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# Dopamine Modulates the Lateral Giant Neuron and Serotonergic Facilitation in Crayfish

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**DOPAMINE MODULATES THE LATERAL GIANT NEURON AND  
SEROTONERGIC FACILITATION IN CRAYFISH**

Thesis submitted to  
the Graduate College of  
Marshall University

In partial fulfillment of  
the requirements for the degree of  
**Master of Science**

Biological Science

By

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Approved by

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Marshall University  
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## LIST OF ABBREVIATIONS

5-HT	serotonin
5-HT <sub>1</sub>	type 1 serotonin receptor
AP	action potential
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
DA	dopamine
D <sub>1</sub>	type 1 dopamine receptor
EGTA	ethylene glycol tetraacetic acid
EPSP	excitatory post synaptic potential
A6	6 <sup>th</sup> abdominal ganglion
GPCR	G protein-coupled receptor
IP <sub>3</sub>	inositol triphosphate
LG	lateral giant interneuron
LTP	long term potentiation
PDE	phosphodiesterase
PKC	protein kinase C
PLC	phospholipase C

# DOPAMINE MODULATES THE LATERAL GIANT NEURON AND SEROTONERGIC FACILITATION IN CRAYFISH

Joshua S. Titlow

## ABSTRACT

The lateral giant (LG) neural circuit is a model system used to study the function of individual neurons. The LG circuit is part of a tail flip escape reflex that is a defensive behavior for crayfish. This thesis begins by addressing the effects of dopamine (DA), a neurotransmitter involved in normal and abnormal behaviors of most animals. Here it is shown that dopamine decreases the excitability of the LG neuron, a trigger for the escape reflex.

An electrophysiology protocol was used to mimic sensory input to the LG neuron. Stimulating a sensory nerve in the last ganglion with an electrode evoked an excitatory post synaptic potential (EPSP) in the LG similar to a real tactile stimulus. Exogenous dopamine was applied to the mostly intact nervous system at different concentrations and different bath temperatures while EPSPs were recorded from inside the LG's axon. Dopamine consistently caused EPSPs to decrease 10% in bath temperatures near 20°C. Below 17°C the depressive effects of dopamine were more variable.

The second part of this thesis addresses the effects of dopamine and serotonin (5-HT) on the LG. The influence of serotonin on behavior and physiology has been shown to overlap with that of dopamine. Both amines are present in the terminal ganglion near the LG, and both are expected to reach the LG through classical synapses, volume transmission, and through the hemolymph. Therefore the effects of dopamine and serotonin on excitability of the LG were tested. Together the amines increased the amplitude of EPSPs



beyond threshold to evoke action potentials in the LG. However, when dopamine was applied long before serotonin, EPSP amplitude did not increase. These data indicate that there is a temporal component to the summation of modulatory effects in neurons. Cross talk between the two systems should be considered to gain a better understanding of diseases associated with either amine. Monoamine receptors and second messengers that mediate the effects of dopamine and serotonin are highly conserved.

KEY WORDS: modulation, metamodulation, dopamine, serotonin, crayfish, lateral giant neuron, facilitation, depression, excitatory post synaptic potential, plasticity

## **Chapter 1- General Introduction**

*Dopamine and serotonin act as neuromodulators to fine tune behavior.*

Without dopamine (DA) in the central nervous system it would be nearly impossible to control basic motor function or experience gratification. Dopaminergic neurons projecting into a particular area of the nervous system give an indication that the monoamine is involved in the function of that area. Thus, dopaminergic cells innervating the striatum are associated with motor control, those innervating the limbic system have autonomic and memory-related functions, and dopaminergic cells in the cerebral cortex are involved with cognition. This simplified paradigm of dopamine pathways in the brain reflects decades of immunohistochemistry and functional imaging in rodents and primates that has provided insight into how dopamine is involved in addiction, schizophrenia, Parkinson's disease, and depression. Still there are aspects of dopaminergic modulation that are not completely understood.

Serotonin (5-HT) is a similar monoamine with distribution in the brain that overlaps with dopamine, making it likely that dopamine is not the only neurochemical involved in those functions (Baskerville and Douglas, 2010). Manipulation of neural circuits with selective serotonin reuptake inhibitors is a common approach for the treatment of depression. Serotonergic neurons innervate many of the same nuclei that are targets of the dopamine pathways and have been shown to modulate dopaminergic neurons (Olijslagers et al., 2006; Di Giovanni et al., 2008). In the mouse striatum, serotonin is taken up via the dopamine transporter into dopaminergic neurons, and the two amines are coreleased

(Zhou et al., 2005). The neocortex and striatum, which are involved in conscious thought and memory, are examples of nuclei that are potentially affected by both molecules. They receive input from dopaminergic and serotonergic cells and express receptors for both amines (Haghir et al., 2009). Working memory is modulated by dopamine and serotonin in humans (Lucianna et al., 1998). The amines are also deactivated by the same enzyme, monoamine oxidase A (Meyer et al., 2006), giving further reason to believe that their function is related. Given that dopamine and serotonin systems overlap anatomically, this study addresses overlap in their physiological effects at the level of individual neurons.

#### *Dopamine, serotonin, and sensory motor function*

Dopamine and serotonin are studied in a number of animal model systems. Because the goal here was to describe how neuronal function is affected by dopamine and serotonin, the intact mammalian brain would be a difficult place to start. Complex networks and heterogeneous clusters of neurons make it difficult to study the physiology of single cells using intact vertebrate brains. This is one reason invertebrates are often used in neurobiology research.

Movement is an important part of an animal's behavioral repertoire. Foraging, mating, and defense strategies are all based on the execution of fine movements. Avoidance reflexes are particularly well understood and are informative model systems, e.g., the sea slug gill withdraw reflex and the escape response of crickets, cockroaches, and crayfish (Kandel and Tauc, 1965). Complex neurobiological questions can be addressed in these systems, e.g., how are individual neurons affected by sensory stimuli? How do

combinations of molecules or individual molecules at different concentrations affect sensory motor function? These are questions that were addressed in this work using an invertebrate reflex system.

