to 90 minutes of saline exposure. * indicates values significantly different from saline controls (MWU: $p < 0.05$).



Superfusion of GABA can activate different types of GABA receptors and PTX is known as a “dirty drug” that can have a large number of different effects (Lingle & Marder, 1981; Etter et al., 1999). Therefore, using a more specific agonist for the receptors likely to be involved in inhibition of the MG may allow for a greater understanding of the inhibition present in the circuit. Muscimol is a GABA_A receptor agonist that activates these ionotropic receptor channels with significantly higher potency than GABA. For this experiment, 25 μM muscimol was superfused over MG preparations during electrically-evoked antennal II nerve EPSPs. The expectation was that if components of the MG circuit are modulated through GABAergic inhibition, superfusion of a GABA_A agonist would have significant effects on MG EPSPs. However, muscimol superfusion produced little to no change in early and late EPSP components in neither 15 nor 30 minutes of exposure. Amplitudes measured after 15 minutes of exposure were 109.5 ± 12.95 % and 97.74 ± 10.34 % for the early and late EPSP component, respectively. Following 30 minutes of muscimol, amplitudes were 108.91 ± 11.43 % for the early component and 96.83 ± 6.49 % for the late component. A Wilcoxon signed-rank test comparing the muscimol values against baseline revealed no statistical significance ($p > 0.05$ for early and late EPSP component). Interestingly, the facilitation observed in the early component of the muscimol-exposed group is also not significantly different from amplitudes measured in the PTX-exposed group (MWU, $p > 0.05$). Because average amplitudes were recorded for each 5 minute time bins, analysis can be applied to more time points than those shown in figure 6.7. When observing these time bins, there was a statistically significant effect in the late EPSP component (MWU, $p < 0.05$) for both 20 and 25

minute time points of exposure where PTX exposed amplitudes were measured as 133.18 ± 8.71 % and 129.63 ± 13.48 %, respectively, while muscimol-exposed preparations were measured as 96.57 ± 6.38 % and 96.411 ± 5.59 %, respectively. While there wasn't any significant change during the muscimol exposure itself (MWU: $p > 0.05$), values were significantly lower following 60 minutes of washout in the early component when compared to saline controls, with an average of 56.36 ± 12.05 % (MWU, $p < 0.05$). The late EPSP component was not significantly reduced compared to controls after 60 minutes, with an average amplitude of 44.27 ± 17.61 % of baseline (MWU, $p > 0.05$). While this value is much lower than those recorded in saline (88.32 ± 19.45 %), both groups had high standard error values.

During muscimol exposure, I observed an interesting biphasic effect in some of the animals (**Fig. 6.8**). In these semi-intact preparations, EPSPs were at first facilitated, increasing in amplitude in both early and late components. Because this increase was only observed in two preparations of the seven, the average increase for the entire sample was only slightly above baseline. The two preparations, however, reached early component amplitudes greater than 150% of their saline baseline amplitudes. It is important to note that while these preparations increased during muscimol, they decreased in washout like the other five preparations. This increase could indicate a different distribution of GABA receptors that might more greatly enhance inhibition of pre-synaptic inhibitory components, resulting in an enhancement of the MG EPSP.

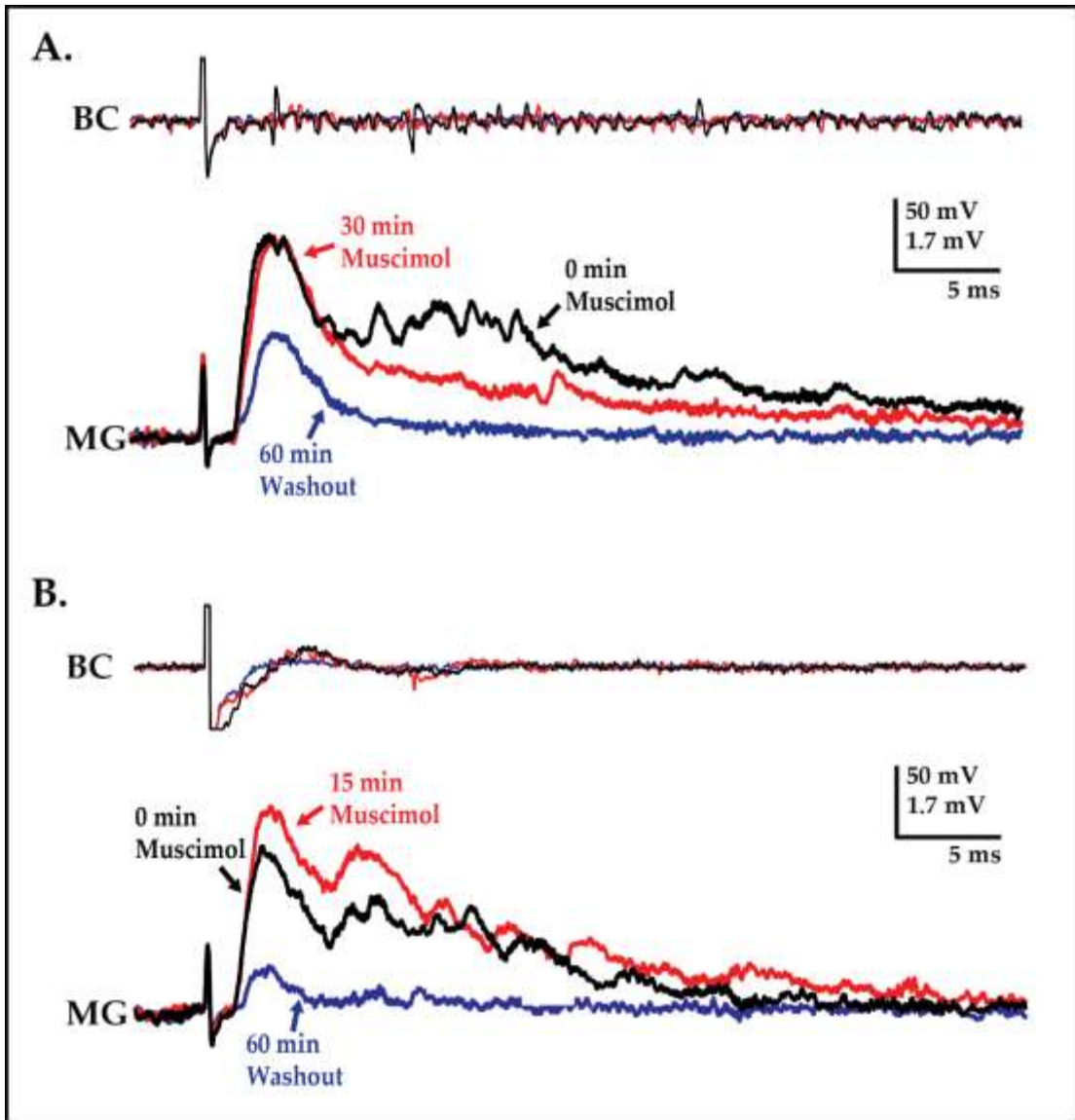


Fig. 6.8: Different examples of MG EPSPs exposed to 25 μ M muscimol. A) The effect of muscimol exposure is most apparent during the saline superfusion following the 30 minutes of muscimol exposure. Later EPSP activity is shown to decrease during muscimol exposure, but the earlier excitatory component of the EPSP remains unchanged until the preparation is washed out with normal crayfish saline. B) In some experiments, muscimol superfusion results in a facilitation of both early (3 ms

after stimulus) and late (6 ms after stimulus) MG EPSP components. Like in (A), these examples are also substantially reduced during the saline washout.

Experiment 6.5: MG Input Resistance and Muscimol

In the previous experiment, I found that in several preparations, muscimol exposure led to an unexpected increase in the MG EPSP amplitude. Following this facilitation, these preparations would typically show decreases below baseline amplitude during the saline washout. Because it seems likely that this biphasic effect could be mediated through a pre-synaptic effect, I repeated the muscimol experiments and measured the input resistance of the MG during muscimol exposure. This involved impaling the same MG neuron with two intracellular electrodes, one to measure membrane voltage and the other to inject low amounts of positive and negative current to measure the change in the cell's input resistance. Measuring changes to the input resistance of a cell can indicate the location of inputs as pre-synaptic or post-synaptic (Valentino & Dingledine, 1981). The number of open ion channels of a cell determines the value of the input resistance. If the cell being measured experiences an effect from the exposure to a drug, that effect may be due to directly acting on the cell or by affecting the inputs to that cell. If the change in input resistance of the cell changes during drug exposure and coincides with changes to the drug-induced change of the EPSP, then the drug is activating channels on the cell itself. If the input resistance remains unchanged compared to the EPSP, then the effect of the drug is likely pre-synaptic, affecting inputs to the cell to create the change observed in the EPSP. For the muscimol-induced effect of the MG; if the

facilitating effect of muscimol is mediated through a pre-synaptic effect then the input resistance of the MG should remain relatively unchanged as the EPSP amplitude increases; if the effect is post-synaptic then the input resistance should change as the MG EPSP changes.