A neurobiological role for dopamine and serotonin appears early in evolution. In *Caenorhabditis elegans* and other roundworms both amines modulate neurons that are involved in movement (Rao et al., 2010). Dopamine and serotonin also affect movement in crustaceans, rodents, and primates. Because *C. elegans* has on the order of 300 neurons (humans have billions), the function of individual neurons and distribution of the amines is known. Immunochemical studies have identified dopamine in eight mechanosensory neurons (Sawin et al., 2000), cells that transmit electrical signals in response to mechanical energy (Goodman, 2006). Serotonin has been identified in nine different neurons that control motor function and feeding behavior (Rao et al., 2010). Behavioral studies using exogenous applications of the amines and genetic mutations to the amine systems have reiterated that movement is affected by both amines (Sawin et al., 2000). At least five *C. elegans* neurons express metabotropic receptors for both amines (Chase and Koelle, 2007). That the amine systems interact at the single cell level justifies a need to understand the functional significance of those interactions if we are to comprehend their role in this behavior.

An avoidance reflex is a behavior that generally consists of five parts: receptor, sensory neuron, interneuron, motor neuron, and muscle. Neural components of the reflex are important because they exhibit plasticity. Plasticity is the capacity of neurons to change

their function and enable learning and adaptation. Neurons do this by forming new synapses or altering their chemical makeup. The different mechanisms allow for plasticity that is appropriate in different temporal contexts, situations that are immediate, chronic, or recurring. Activity-dependent changes in neural circuits are considered a cellular mechanism for learning and memory (Pinsker et al., 1973; Izquierdo, and Medina, 1997). When a neuron fires repeatedly over a short period of time, cells adjust to the input such that subsequent input of similar intensity will evoke a greater or lesser response (Hebb, 1949).

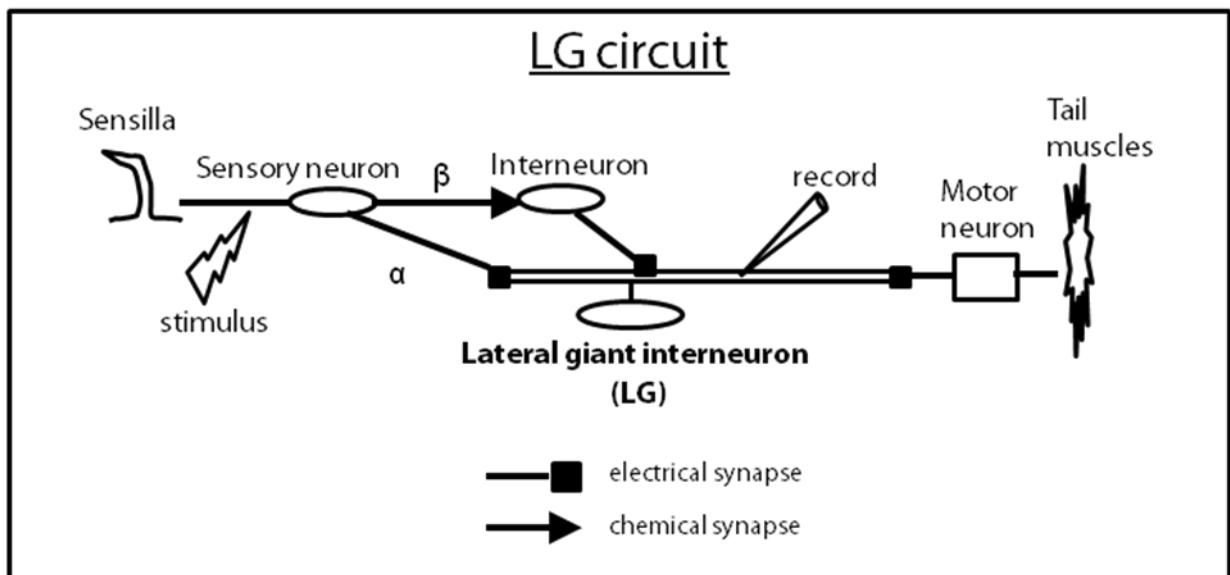
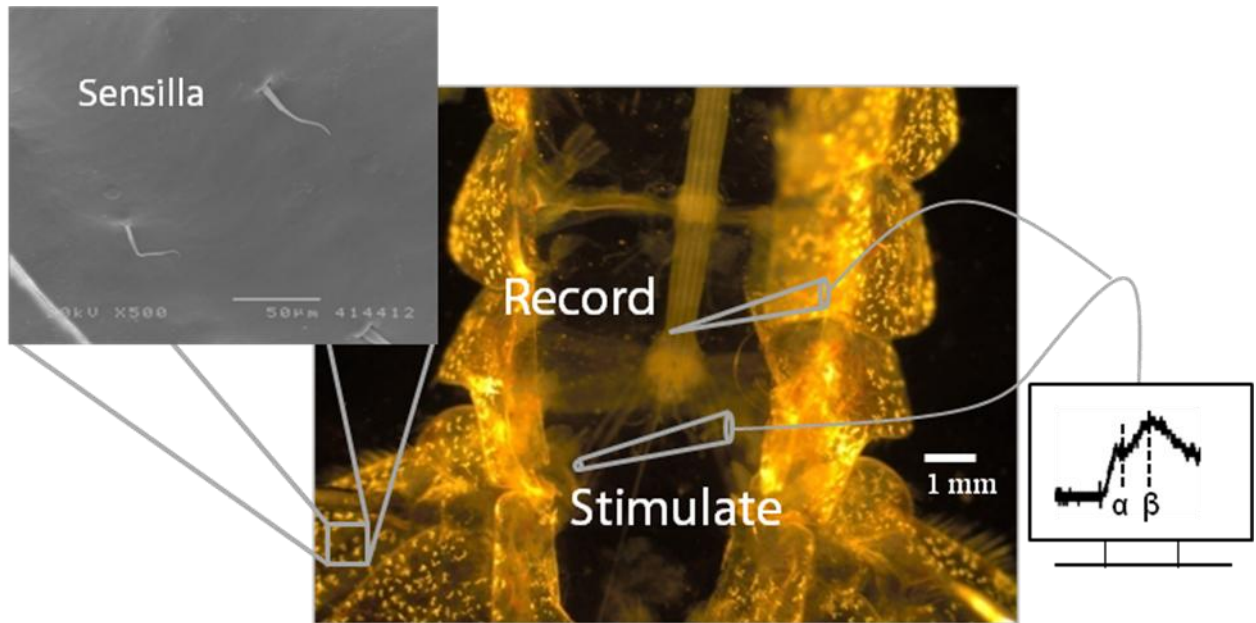
The focus of this thesis is on chemical modulation that changes the function of a neuron on the order of 15-60 minutes, changes that enable learning during agonistic encounters or other recurring situations such as foraging or mating (González-Burgos and Feria-Velasco, 2008). In this regard, dopamine and serotonin act as neuromodulators to bias a neural circuit towards the most appropriate response based on previous outcomes (Kravitz, 1988; Edwards et al., 2002). The individual change in neural circuit activity caused by either molecule is referred to as modulation; the combined effects are called metamodulation (Katz and Edwards, 1999, Mesce, 2002). Cells can be modulated by many molecules in addition to serotonin and dopamine. Due to the broad range of effects a single modulator has on a given cell, metamodulation has been overlooked, despite all indications that cells respond to more than one modulator. This combination of amines is being studied first because there are known interactions between the two systems, both in vertebrate and invertebrate animal models. The effects of serotonin in the crayfish lateral giant reflex

circuit have been thoroughly described. Two serotonin receptors (5-HT<sub>1α</sub> and 5-HT<sub>2β</sub>) have been characterized in the swamp crayfish, *Procambarus clarkii*. Their protein structure, second messenger coupling, and pharmacology are conserved between mammalian and other crustacean serotonin receptors (Spitzer et al., 2007). The 5-HT<sub>1α</sub> receptor is expressed in somata and neuropil throughout the crayfish nerve cord (Spitzer et al., 2005). It is therefore ideal to use this model system to learn if serotonin's effects are altered by dopamine.

#### *Neuroanatomy and physiology of the crayfish lateral giant circuit*

*P. clarkii* has been an enlightening model organism because it has stereotypical behaviors that can be traced to single neurons (Antonsen and Edwards, 2003). The tail flip reflex is one behavior that has been characterized down to the level of individual cells (Edwards et al., 1999; Herberholz, 2009). The lateral giant (LG) neuron is a command neuron for the tail flip reflex. The idea of a command neuron is that it is “necessary and sufficient” for producing a particular behavior (Kupfermann and Weiss, 1978). A command neuron is essentially the trigger for a behavior. When the LG fires an action potential, the impulse is sent to motoneurons that excite flexor muscles in the tail. The tail muscles contract and cause the tail to flip the animal away from predators. This is an effective defense strategy seen in lobsters and other aquatic organisms (Arnott et al., 1998).

*P. clarkii* has a nerve cord that runs the length of its body with segmented ganglia making up a brain, thoracic, and abdominal regions. The abdominal region has six ganglia, which contain motor and sensory neurons innervating the tail, and interneurons that



**Figure 1-1.** Basic structures of the lateral giant escape circuit in crayfish.

connect sensory and motor neurons. The LG interneuron integrates sensory information from tactile hairs and proprioceptors in the tail fan (see diagram and images in Figure 1-1). Primary afferent neurons convey information directly to the LG and through the giant sensory interneuron (Herberholz et al., 2002). There is a pair of LG neurons in each ganglion and two pairs in the last abdominal ganglion (A6). When one LG fires, they all fire to initiate the tail flip.

### *Serotonin and dopamine in crayfish*

Biologists interested in the neural and molecular basis of behavior began experimenting with serotonin and dopamine in crayfish and other invertebrates. Initial studies sought to characterize distribution of the serotonin in the nervous system (Elofsson, 1983; Sandeman et al., 1988; Beltz, 1999), and their effects at the organism level (Krasne, 1969; Krasne and Wine, 1975; Krasne et al., 1997). Injecting serotonin into the hemolymph (crayfish have an open circulatory system wherein the amines modulate the nervous system as hormones) caused crayfish and lobster to assume a noticeably flexed posture (Livingstone et al., 1980). This posture resembled a dominant stance, which led to the idea that serotonin plays a role in social interactions. Crayfish were an optimal model to pursue the idea because they form dominant-subordinate dyad relationships in which the pair avoids conflict to minimize stress. The idea was supported when Yeh et al. (1996, 1997) demonstrated that serotonin has opposite effects on the LG neuron in dominant and subordinate crayfish. A study by Huber et al. (1997) later showed that subordinate crayfish



(who ordinarily avoid conflict with the dominant conspecific) engaged in longer, more intense altercations with dominant crayfish following serotonin injections.

Serotonergic modulation is complex even in animals with similar social experience. Figure 1-2 shows how different doses of serotonin affect the LG's response to sensory stimulation (Teshiba et al., 2001). The amplitude of evoked excitatory post synaptic potentials (EPSPs) normalized to baseline are plotted over time. LOW concentrations of serotonin increase the amplitude of EPSPs (serotonergic facilitation), whereas HIGH concentrations decrease them (serotonergic depression). The effects of SHORT treatments are transient, whereas LONG treatments have persistent effects.