Methods

A small subset ($N = 4$, 3.58 ± 0.25 cm) of the MG muscimol experiments were performed while using two intracellular electrodes placed in one MG axon to measure input resistance. The input resistance of the MG was measured through injection of positive current (20- 40 nA) for 30 ms followed by negative current (-20- -40 nA) for 30 ms. After the membrane potential had returned to baseline, the ipsilateral antenna was electrically stimulated to produce a measurable EPSP. Because much of muscimol's inhibitory effects were observed in the saline washout, all preparations received at least 60 minutes of normal crayfish saline following muscimol exposure.

The change in input resistance was calculated by dividing the recorded amplitude of the voltage change before the end of the current injection by the current injected. Much like the change in EPSP amplitude, this input resistance was normalized in relation to the pre-drug saline baseline values. The early EPSP component was compared to the calculated input resistance to determine if changes to the early EPSP amplitude were similar to changes in the input resistance.

Results

Input resistance (IR) measurements can be an indicator of whether the effects of a pharmacological agent are pre or post-synaptic. Input resistance has been measured in previous studies to be approximately 3.6 ± 0.2 ($\times 10^4$) Ω in an adult MG near the subesophageal ganglion (Glantz & Viancour, 1983). I recorded baseline input resistances from one of the juvenile MG neurons used for this experiment. During the saline baseline, the MG had average input resistance values of 11.7 ± 0.06 ($\times 10^4$) Ω . The difference between my recorded input resistance value and the previous work by Glantz & Viancour (1983) is likely the result of the different axonal diameters between an adult and juvenile MG. With increasing axonal diameter, input resistance values would be expected to decrease. Alterations in the EPSP while the input resistance remains constant would suggest that the effect is largely pre-synaptic and that the effects of the drug are largely on neurons upstream from the MG rather than through direct activation of MG GABA_A receptors. While the effect of 25 μ M muscimol was variable across the preparations used for the IR experiments (N = 4), the input resistance seems to correlate with the changes in the EPSP. The EPSP average amplitude over the course of 30 minutes of muscimol exposure, much like in the previous experiment, show little change in the amplitude with averages after 30 minutes of 94.25 ± 12.8 % of baseline (mean \pm SE). Similarly both positive and negative input resistance measurements experienced little change with averages of 102.68 ± 6.81 % and 106.55 ± 7.33 % of the baseline input resistance. Interestingly, in one preparation the EPSP amplitude increased to roughly 125 % of its baseline value and the input resistance measurements increased accordingly to 122.99 % and 127.27 % (positive and negative IR measurement, respectively) indicating that the

increase in amplitude was likely mediated through an MG post-synaptic change (**Fig. 6.9**).

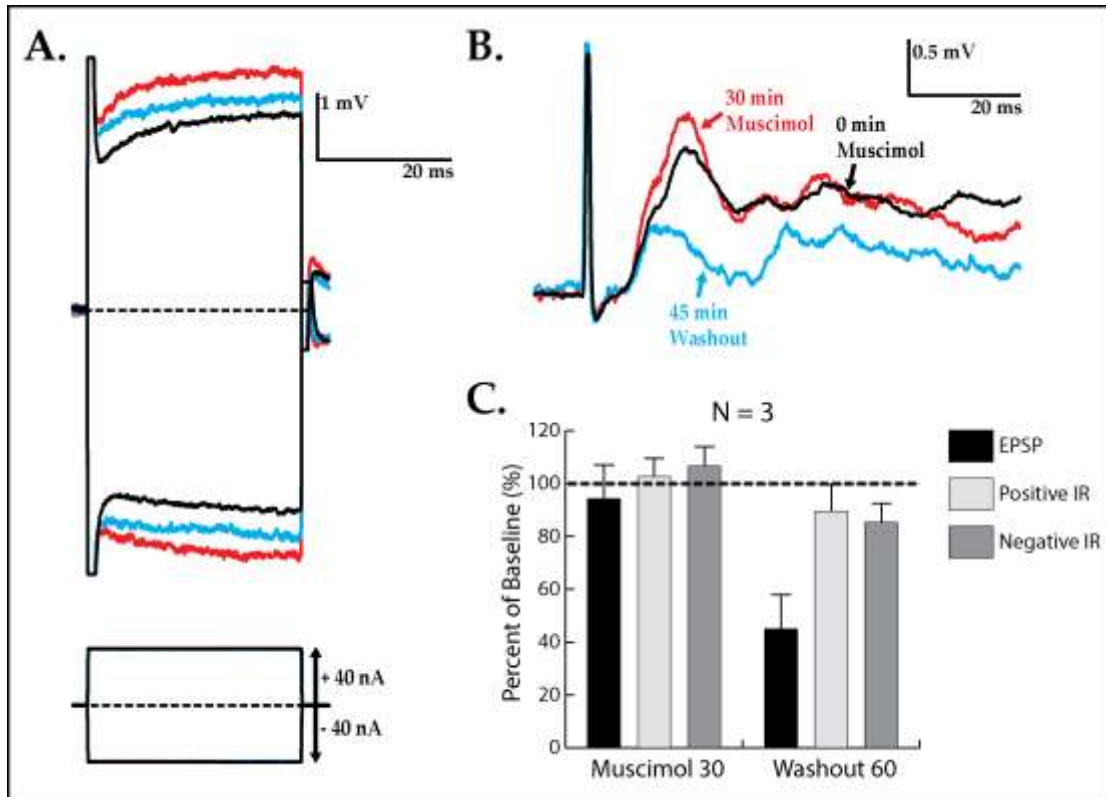


Fig. 6.9: Input resistance of the MG decreases during saline washout while EPSP amplitude has also diminished. The same MG axon was impaled with two different intracellular electrodes and a small amount of current (+40/-40 nA for 30 ms) was injected through one electrode (A) while voltage changes were measured with the other electrode (B). This current was well below the amount of current that would be needed to activate the MG neuron. Pre-muscimol baselines (Black sweep), 30 minutes of 25 μ M muscimol superfusion (Red Sweep), and 45 minutes of washout (Blue sweep) are shown. C) Average percent of baseline values for EPSP amplitude and positive (Positive IR) and negative (Negative IR) values.

This experiment was also performed to measure the changes of the input resistance during the decrease in EPSP amplitude typically seen during washout of muscimol. Much like the previous experiment, preparations displayed average EPSP amplitudes of 44.95 ± 12.68 % of baseline after 60 minutes of saline (following muscimol), and input resistances also showed decline compared to baseline, with average measurements of 89.49 ± 9.83 % and 85.27 ± 7.17 % for the positive and negative current injections, respectively. The increased input resistance in preparations that were facilitated and decreased input resistances measured when the EPSP was diminished both indicate a post-synaptic effect. While the post-synaptic effect of muscimol during exposure needs little explanation, the post-synaptic effect observed when muscimol is washed out of the preparation is more complicated. As the muscimol is washed off, the input resistance is shown to decrease to average levels below the starting values. A component of the EPSP and input resistance decline may be due to muscimol washing off GABA_A receptors of the MG. Additionally, it can be expected that this lengthy recording could lead to a leakier MG axon, which would also result in the observed decreases. To account for this, input resistance changes could be measured when the preparation is exposed only to normal crayfish saline.

Chapter Discussion

All the experiments performed here suggest the importance of inhibitory regulation of MG excitability. I have shown that MG excitatory post-synaptic potentials (EPSPs) evoked through antennal activation have numerous similarities to

mechanosensory-evoked LG EPSPs. I found that components of the MG EPSP are highly resistant to habituation, even following a minute-long barrage of 10 Hz electrical stimuli to the antenna II nerve. The β component of the LG EPSP is also susceptible to a decrease in amplitude when presented with repeated stimulations while the earlier α component is more resistant. In this circuit, the β is known to be the result of chemical synapses in the afferent network while the α component is purely electrical. My result suggests that the MG EPSP, like the LG EPSP, consists of both chemical and electrical inputs.

The presence of GABAergic inhibition in the circuit occurring after the earliest component of the EPSP is confirmed through experiments depolarizing the MG resting membrane potential and separately through superfusion with a saline solution that contains low amounts of chloride ions. These results are expected as the intracellular concentration of Cl^- in the MG, if consistent with the LG, would be higher than the external concentration. Under normal circumstances this would result in depolarizing inhibition as GABA_A receptor activation will produce depolarizing currents as Cl^- leaves the cell (Cattaert & Manira, 1999). I found that this large Cl^- mediated current occurs during the normal course of the EPSP, but also continues long after, sometimes for tens of milliseconds following the stimulus, implying that this very late component of the EPSP is almost entirely mediated by Cl^- currents. Similar to the LG, the MG experiences prolonged depolarization when exposed to PTX, a GABA_A receptor blocker. Also similar to the LG, the MG would at times fire action potentials during the course of this prolonged excitation (not shown). This result suggests the presence of GABAergic inhibition that is blocked by PTX,

allowing the cell to experience extended excitation. As described earlier, the EPSP evoked through antenna II nerve stimulation is rarely sufficient to drive the MG to activation, but the superfusion of PTX allows the MG to overcome this in some cases. These results imply that the late MG EPSP component is under significant inhibitory control, like the LG EPSP.