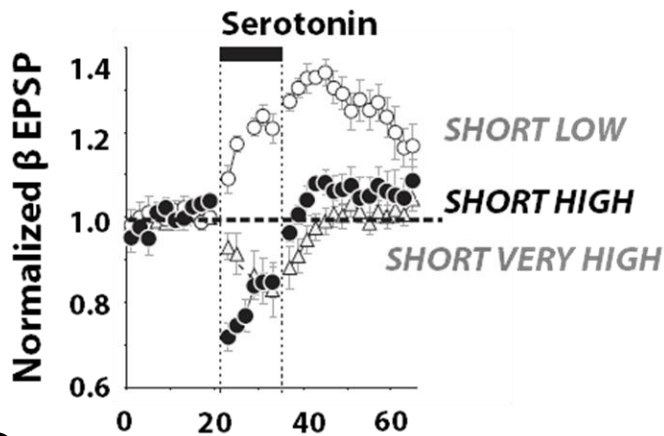
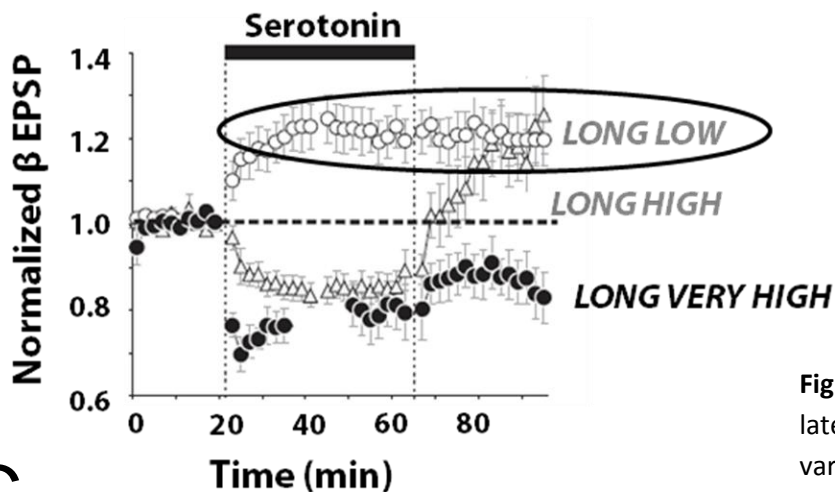
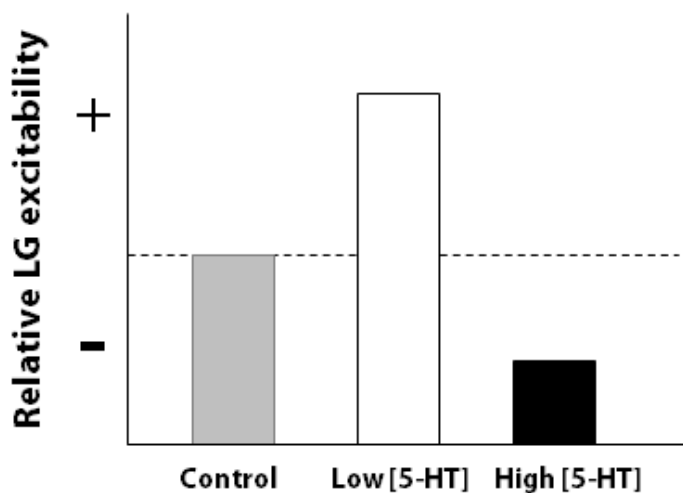
One hypothesis tested in this thesis was that dopamine interferes with serotonergic facilitation. The basis for this notion is that both amines are present in the crayfish ventral nerve cord and hemolymph where it is likely that they both have a role in shaping the function of the LG.

Dopamine has not been studied as extensively as serotonin in the LG circuit (for review of DA in crustaceans see Tierney et al., 2003). Antonsen (2008) has shown that dopamine depresses sensory evoked EPSPs in the LG neuron. He has also tested the combined effects of dopamine and serotonin on the LG neuron (Figure 1-3). Serotonin increases the amplitude of EPSPs and dopamine enhances the facilitatory effect of serotonin (Figure 1-3A,B). Combined, the amines increase EPSP amplitude 20% more than serotonin alone. Dopamine can also block facilitation if the dopamine dose substantially precedes serotonin application (Figure 1-3C). Dopamine doses that do not substantially

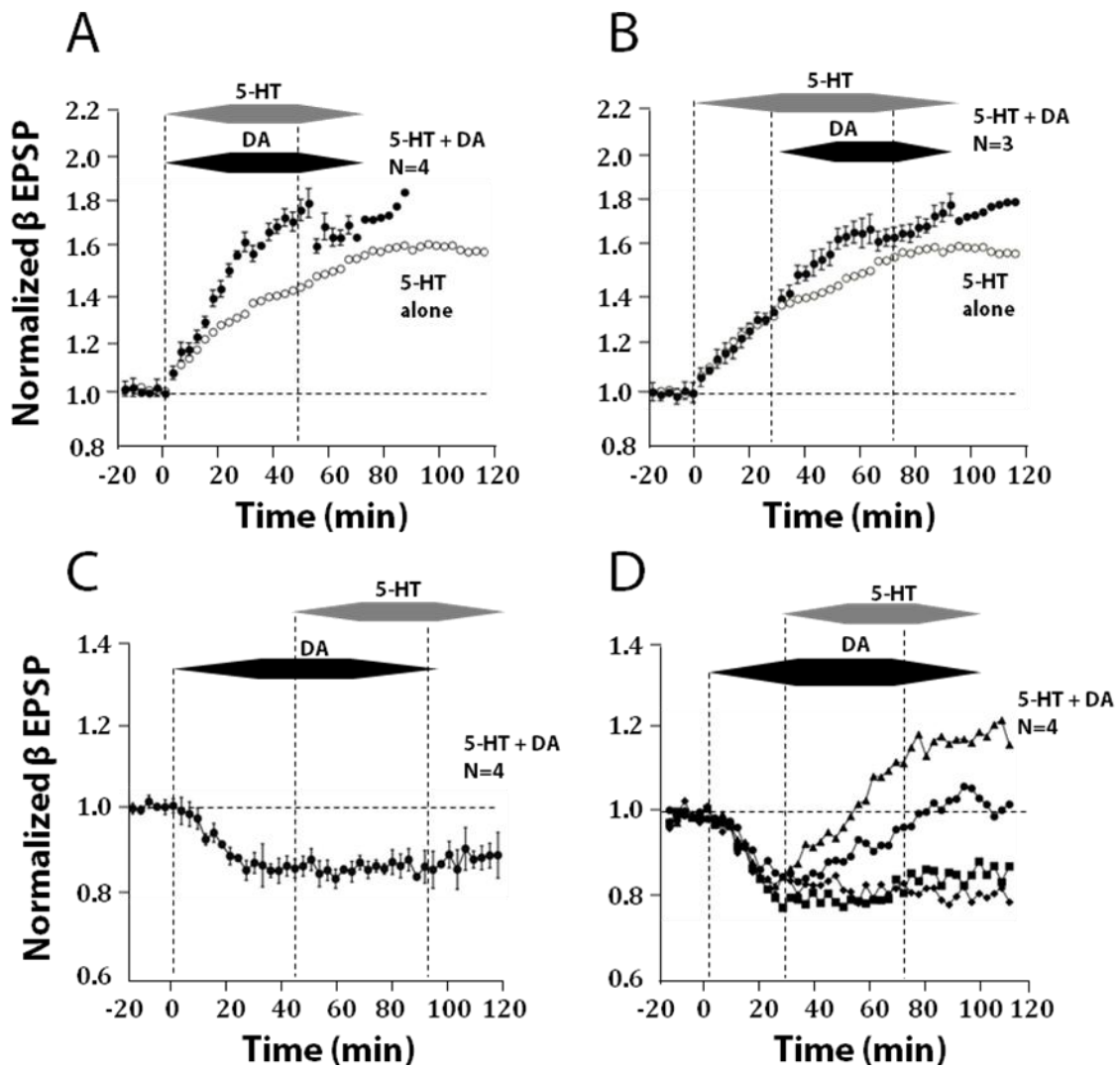
precede serotonin application have inconsistent effects on serotonergic facilitation (Figure 1-3D). This thesis adds to these data by demonstrating that dopamine indeed has two distinct effects on serotonergic facilitation.

### *Summary*

Serotonin and dopamine are biogenic amines that modulate neural circuits. In the central nervous system serotonin and/or dopamine modulate neural circuits underlying emotion, pain perception, sleep, learning, and motor activity (Arias-Carrión and Pöppel, 2007; Burkey et al. 1999; Ursin, 2002, Giménez-Llort et al., 2002). This thesis addresses the effects that the two amines have together on a well known reflex circuit. In Chapter 2, dopaminergic modulation in the crayfish LG circuit is characterized over a range of concentrations and temperatures. The combined effects of dopamine and serotonin on sensory evoked EPSPs in the LG circuit are presented in Chapter 3. Data from the novel experiments in Chapter 4 show that the dopamine system has two distinct effects on serotonergic facilitation, i.e., dopamine can either block or enhance facilitation. An appendix is included to elaborate on these findings with regard to temperature, and to present further new data on mechanosensory integration in the blue crayfish, *Cambarus monongalensis*.

**A****B****C**

**Figure 1-2.** Serotonergic modulation of the lateral giant escape circuit with respect to variations in dosage. *A-* Average EPSP values from six different experiments. SHORT LOW applications of serotonin (5-HT) increase EPSPs and SHORT HIGH applications decrease them. The effects of short applications diminish when 5-HT is removed (black bar indicates length of treatment). *B-* LONG LOW applications increase EPSPs and LONG HIGH applications decrease them. The effects of LONG applications persist after 5-HT is removed (Teshiba et al., 2001). The circled 5-HT regimen was used in chapters 3 and 4 of this study. *C-* A summary of how excitability in the LG neuron is affected by serotonin.



**Figure 1-3.** Preliminary data from the Antonsen lab. Serotonergic facilitation was enhanced when dopamine (DA) was applied with serotonin (5-HT), *A*, or when serotonin was applied first, *B*. Serotonin facilitation was inhibited when dopamine was applied 45 minutes before serotonin, *C*. The effects of dopamine on serotonergic facilitation were inconsistent when dopamine was applied 30 minutes prior to serotonin, *D*, (modified from Antonsen, 2008).

## **Chapter 2- Dopaminergic modulation of the lateral giant neuron in crayfish**

### **ABSTRACT**

The lateral giant escape circuitry in crayfish has yielded experimental data that show how dopamine, other monoamines, and neuropeptides modulate synaptic transmission at the level of individual cells. The pharmacological tools for this system are improving as dopamine receptors have recently been cloned and characterized in crayfish and in other invertebrates. Still, this system is most useful for describing physiological effects in an intact preparation because the circuitry has been identified at the level of individual cells and synapses. Using an electrophysiological approach, this chapter adds experimental data with regard to dopamine modulation in the lateral giant (LG) neuron. Dopaminergic modulation in the LG was characterized at different concentrations and different bath temperatures.

At 10  $\mu\text{M}$ , dopamine was found to depress the LG's response to sensory input. Dopamine concentrations greater and less than 10  $\mu\text{M}$  did not consistently depress the LG's response, and in some experiments elicited facilitation. It would not be unordinary for dopamine to have different effects on the LG at different concentrations. The effect of serotonin on the LG and other systems varies at different concentrations.

## Introduction

Dopamine has been identified in the nervous systems of most invertebrate groups (Alvarez et al., 2005). The simplicity of those systems makes them ideal for studying the physiological effects of dopamine, and, in many cases, behavioral effects can be studied by manipulating the dopaminergic systems *in vivo*. For example, injecting dopamine into the honeybee brain leads to a reversible decrease in conditioned responses, an element of associative learning that is conserved across species (Scheiner et al., 2006). Likewise courtship behavior is modulated by dopamine in the blue crab (Wood et al., 1995). In *Aplysia*, dopamine modulates the gill withdrawal reflex (Ruben and Lukowiak, 1983).