The increased depolarization of the MG that was observed when some preparations were exposed to muscimol seems to be at odds with the expectation of a GABA_A agonist, but there are several possible explanations. First, this only occurred in a few preparations and, when comparing the average change during muscimol exposure, it was not statistically significant. However, this GABA_A agonist-induced facilitation might be explained by the afferent pathway that provides excitatory input to the MG. It is possible that this pathway contains inhibitory interneurons that inhibit pre-synaptic input to the MG. If these interneurons have a higher binding affinity for muscimol than the MG itself, then muscimol may bind to these cells, reducing the amount of inhibition of excitatory MG inputs. This may relieve some inhibition from the MG, causing an increase in the early EPSP amplitude, which is excitatory. However, the results from the input resistance measurement experiments somewhat contradict this possibility. In the few samples where I noted an increase of the MG EPSP, the input resistance also increased, suggesting that the increase in EPSP amplitude coincided with post-synaptic changes. The results here indicate a biphasic effect where some preparations showed increased EPSP amplitudes during muscimol followed by a decrease in amplitude below baseline in the washout.

It is interesting to compare the effects of PTX and muscimol, agents that should have opposite effects with PTX being a potent GABA_A channel blocker (Newland & Cull-Candy, 1992) and muscimol being an extremely potent GABA_A agonist. What is actually observed in my experiments is a very similar effect between the two drugs. In some preparations, superfusion of these two agents produce facilitation of the excitatory early EPSP component, although PTX does appear to have a stronger effect overall. Compared to the previous work performed in the LG circuit, PTX appears to strongly increase both the early and the late EPSP component of the MG. Based on the membrane depolarization experiment and the low Cl⁻ saline experiment, the early MG EPSP component appears to be primarily excitatory while the late component is largely inhibitory. PTX exposure in the LG has little effect on the β component (most similar to the early component of the MG), instead more greatly increasing the EPSP region immediately following the β . This may indicate that there is more presynaptic GABAergic inhibition occurring during the MG EPSP peak as compared to the LG. While the effect on the late component of the MG was more variable than the early component, the enhancement was higher on average than at the early component. In contrast to PTX, muscimol has no effect on both the early and late component, but leads to significant decreases in the amplitudes of both components during the saline washout. The muscimol-induced decrease observed in washout may be due to the removal of muscimol from the preparation. Because muscimol is an agonist to ionotropic GABAergic receptors, the effect would be expected to manifest earlier during muscimol exposure itself. Perhaps the muscimol

concentration was too low to elicit significant changes initially at lower concentrations. .

In the LG chapter (4), socially isolated preparations were more likely to be excitable under control saline exposure. In this chapter, socially isolated preparations seem more excitable as the recording continues, as can be seen when the MG preparation is exposed to normal crayfish saline for an extended duration in the absence of other pharmacological agents. While none of the saline-exposed averages showed statistical significance from baseline, some preparations showed increases beyond what may be expected from normal crayfish saline. This facilitation may indicate the presence of some form of potentiation as the antennal II nerve is repeatedly stimulated for almost two hours. However, samples exposed to 90 minutes of saline did not display a similar drop in potential amplitude when compared to PTX or muscimol exposed samples, suggesting that normal preparations remain viable for the duration of the recording.

Although there are differences between MG and the well-understood LG, it makes sense that many similarities are observed in both escape circuits. Both behaviors are reflexive behaviors that must be specialized for responding to phasic stimuli and for speed. The mechanisms that have been described in the LG circuit, and now for the first time in the MG as well, allow for this specialization.

An adjustment in MG excitability is a possible candidate for modulating the crayfish's ability to perform value-based decision making, previously seen in the shadow experiments performed in our laboratory (Liden et al., 2010). It is important to note that the type of stimulus provided to excite the MG in the two experiments

(the shadow experiment and the electrically-evoked EPSPs described here) belong to two different sensory systems. The MG is multi-sensory, receiving excitatory input from both mechanosensory and visual stimuli (and possibly olfactory as well). The shadow experiment elicits a response without directly touching the animal, instead exciting the MG through the presentation of a visual danger stimulus in the form of a passing shadow. In contrast, the electrical stimulation used in my experiments presented here mimics a phasic mechanosensory stimulus to the antenna. While the distinction is important and more work needs to be done linking GABAergic inhibition to this decision-making process, the inhibitory characteristics described here will regulate the excitability of the MG and will likely play a role in determining the likelihood of a tail-flip response to make a behavioral choice between freezing or tail-flipping. Additionally, mechanosensory inputs must play a role in crayfish agonistic encounters, therefore the degree of inhibition of these excitatory inputs could dramatically alter the result of a fight, influencing dominance hierarchies.

Chapter 7: Muscimol, Alcohol and the MG

Summary

In previous chapters I have demonstrated the medial giant's (MG) physiological sensitivity to acute EtOH exposure (Ch. 5) and that the MG is inhibited through GABAergic mechanisms (Ch. 6). In this chapter, I explore how EtOH interacts with the MG through pre-treatment with a potent GABA_A agonist, muscimol. I show here that when exposed to muscimol *before* EtOH, the large increases to excitatory post-synaptic potential (EPSP) amplitudes seen after EtOH exposure (experiment 5.1) are no longer present. In addition, these muscimol preparations showed none of the decreases typically seen during washout (experiment 6.4). These results can be interpreted in several different ways. Comparing the muscimol pre-treatment preparations to those pre-treated with saline may indicate that muscimol blocks the effect of EtOH. Because EtOH exposure appears to prolong the first phase of muscimol exposure it could also be interpreted that EtOH facilitates the action of muscimol. Additionally, it is also possible that muscimol and EtOH are operating independently of each other, and that the second muscimol phase that normally decreases the MG EPSP offsets the normal increase observed in the saline pre-treated samples.

Background

From the previous experiments, I have shown that the MG EPSP is increased by EtOH (Ch. 5) and inhibited through GABAergic mechanisms (Ch. 6). Other research has indicated that the GABAergic system is a major target for alcohol (Mehta & Ticku, 1988; Mihic et al., 1997; Lobo & Harris, 2008).

Previous work has shown interactions of muscimol and EtOH in various models. Behaviorally, activation of GABA_A receptors through systemic injection of muscimol has been shown to reduce EtOH consumption in mice (Quoilin & Boehm II, 2016) and rats (Wilden et al., 2014). Another study in rats has shown that EtOH consumption can be increased through muscimol injections to the caudal nucleus of the raphe (Dudek & Hyttia, 2016). In 2012, Wakita et al. showed that co-application of muscimol with 10 mM EtOH increased the spontaneous occurrence of inhibitory and excitatory post-synaptic currents (IPSCs and EPSCs), but reduced evoked currents in a reduced rat hippocampal “synaptic bouton” preparation. In this study, 10 mM EtOH alone produced none of these changes, implying that muscimol increased the effects of EtOH. Additionally, EtOH induces inhibition of excitatory neurotransmission in the nucleus accumbens and this inhibition increases in strength and duration when muscimol is presented (Mishra & Chergui, 2011). The trend across many studies on the interaction of muscimol and EtOH is that EtOH-induced effects are facilitated when muscimol is co-applied. This suggests that muscimol would have a facilitating effect on the GABAergic mechanisms of EtOH exposure. However, a study by Liljequist & Engel (1982) has shown that EtOH increased locomotor activity in mice when exposed to low EtOH concentrations, and this excitation could be

suppressed by muscimol pre-treatment. In the same study, muscimol was shown to also increase the sedative effects of higher EtOH concentrations as co-application of muscimol and high EtOH concentrations led to increased EtOH-induced sleep-time. Together, these studies suggest that the interaction of muscimol and EtOH might be dose-dependent, with muscimol blocking behaviors induced by low EtOH concentrations and facilitating behaviors induced by higher concentrations.

For my experiment, I treated MG preparations with 25 μ M muscimol and then exposed the preparation to 100 mM EtOH for 30 minutes. I found that muscimol pre-treated EtOH preparations showed little enhancement of both early (3 ms after the stimulus) and late (6 ms after the stimulus) EPSP components when compared to EtOH preparations that were pre-treated with normal crayfish saline. These preparations also failed to exhibit the muscimol-induced decrease observed in preparations that only received muscimol (Ch. 6). These data suggest that some of the EtOH-induced EPSP facilitation is counteracted by the GABA_A receptor agonist muscimol.

Experiment I: Muscimol Pre-treatment Blocks EtOH-induced Facilitation

The only experiment for this dissertation chapter is the combination of the experiments performed in chapter 5 and chapter 6. In the last chapter, I had shown that the MG neuron is inhibited through GABAergic mechanisms and that the GABA_A agonist, muscimol, can have significant effects, facilitating some EPSP amplitudes early on before leading to a significant decrease after muscimol was replaced with normal crayfish saline during washout. Because of the results from the

low Cl^- saline (experiment 6.3) and MG membrane depolarization (experiment 6.2) experiments, I know that the late EPSP component (6 ms after the stimulus) is mediated by GABAergic inhibition and because GABA is a primary target of alcohol exposure (Mehta & Ticku, 1988; Mihic et al., 1997; Lobo & Harris, 2008), it can be reasoned that pre-treatment using a GABA agonist will alter the effect EtOH has on the MG EPSPs.

Methods

Socially isolated (7-10 days) juvenile crayfish ($N = 6$, 3.62 ± 0.16 cm) were pinned to a sylgard lined dish and the brain connectives were exposed in the head. The MG was impaled using an intracellular electrode and EPSPs were generated through electrical stimulation of the antenna II nerve ipsilateral to the impaled MG. The interaction of GABAergic neurotransmitter systems and EtOH were investigated by exposing MG preparations to 25 μM Muscimol superfusion followed by 100 mM EtOH. Because the EtOH-induced facilitation of antenna II evoked MG EPSPs appears to plateau after 30 minutes, the preparations were exposed to 30 minutes of muscimol (phase 1) followed by 30 minutes of EtOH exposure (phase 2). Phase 2 was followed by at least 60 minutes of saline washout. While in other experiments, EPSP amplitudes were compared to the baseline amplitude, this experiment involves the exposure of the preparation to two different drugs. Because of this, the EPSP amplitudes measured during the EtOH phase were normalized to the muscimol values recorded at the 30th minute of exposure. By doing this, I was able to compare the effect that muscimol has on EtOH-induced facilitation when the preparation is pre-treated with saline before EtOH (Ch. 5).

Additionally, while there may be a significant effect between muscimol pre-treated and saline pre-treated EtOH exposed animals, the effect that EtOH has on the normal progression of muscimol exposure was also of interest. In order to compare the effect that EtOH has on the muscimol effect shown in the previous chapter, comparisons will also be made between preparations that were exposed to 25 μ M muscimol followed by saline and those followed by EtOH.

Results

Of primary interest in these experiments was how muscimol pre-treatment (MP) affected the EtOH-induced facilitation of the MG. This was done by normalizing the EPSP amplitude of the MP preparations during EtOH exposure to the last measured EPSP amplitude of muscimol exposure and comparing these 30 minutes of EtOH exposure to the first 30 minutes of MG preparations that were saline pre-treated (SP; experiment 5.1). Because of the significant effect that EtOH has within the first 30 minutes of exposure (**Fig. 7.1**), MP preparations were only exposed to 30 minutes of 100 mM EtOH after 30 minutes of 25 μ M muscimol superfusion. In chapter 5, I showed that 30 minutes of EtOH exposure leads to significant increases in MG EPSP amplitudes with an average early component of 159.62 ± 15.52 % of the baseline amplitude (mean \pm SE). In contrast, the change that EtOH exposure has on the early component when pre-treated with muscimol appears to be minimal after 30 minutes with average amplitude only 102.84 ± 9.87 % of the muscimol baseline (**Fig. 7.2**). Using a MWU non-parametric test for independent groups, there is a significant difference ($p < 0.05$) between the 30 minute time points of saline pre-treated and

muscimol pretreated preparations. The effect is largely similar for the late component with SP preparations measuring average late component amplitude of 183.22 ± 32.87 % while the MP group had an average amplitude of 105.55 ± 12.66 % of baseline.

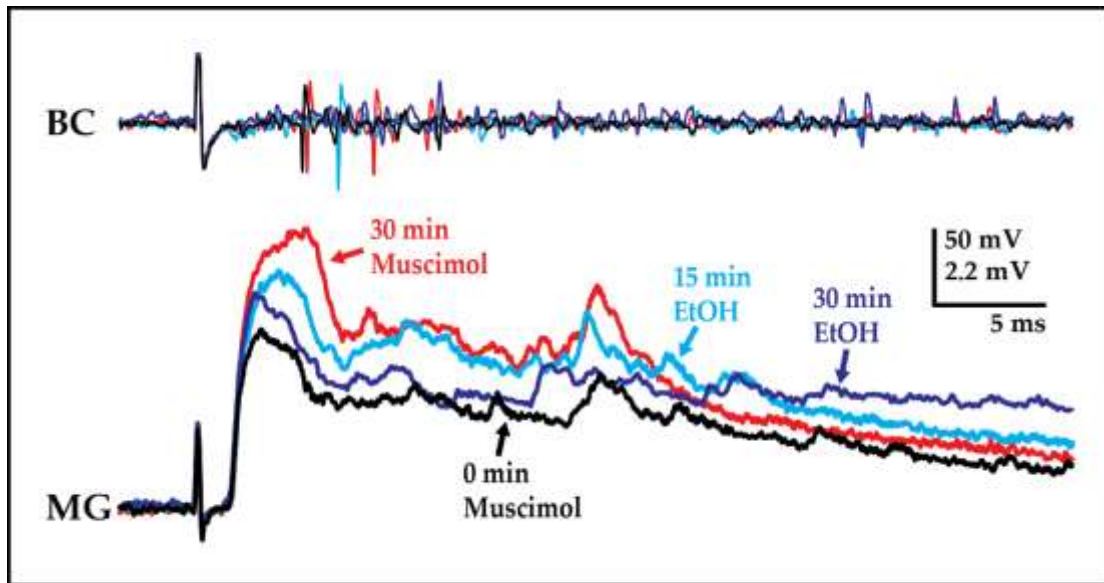
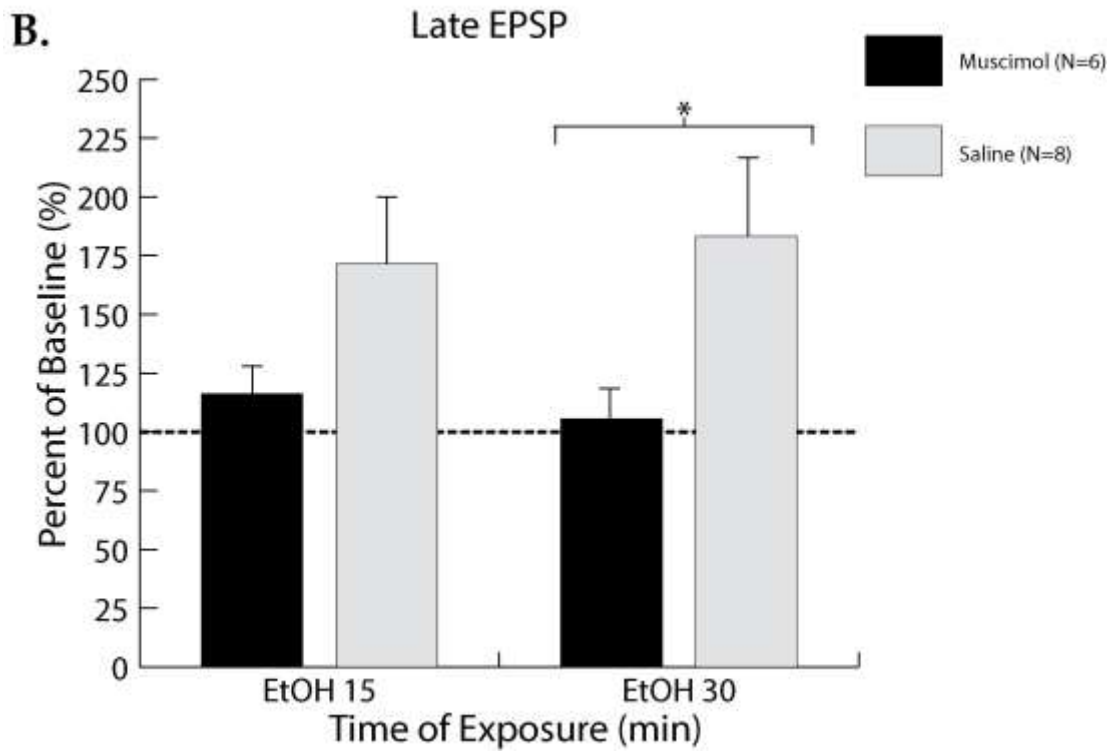
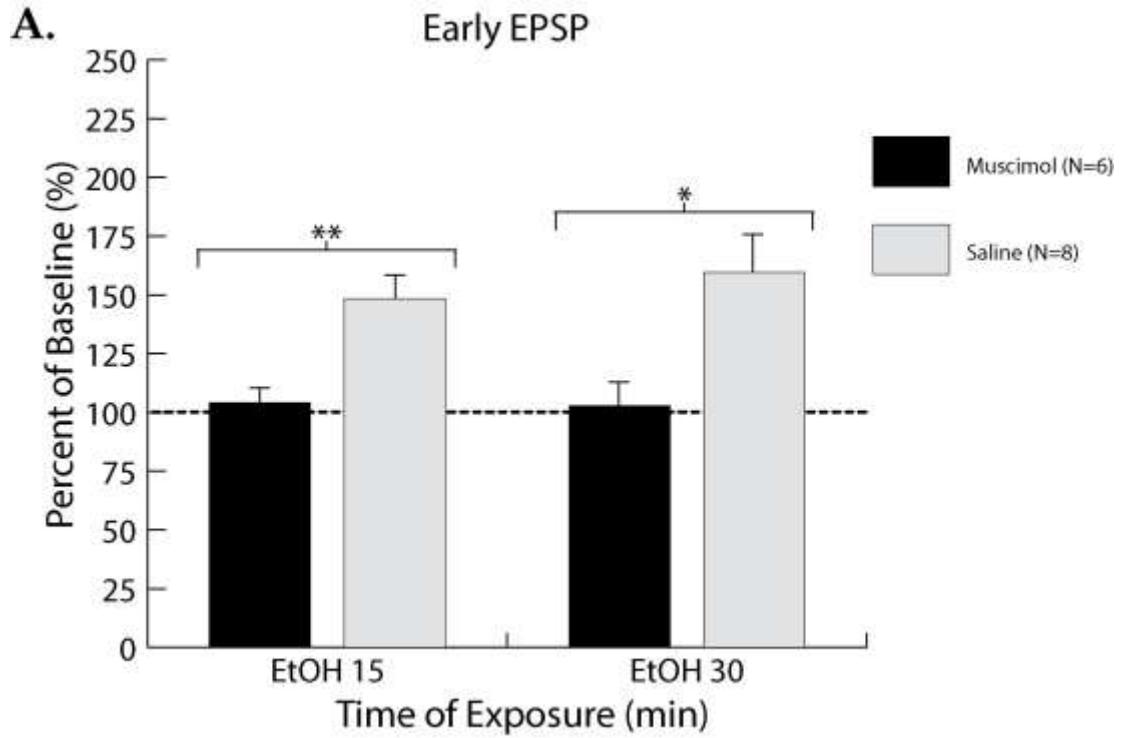