At the cellular level, dopamine has been shown to modulate neural circuits in a variety of animal models. Crustacean dopamine receptors are orthologs of human receptors (Clark and Baro, 2007). In the lobster pyloric network, exogenous dopamine enhances synaptic inhibition and weakens electrical coupling (Johnson et al., 2005). The monoamine has recently been shown to increase firing frequency of motoneurons, giant interneurons, and edge cells in the lamprey spinal cord (Kemnitz, 1997). Finally, dopamine increases excitability and synaptic transmission in the intact rat spinal cord (Han et al., 2007).

Here the effects of exogenous dopamine on the crayfish lateral giant escape circuit were tested. The experiments were conducted over a range of dopamine concentrations. The reason for this is two-fold: 1) physiological concentrations of the modulator vary between specimens and in neural networks within the same specimen, and 2) within a

given neural network, the same modulator can evoke different effects (Teshiba et al., 2001; Harris-Warrick and Johnson, 2010).

Because crayfish are ectotherms and startle responses in other aquatic organisms are sensitive to changes in temperature (Szabo et al., 2008), the effects of dopamine were also studied at different temperatures. With the nerve cord still attached to the abdomen in a bath solution of 20°C, slowly raising the bath concentration to 10  $\mu$ M dopamine depressed sensory evoked EPSPs in the LG neuron. Dopaminergic depression was less consistent below 17°C and at concentrations of 1  $\mu$ M or 50  $\mu$ M.

## **Methods**

### *Specimens and husbandry*

Crayfish, *Procambarus clarkii*, were obtained from Atchafalaya Biological Supply, Raceland, LA. They were kept in individual 1 L aquatic tanks lined with pebbles, so the animal could burrow, and a small pipe for them to use as a den. Lights were on at 6 am, off at 6 pm. Animals were fed shrimp pellets on Fridays, and experiments were performed during the week to prevent feeding effects. Specimens were isolated at least two weeks prior to physiology experiments to eliminate social status as a confounding variable (see Yeh et al., 1996, 1997).

### *Dissection*

Animals were anesthetized for 20 minutes in an ice bath and decapitated by transecting the circumesophageal connective posterior to the brain. The carapace, visceral organs, gill chambers, limbs and antennae were excised prior to placing animals in a dish

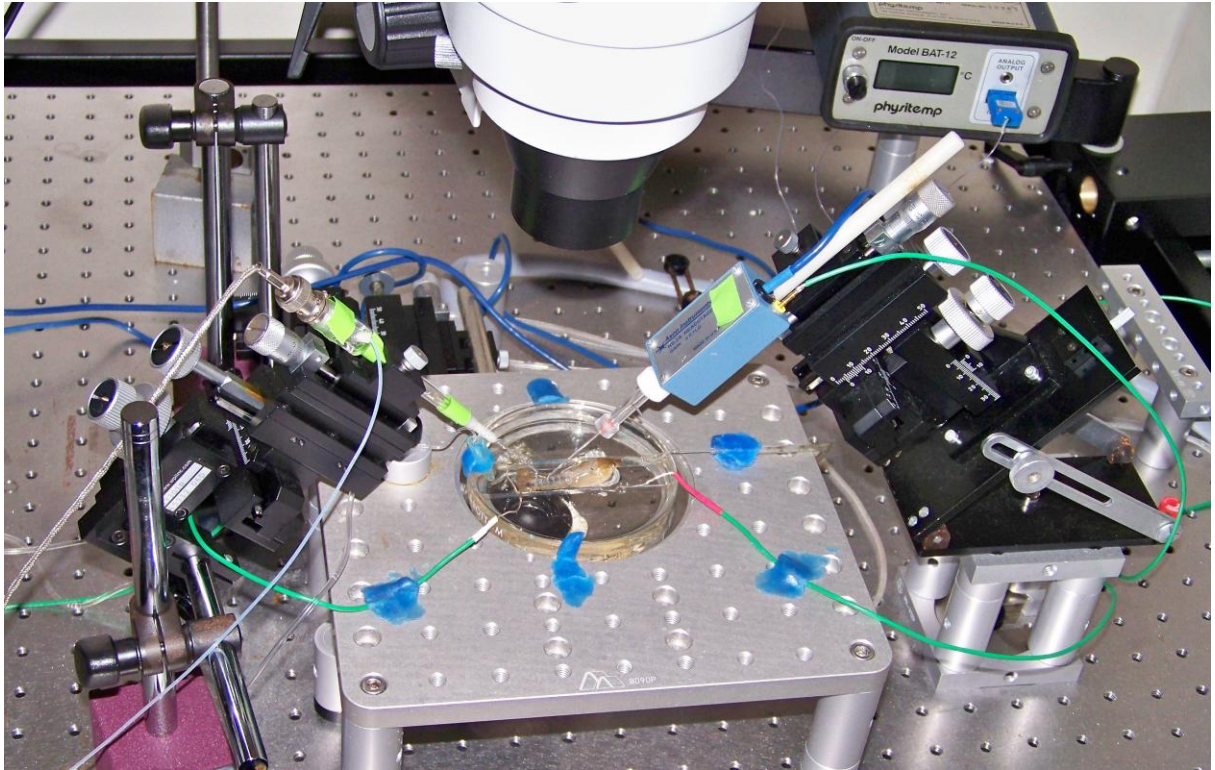
filled with oxygenated crayfish saline (202mM NaCl, 5.37mM KCl, 13.53mM CaCl<sub>2</sub>, 2.6 MgCl<sub>2</sub>, 2.4mM HEPES; pH 7.4). A portion of the distal cuticle was removed so flexor and extensor muscles could be excised, leaving the nerve cord with as many sensory nerves intact as possible (Figure 1-1).