Fig. 7.1: Sample MG EPSP when preparation is pre-treated with muscimol before EtOH exposure. Sample electrophysiology traces recorded intracellularly from the medial giant (MG) neuron of the brain connectives (BC). An extracellular recording hook was placed on the ipsilateral BC to the stimulated antennal II nerve stimulated through extracellular hook electrode. MG recording was performed in the ipsilateral BC. All preparations were exposed to a saline baseline (Black sweep), then 30 minutes of 25 μ M muscimol exposure (Red sweep), and followed by 30 minutes of 100 mM EtOH exposure (Blue sweeps). All preparations also received a saline washout for 60 minutes (sweep not pictured).

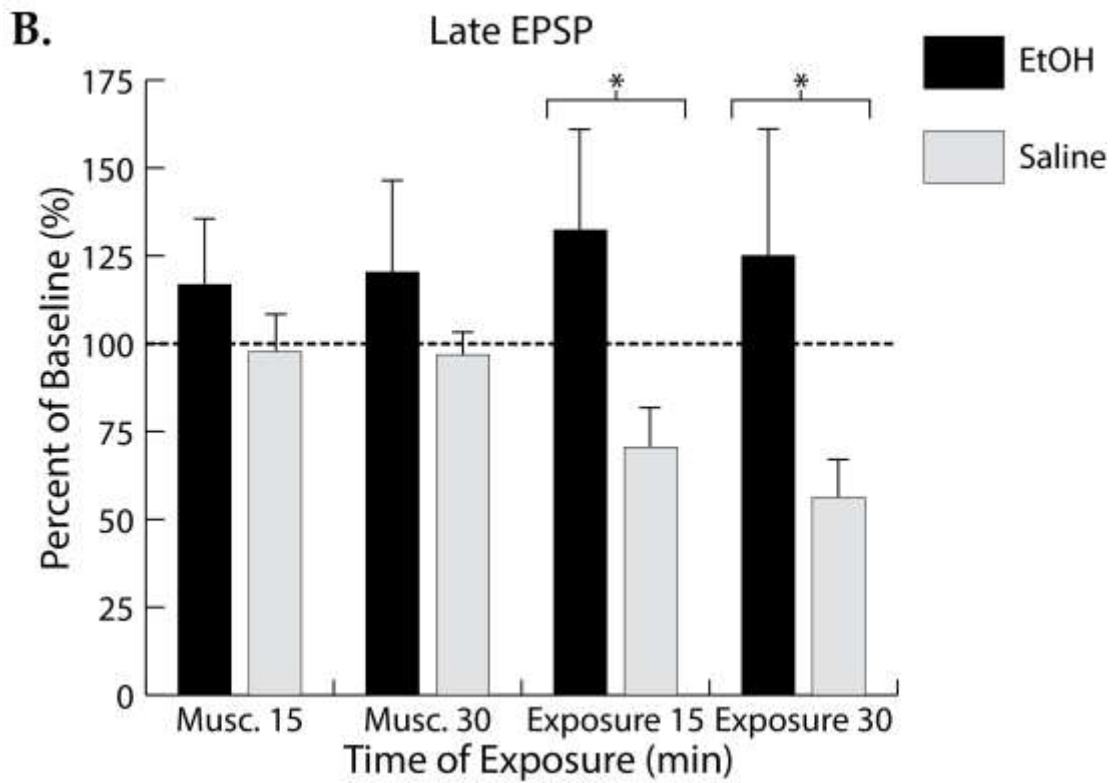
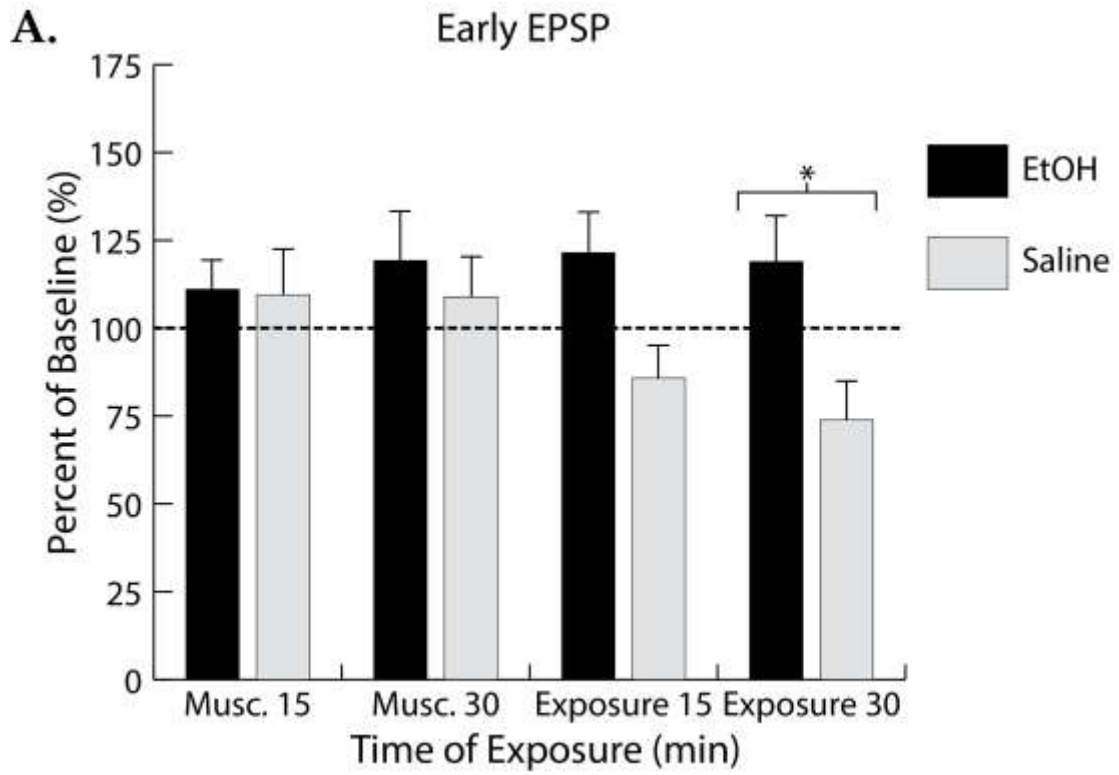
Fig. 7.2: Comparison of early (A) and late (B) MG EPSP components exposed to 100 mM EtOH when pre-treated with saline (Saline) or 25 μ M muscimol (Muscimol). Preparations that are exposed to saline prior to EtOH exposure (Ch. 5) are increased over those that have been pre-treated with muscimol in both early and late EPSP components. Muscimol preparations received 30 minutes of muscimol prior to EtOH exposure and had their EtOH EPSP amplitudes normalized to the 30 minute value of muscimol exposure as it directly preceded EtOH exposure. As in other experiments, Saline preparations were normalized to the saline baseline values that directly preceded EtOH superfusion. * indicate time points where the EPSP amplitudes were significantly different using a MWU test with a p-value of $p < 0.05$. ** indicate time points where the difference was significantly different with p-values of $p < 0.01$.



This implies that the EtOH facilitation of the MG EPSP is not observed after the preparation was first exposed to muscimol. Wilcoxon signed-rank test yielded no significance between either 15 minutes or 30 minutes EtOH when compared to muscimol baseline for the early EPSP component. This provides further evidence that the enhancing effect seen when the MG is exposed to EtOH may have been counteracted by the effect of muscimol. When compared to the results of muscimol exposure after normal crayfish saline performed in Chapter 6, it can also be seen that EtOH prevents the decrease of the EPSP amplitude that was observed when preparations were not exposed to the combination of the two drugs (**Fig. 7.3**).