### *Electrophysiology preparation*

After dissection, specimens were pinned to a Sylgard-lined flow chamber fitted with tubing and peristaltic pumps to circulate fresh saline and modulator solutions (Figure 2-1). For all experiments saline was dripped into the perfusion chamber at 0.35ml/min. The suction electrode (for stimulus) was attached to the second nerve root as far away as possible from the ganglion. Borosilicate glass capillary tubes (O.D. 0.9mm, I.D. 0.7 mm) were pulled so that the end of the glass had a diameter equal to the size of the second nerve, which was critical for delivering a consistent shock with minimal artifact. Recording electrodes were pulled from the same glass to resistances of 10-40 M $\Omega$  and filled with 3M KCl.

Voltage shocks were delivered with a pulse stimulator (AM systems- 2100) passing through a differential AC amplifier (A-M Systems-1700). Membrane potential was recorded and digitized through a Digidata 1200 acquisition board with pClamp 9 software (both from Axon Instruments). Temperature of the bath solution was recorded with a digital probe inserted directly into the bath solution.





**Figure 2-1.** Preparation of the crayfish for neurophysiological studies. Apparatus for electrophysiology experiments. Suction electrode on the left passes voltage shock through the second nerve, while electrode on the right records voltage from inside the ipsilateral LG. DA, 5-HT, and oxygenated saline are pumped through the recording chamber at a controlled rate and temperature.

## *Analysis*

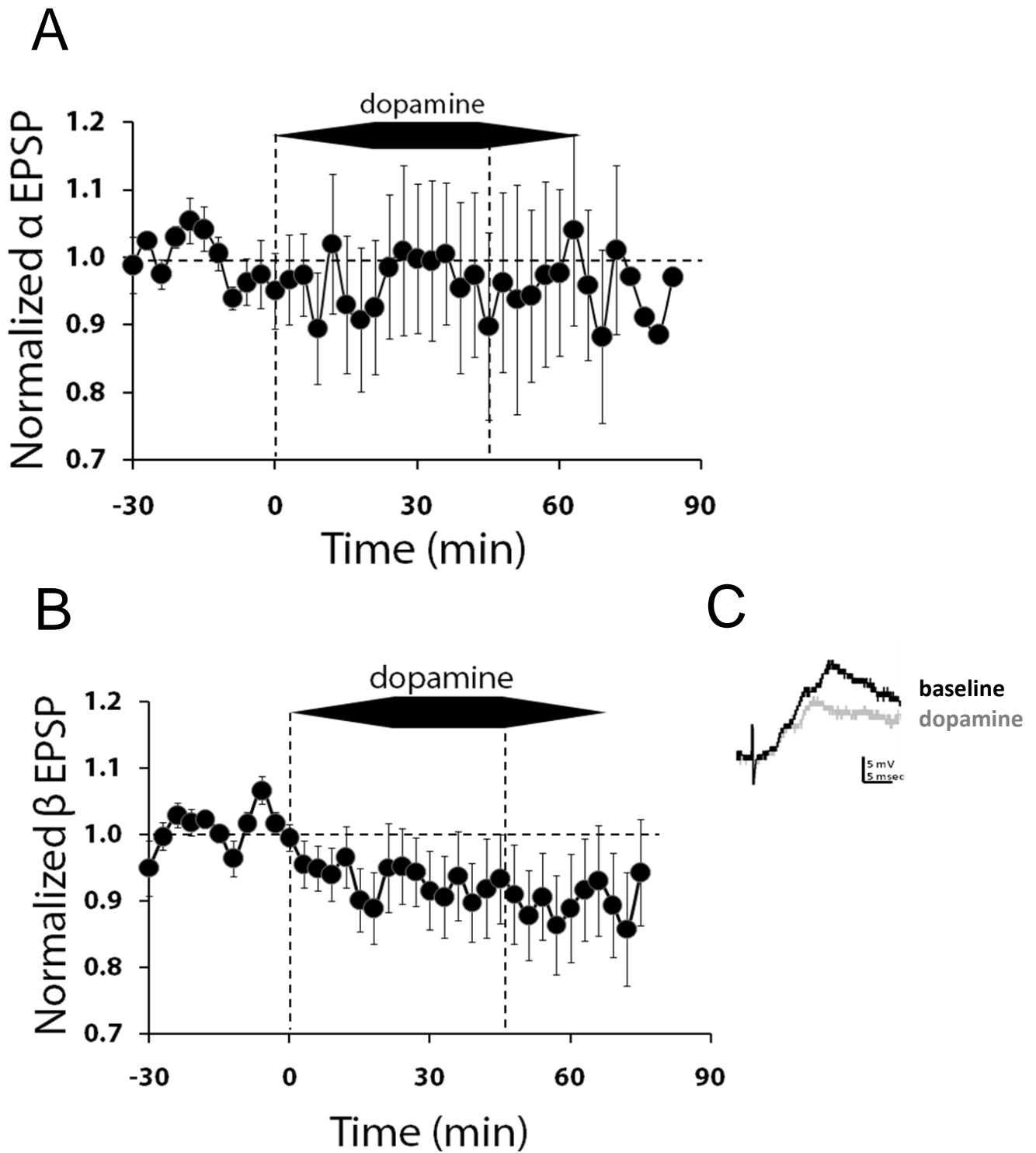
After digitization, data were stored as binary files and analyzed using Clampfit v. 10.0. Resting membrane potential and EPSP peaks were measured using cursors in the software and stored in a spreadsheet. Formulas were then written in Microsoft Excel to determine the raw amplitude and normalize the data set. Normalized data were analyzed using Friedman's test with Dunn's post-test for multiple comparison between treatments. Only  $\beta$  amplitudes were analyzed for statistical significance. This procedure is common in the literature because  $\beta$  waveforms are more robust and easier to distinguish (Lee et al., 2008), and spike threshold is normally reached at the  $\beta$  synapse.