Fig. 7.3: EtOH following muscimol prevents muscimol-induced decrease.

*Preparations were exposed to 30 minutes of 25 μ M muscimol followed by either 30 minutes of 100 mM EtOH (EtOH, N = 6) or 30 minutes of normal saline (Saline, N = 8). All experiments were then washed out for 60 minutes (not shown). * indicates time points where the EPSP amplitudes were significantly different between preparations exposed to EtOH following muscimol and those exposed to saline following muscimol (MWU test: $p < 0.05$).*



While superfusion of EtOH following 30 minutes of muscimol exposure doesn't seem to further enhance the MG EPSP, this does not mean that EtOH has no effect on the MG EPSP. In fact, superfusion of 100 mM EtOH following muscimol produced a measurable effect. Both early and late components were facilitated compared to preparations that were exposed to muscimol only. When tested for significance using a MWU test, both 15 and 30 minute time points in EtOH exposure for both early and late components were significantly higher than those components recorded in saline after muscimol in groups from Chapter 6 (MWU, $p < 0.05$).

Both groups showed similar muscimol related effects of the early component during the first 30 minutes, which is expected as both groups were treated identically until EtOH was superfused. However, muscimol appears to have different effects on the late component between the two groups. After 30 minutes of muscimol, the average amplitude of this component in preparations that would then go on to be exposed to EtOH was 136.20 ± 25.46 % of baseline while preparations that would then be exposed to saline were 96.83 ± 6.49 % of baseline. Although these two values are not statistically significant (MWU, $p > 0.05$), this might be related to a high level of variability in the samples. This can also be seen when these data are represented using a box and whisker plot (**Fig. 7.4**). This variation, however, is not related to differences in stimulus voltages used to elicit EPSP in these two groups. For the ME group the average voltage applied to the antenna II nerve was 1.17 ± 0.62 mV, and for the MS group it was 1.31 ± 0.66 mV.

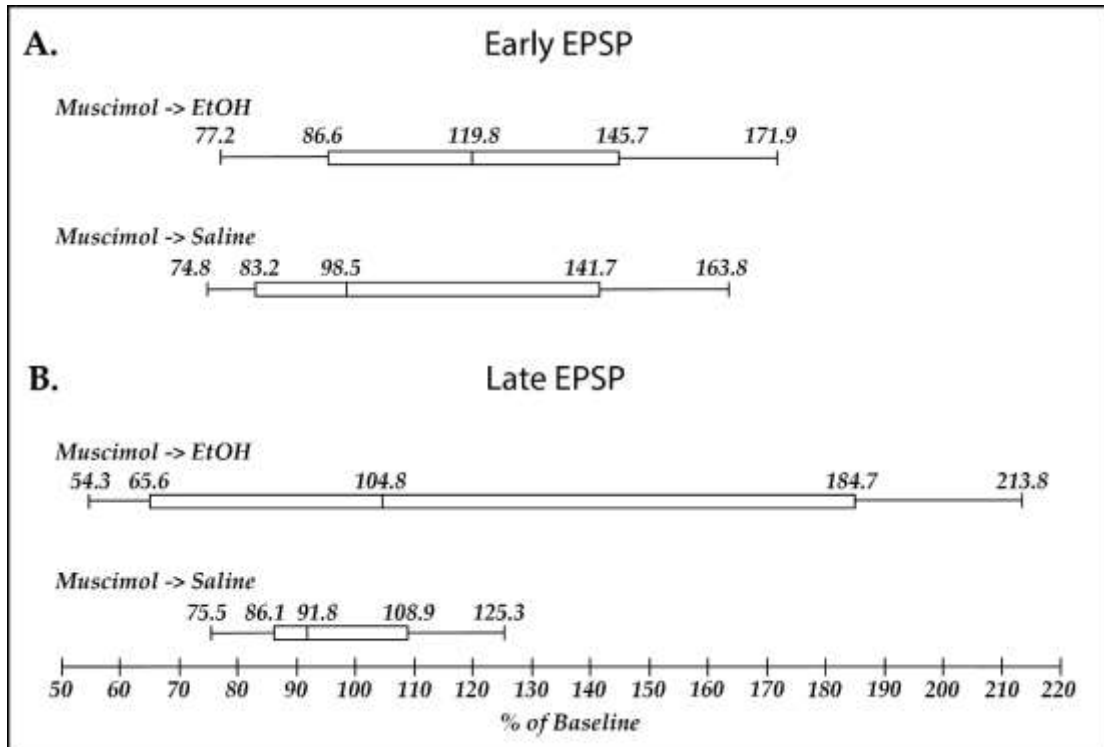


Fig. 7.4: Comparison of 25 μ M muscimol exposure between preparations that would be exposed to EtOH following muscimol (Muscimol -> EtOH) and those that would be exposed to normal crayfish saline after muscimol (Muscimol -> Saline) groups in both early (A) and late (B) MG EPSP components. A. Change in early component of MG EPSP after 30 minutes of muscimol as percentages of the amplitude recorded during the saline baseline. B. Change in the late component of the MG EPSP after 30 minutes of muscimol exposure as percentages of the amplitude recorded during the saline baseline.

Chapter Discussion

Because it is believed that some of EtOH's effects on the nervous system can be attributed to interactions with GABAergic neurotransmitter systems, it was

predicted that muscimol pre-treatment would have a significant effect on the EtOH-induced change of the MG EPSP. As described earlier, much of the previous research describes the interaction of muscimol and EtOH as facilitatory (Mishra & Chergui, 2011; Wakita et al., 2012; Dudek & Hyytia, 2016), but work in mice has also shown that muscimol pre-treatment before low doses of EtOH suppressed EtOH-induced increases in locomotor behavior (Liljequist & Engel, 1982). In this study, EtOH exposure without muscimol increased locomotor behavior, and muscimol injected in the absence of EtOH had no effect on locomotion. When combined (muscimol followed by EtOH), however, locomotor behavior was decreased below normal levels. In many ways these results parallel what I have found for the MG circuit. In experiment 5.1, I showed that the MG is highly responsive to acute EtOH exposure, increasing early (excitatory) and late (inhibitory) EPSP component amplitudes to more than 150% of baseline. In those experiments, EtOH exposure was preceded by normal crayfish saline (saline pre-treatment), while in this chapter, preparations received muscimol pre-treatment before EtOH superfusion and this resulted in no increase of the MG EPSP during EtOH exposure. Based on these results, it seems likely that muscimol blocks EtOH's effect on the MG, possibly by occupying the same receptors that EtOH would normally bind to in muscimol's absence.

Previous work indicates that EtOH and muscimol typically have significant positive interactions; one enhances the function of the other. It is important to note that while there was little enhancement in my study, there was also little decrease in EPSP amplitudes during washout, which is typically observed after muscimol exposure (in no EtOH conditions, Ch. 6). In the previous chapter, I showed that

muscimol exposure increased the early EPSP component and strongly decreased it during saline washout (experiment 6.4). Late EPSP components showed little change during muscimol exposure itself, but like the early component, decreased significantly during washout. In the current experiment, preparations that received muscimol pre-treatment before EtOH displayed the same increase of the early EPSP component during muscimol, but these preparations did not decrease after the muscimol phase ended. This prolonged muscimol-induced increase could be seen as a potentiating effect of EtOH, something that is predicted based on some previous work. As mentioned earlier, it is also possible that muscimol and EtOH bind to the same GABA_A receptors, which may explain why the EPSP remains elevated while non-EtOH muscimol preparations show significant EPSP decline. If they both bind to the same receptors and the muscimol-induced decrease observed in washout is the result of muscimol being washed off receptors, perhaps the GABA agonist is replaced by EtOH binding to the now vacant receptors.

Additionally, it must be considered that EtOH has been shown to act on other systems, including serotonergic neurotransmitter systems (Barr et al., 2003; Ferraz & Boerngen-Lacerda, 2008). This may be one reason for the complexity in interpreting the effects of EtOH. Many EtOH studies indicate varying effects based on the concentration of the drug, and it is likely that the MG circuit is no different. Altering the EtOH concentration following muscimol pre-treatment could alter the type of response and neurotransmitter systems involved instead of simply altering the degree of response.

Thus, it is possible that muscimol and EtOH are working through completely different mechanisms. For example, the prolonged muscimol-induced EPSP increase that is seen during EtOH exposure may be the result of the muscimol-induced EPSP decrease, typically observed in washout, being offset by the simultaneously occurring EtOH-induced increase. However, given the history of research that indicates an interaction between muscimol and EtOH, it seems likely that at least some of the interactions are mediated through the GABAergic system.

Chapter 8: General Discussion

Discussion

Since I have already discussed the results of my dissertation at the end of each chapter, I will only provide a broad, general discussion here as well as some ideas for future research that can build on my work.

Throughout the course of this dissertation research, I have shown that the crayfish, *Procambarus clarkii*, can serve as an effective model for the physiological study of acute EtOH exposure. Previous work in the Crustacean Neurobiology and Behavior Lab (PI: Herberholz) has shown that crayfish are behaviorally sensitive to EtOH and produce intoxicated behaviors that are easily compared to vertebrate models including humans. I have now provided evidence that the lateral giant (LG) neuron, a single neuron that has been the focus of electrophysiologists for more than 70 years (Wiersma, 1947; Furshpan and Potter, 1959), becomes more excitable after EtOH exposure and that this effect can be altered by the recent social experience of the crayfish. Additionally, I have shown that descending inputs from the crayfish brain regulate the local sensitivity of the LG circuit as higher concentrations of EtOH are required to elicit a neural response in preparations where brain inputs are removed. Since the mechanisms underlying sensory activation of the medial giant (MG) circuit have not been explored, but MG activity is behaviorally more relevant than LG activity (Herberholz et al., 2001), and the MG is implicated in social-based decision making (Edwards et al., 2003) and value-based decision making (Liden et al., 2010), I characterized the physiological characteristics of this single neuron. This

included its response to EtOH and the corresponding GABAergic inhibition. Using neuropharmacology and intracellular electrophysiology, I found that the MG, much like the LG, receives significant GABAergic inhibition after stimulation of mechanosensory antenna II afferents that dominate the later components of the excitatory post-synaptic potential (EPSP). Furthermore, I was able to show that the MG EPSP is facilitated by EtOH exposure, and that the application of a GABA agonist, muscimol, reduces this EtOH-induced facilitation. Together this shows that the facilitation induced by EtOH exposure is, at least in part, due to the alterations of the GABAergic system.

It can be argued that the major societal problems induced by alcohol are largely the result of chronic alcohol abuse (NIAAA, 2012), while the research presented here is entirely the result of acute alcohol exposure. Although these acute exposure experiments have value in identifying the cellular mechanisms underlying alcohol intoxication, they do not address chronic abuse. In the future, it would be possible to house animals in low-concentrations of alcohol for extended periods of time to test the effects of chronic exposure, as well as other drug related symptoms such as withdrawal and tolerance. However, acute exposure does have significant human-health and societal implications. As discussed in the introduction, the number of alcohol-related fatalities in vehicular collisions is incredibly high, with more than 10,000 deaths per year in the United States (NIAAA, 2012). While understanding chronic exposure is important for improving treatment of alcohol use disorders, many negative behavioral effects are the result of acute exposure. Additionally, acute doses of alcohol generally result in disinhibition in various human and non-human models.

The physiological mechanisms underlying these effects are still unknown and a model capable of using well-understood neural circuits is extremely valuable. Most importantly, I have found that the effects of acute alcohol exposure depend on the previous social experience of the animal, and this has several broad implications for possible alcohol disorder treatments. As I have shown in chapter 4, social experience in crayfish can alter the effect of acute alcohol exposure. The implication for this finding is that the importance of social history of an individual might have to be taken into account in alcohol treatment. Perhaps treatments (both pharmacological and behavioral) vary in efficacy due to the individual social backgrounds of each patient seeking treatment. Incorporating social history into treatment may allow a more targeted approach that could improve abstinence and decrease relapse in patients with alcohol use disorders. If my hypothesis is correct, and future work confirms, that social experience alters receptors in the brain (e.g., GABA), which then changes the cellular targets for alcohol, more specific pharmacological treatments can be developed for these receptors types.

Future Directions

While the research discussed in this dissertation has shown neurophysiological effects of EtOH on the two giant-mediated tail-flip circuits, full exploration of the mechanisms underlying acute EtOH exposure requires more work. Additionally, the work here can be taken in numerous directions exploring the interactions of EtOH with value-based decision making, agonistic behavior, and the mechanisms of chronic EtOH exposure. For the remainder of this chapter, I will

discuss some of these possible directions that seem immediately apparent based on the research that I have conducted.

Mechanisms of Acute EtOH Exposure

It is known that EtOH operates through many different systems, including GABA and serotonin, but my research has focused on GABA. This is due to a large body of existing work linking tail-flip escape circuits to GABAergic inhibition (Roberts, 1968; Edwards et al., 1999; Krasne et al., 2015). I have shown that EtOH interacts with GABAergic inhibition, possibly through occupying the same receptors as muscimol in the MG circuit, but the specific mechanisms by which EtOH exerts its effects requires further exploration. Additionally, I have shown that acute EtOH exposure of the isolated tail preparations suggests that descending inputs from the crayfish brain, possibly descending tonic inhibitory inputs to the LG, are important for EtOH sensitivity. While important for EtOH sensitivity, I have also shown by removing these inputs that EtOH has a local effect on the LG circuit as higher EtOH concentrations still produce a change even after these inputs are removed. Future experiments would be necessary to determine the specific role of tonic inhibition in EtOH sensitivity. Injections of GABA agonists or antagonists into EtOH-exposed freely behaving crayfish can also directly tie EtOH-induced behaviors to interactions with GABAergic systems.

While my research has targeted the interaction of EtOH and GABA, crayfish escape circuitry can also serve as a model for the interaction of EtOH and serotonin neurotransmitter systems (Yeh et al., 1996; Yeh et al., 1997; Edwards et al., 1999; Teshiba et al., 2001; Krasne et al., 2015). It has been shown that distributions of

serotonin receptors may be altered in animals of different social experience, which may mediate the various behavioral and cellular effects after serotonin application. Given this information and the fact that serotonin is a known target of EtOH (Barr et al., 2003; Ferraz & Boerngen-Lacerda, 2008), it seems likely that the serotonergic neurotransmitter system in crayfish may also be implicated in socially-mediated differences in EtOH sensitivity.

Crayfish Value-Based Decision-Making and Alcohol

I have shown that the MG neuron is facilitated through exposure to 100 mM EtOH. This facilitation could have interesting implications for value-based decision making that crayfish have been previously shown to exhibit using the MG tail-flip (Liden et al., 2010). Crayfish are capable of making value-based decisions when presented with a threatening visual stimulus during food-directed foraging behavior. Crayfish will either freeze in place or perform an MG tail-flip as they move towards an expected food source and perceive an approaching shadow. The proportion of freezes over MG tail-flips changes with increasing value of the food odor, or increasing the hunger state of the animals. Additionally, making the shadow stimulus more threatening can promote more MG tail-flips (Liden & Herberholz, 2008). Liden et al. (2010) suggested that crayfish exhibiting freezing behavior is a better behavioral choice when food is of high importance and risks must be taken. Freezes keep the animal closer to the food than tail-flips, but tail-flips are probably the safer choice to avoid predation.

This established behavioral paradigm allows for an easy adjustment to study the effects of GABAergic inhibition and acute alcohol exposure on decision making. As I have shown in chapter 6, GABAergic inhibition is present in the MG neuron. This inhibition is a possible mechanism for this value-based decision as adjusting MG excitability by increasing or decreasing GABAergic inhibition can change the likelihood of MG activation. Similar to the experiments I performed to describe GABAergic inhibition of the MG circuit, pharmacology can be useful for exploring the interaction of inhibition and decision making by injecting crayfish with GABA agonists or antagonists prior to shadow exposure. These injections can then be used to block any EtOH-induced effects that could be observed. Following these experiments, it would be more approachable to explore the interaction of EtOH, decision-making, and GABAergic inhibition.

The effect that acute intoxication can have on decision making is already an established vein of research in mammals and non-mammalian vertebrates (Luca and Gerlai, 2012). In a looming stimulus paradigm similar to the shadow used in Liden & Herberholz (2008), Luca and Gerlai (2012) have shown that acute alcohol exposure for 60 minutes blocks the fear response in zebrafish to threatening overhead bird stimuli. This work suggested an effect on risk assessment as general locomotor function was unaffected on all but the highest alcohol dose. Similar experiments using the shadow paradigm are currently underway in the CNBL: Crayfish are tested in the shadow apparatus after being exposed to EtOH for a short period of time only, enough that the animals are “mildly” intoxicated, but not long enough that EtOH-induced changes are observed. This experimental approach offers a unique

opportunity to test the effects of EtOH on risk-taking behavior, and the behavioral results can be linked back to a single neuron. The work presented in my dissertation on the properties of the MG neuron can inform these behavioral results and mechanisms can be further explored through blocking or facilitating the EtOH-induced effect through injection of GABAergic agonists and antagonists.

Crayfish Social Behavior

Crayfish engage in well-described agonistic encounters between pairs with one animal emerging as a dominant and the other as a subordinate (Herberholz et al., 2001; Edwards et al., 2003). Throughout chapter 4, I have shown that different social experiences can alter EtOH sensitivity, with communal animals being more sensitive than those that had been socially isolated prior to EtOH exposure. Communal animals in these experiments are likely to have highly variable social backgrounds and would include crayfish that were subordinate and others that were dominant at the time of exposure. It would be of interest how these specific backgrounds could influence EtOH sensitivity. While the animals used in experiments described in chapter 4 are removed from a tank housed with other animals, future experiments would need to pair animals together, possibly multiple different times, for agonistic encounters to determine dominant or subordinate status.

In addition to better understanding the influence of specific social backgrounds on EtOH sensitivity, my work exploring the physiology and GABAergic inhibition of the MG may inform its role in social-based decision making. As described earlier, an animal that will become subordinate following an agonistic

encounter will typically trigger an MG escape response to end the contest. While I have shown that the MG is inhibited through GABAergic mechanisms (experiment 6.4, Chapter 6), it remains unclear if modulation of this inhibition is the underlying reason for triggering an MG escape in this context. While the interaction of EtOH and social behavior is also of major interest, understanding how this social decision is made can be extremely important for understanding any EtOH-induced alterations of social behavior. Alterations of the GABAergic neurotransmitter system through the injection of various agonists and antagonists while the crayfish is engaged in a fight may prove valuable in determining GABA's involvement. Additionally, it allows for the use of pharmacological manipulation of GABAergic neurotransmitter systems to alter EtOH's effect, thereby elucidating possible mechanisms by exacerbating or eliminating the effect depending on the pharmacological agent used.

There is a large volume of research studying the effect of EtOH on social behavior in many different model organisms. It is clear that alcohol influences violent behavior (Leonard, 2001; Norstrom & Pape, 2010) and an aggressive model with a fewer number of neurons like the crayfish could be very valuable to further explore this. While chapter 4 has shown that social experience can influence alcohol sensitivity in the LG circuit with communal animals being more sensitive to lower EtOH concentrations than socially isolated animals, crayfish also have a rich history of research in social behavior and aggression (Edwards & Herberholz 2005, Herberholz 2014). In other species, alcohol has been shown to have significant effects on aggressive behavior. In non-human primates, lower EtOH doses were shown to increase the occurrence of aggressive behaviors in dominant monkeys while

aggression in subordinate monkeys was largely unaffected (Winslow and Miczek, 1985). In addition, research in humans have shown that alcohol increases aggression in a wide gamut of laboratory paradigms including administration of shocks to a fictitious opponent or in tasks where participants can take something of value from an opponent (Heinz et al., 2011). Thus, studying the effect of alcohol on aggression and social dominance formation in crayfish could be complemented by the knowledge gained in my dissertation. Because changes in the excitability of the MG neurons are likely involved during agonistic encounters and when one animal submits to its opponent, an understanding of the underlying cellular mechanism for these changes is now available.

Crayfish interact with other members of their species through highly stereotyped agonistic encounters in order to form dominance hierarchies (Kravitz & Huber, 2003; Herberholz, 2014; Herberholz et al., 2016). These hierarchies are formed to determine first access to important resources such as space, food, and mates (Bovbjerg, 1953; Herberholz et al., 2007). In many of these studies, aggressive behaviors are quantified and calculated into a dominance index, a number used to compare the relative level of aggression of an animal with subordinate behaviors. EtOH-exposed animals could be exposed to a concentration that would not elicit the behavioral symptoms of intoxication described earlier in this dissertation before being placed into size-matched pairing with a non-intoxicated opponent. The proportion of intoxicated animals that won and their average dominance indices would be compared against control animals. If EtOH exposure does have an influence on crayfish aggression, I would predict the following outcome. If EtOH increases

aggression, EtOH-exposed animals would have higher dominance indices and win more fights than non-EtOH exposed controls. In addition, these animals would be likely to obtain dominant status over their non-EtOH pre-exposed opponents. Additionally, manipulation of inhibition in the MG circuit while animals are exposed to GABA could demonstrate important interactions between inhibition, EtOH, and agonistic behavior.

Cellular Model of Tolerance and Withdrawal

As mentioned in the general discussion earlier in this chapter, an interesting avenue that future research could approach would be through the neurophysiological mechanisms of chronic EtOH exposure. While Macmillan et al. (1991) and Hausknecht et al. (1992) largely focused on the behavioral effects of chronic EtOH exposure in crayfish, neither explored the effect of EtOH on individual neurons using single-cell electrophysiology. Macmillan et al. (1991) showed chronic EtOH exposure for one week increased the time it took for the animal to right itself when placed in a supine posture (righting reflex), and this time could be increased by increasing the EtOH concentration in the bath. Physiologically, this study focused on the effect EtOH had on muscle receptor organs of the dorsal abdominal joints through extracellular recordings. In Hausknecht et al. (1992), animals were exposed to four weeks of low concentration EtOH in their housing tanks and showed similar behavioral symptoms to those seen in other species with increased behavioral activity at the beginning of exposure, which was followed by decreased performance in righting behavior. The use of giant tail-flip circuitry (MG or LG), which I have shown to be highly susceptible to acute EtOH application, would allow investigating the

cellular mechanisms underlying tolerance and withdrawal. This could also be compared to existing work that described the effects of other drugs, opiates and amphetamines, on withdrawal and drug-seeking behavior in crayfish (Huber et al., 2011; Imeh-Nathaniel et al., 2014).

Medial Giant Circuit Anatomy & Physiology

While an entire chapter of this dissertation has been dedicated to describing the physiological properties of the MG circuit, questions remain regarding the anatomy and physiology of this escape circuit. While evidence from my studies suggest that GABAergic inhibition alters MG excitability, the locus of this inhibition remains unclear. What is known regarding tonic inhibition in the LG circuit would suggest that GABAergic inhibition likely occurs in the dendrites (Vu & Krasne, 1992). This would suggest that inhibition present in the MG may also be located in the dendrites. Input resistance changes following exposure to the GABA agonist, muscimol, suggests that the influence is at least partly post-synaptic, that is, occurring directly on the MG itself. Immuno-labeling of GABA receptors of the MG could verify the location of this inhibition. Of course, it is important to note that the MG is also functionally different from the LG in that it receives excitatory input from multiple sensory systems, including mechanosensory and visual inputs (Liden & Herberholz, 2008; Liden et al., 2010). It is possible that the GABAergic inhibition varies across the inputs of the two sensory modalities and that inputs from different modalities are differently inhibited. Because of this, future work should determine how the inhibitory properties I have described that govern mechanosensory inputs generalize to visual inputs.

Additionally, the sensory modalities that provide excitatory input to the MG are only recently known, and therefore, the inputs themselves are not mapped and the organization of the afferent pathways is currently unknown. I have presented data which suggest the presence of both electrical and chemically-mediated synapses involved in the MG mechanosensory afferent pathway through electrophysiological experiments, but further work is needed to confirm this. These electrical synapses can be explored through various anatomical techniques including dyes that can pass through gap junctions, such as neurobiotin (Herberholz et al., 2002; Antonsen & Edwards, 2003). Further exploration of the properties of the MG escape circuit is expected to inform well-described behaviors in the crayfish including value-based and social decision making.

Publications

Much of the work presented in this dissertation is in preparation for publication. The effect of acute EtOH exposure on the LG and the influence of social experience project (chapter 4) is in the final stages of preparation. The data I have collected will be combined with behavioral data and freely behaving electrode-implanted electrode animals to form a single paper. The advantage of including these projects together is that it introduces the tail-flip circuit as a model for EtOH from its behavioral sensitivity down to the circuit and single cell. This publication is planned for submission to the *Proceedings of the National Academy of Sciences (PNAS)* once the final manuscript is completed. Since little is known regarding the neurophysiological properties of the MG circuit, the data collected for chapter 6 will

be prepared for another submission. This project will likely not be combined with behavioral data and will likely be submitted to a journal such as *Journal of Neurophysiology*. In regards to the dissertation projects, the experiments showing the effect of EtOH on the MG circuit and the alteration of EtOH's effect using the GABA agonist, muscimol, will be combined with behavioral data involving the described shadow paradigm (Background, Chapter 5). Trials are currently being run in the Crustacean Neurobiology and Behavior Laboratory that exposes juvenile crayfish to 1M EtOH before their responses to the shadow danger signal are tested.

In addition to the publications in preparation for this dissertation work, I have co-authored a paper discussing the dynamics of crayfish agonistic behavior, earning the cover of *Biological Bulletin* in its April, 2016 issue (Herberholz et al., 2016).

When equally-sized crayfish are paired, they engage in agonistic behaviors resulting in one animal being the dominant and one animal being the subordinate leading to a stable dominance relationship between both animals (Graham & Herberholz, 2009; Herberholz et al., 2016). The major finding of this paper is that established dominance relationships between two crayfish can be destabilized by the presence of a larger intruder that defeats both original opponents, but this destabilization only occurs when the original subordinate is witnessing the dominant's defeat.

In addition to being in the final stages of preparing the LG EtOH work (Chapter 4), I am also involved in the final preparations for another paper focused on the coordination of antennal scales in tail-flip escape behavior. Crayfish possess antennal scales located near their rostrum that undergo rapid extension during tail-flip escape behavior with a high degree of temporal coordination. These scales are extended at

different time points during a tail-flip depending on the type of escape circuit activated, LG or MG. Interestingly, these differences are observed when activating the giant neurons directly through intracellular current injections implicating a new and previously unknown role for these circuits.

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