## **Results**

### *Sensory-evoked EPSPs in the lateral giant neuron are depressed by dopamine*

This experiment was designed to mimic sensory input and test the modulatory effects of dopamine. The effect of dopamine on sensory evoked  $\alpha$  EPSPs in this data set was variable (Figure 2-2A). Deviation in  $\alpha$  EPSP amplitude was high, but many of the peaks were below the average.

During a 45-minute application of 10  $\mu$ M dopamine, sensory evoked  $\beta$  EPSPs in the LG were depressed 10% on average (Figure 2-2B). Depression persisted through the thirty minute wash and grew to 15%. The onset of dopaminergic depression was slow, though consistent with the rate in which the amine was applied. Saline in the flow chamber was completely replaced with 10  $\mu$ M dopamine within approximately 25 minutes, which was how long it took to observe the maximum change in EPSP size.



**Figure 2-2.** Ten  $\mu$ M dopamine causes depression of sensory evoked  $\beta$  EPSPs in the lateral giant neuron. *A*- alpha EPSPs were erratic and showed no obvious trends. *B*- the average decrease of  $\beta$  EPSP amplitude was 10%. Values are normalized to the first 10 trials (mean  $\pm$  SEM,  $n = 9$ ). *C*- A representative trace depicting dopaminergic depression of an EPSP.

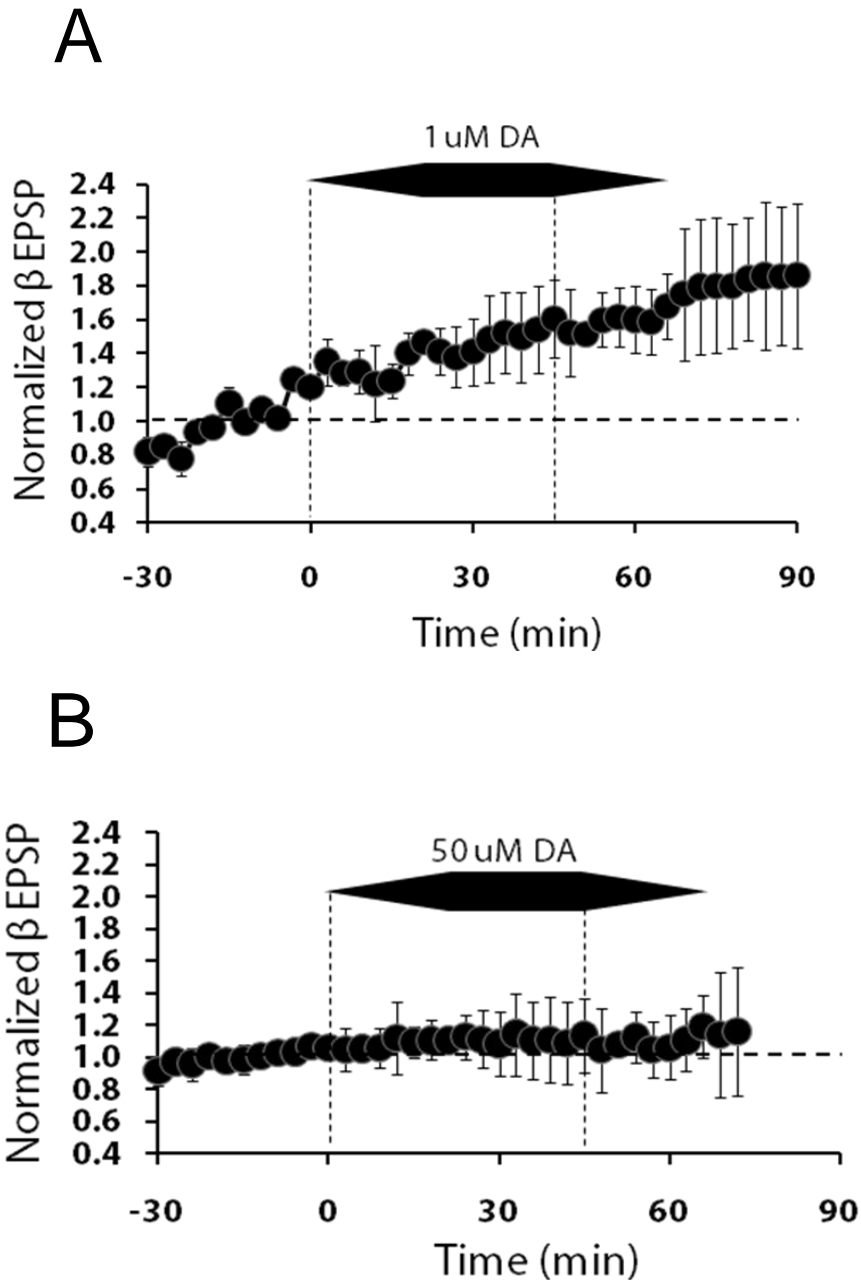
### *Dopaminergic modulation of the LG may be sensitive to concentration*

The modulatory effects of monoamines in the LG circuit have been shown to vary with concentration (Teshiba et al., 2001). Dopamine has opposing effects in the lobster pyloric network (Harris-Warrick and Johnson, 2010). After determining that a 10  $\mu\text{M}$  application of dopamine caused depression of sensory evoked  $\beta$  EPSPs, higher and lower concentrations were tested. Neither concentration effectively depressed EPSPs. If anything the 1  $\mu\text{M}$  dopamine application caused facilitation of  $\beta$  EPSPs (Figure 2-3A). In all three experiments the amplitude of the last EPSP was at least 30% larger than the baseline.

Facilitation was also observed in two experiments in which 50  $\mu\text{M}$  dopamine was applied (Figure 2-3B). However, depression occurred in two experiments, and no change was observed in the other experiment with 50  $\mu\text{M}$  dopamine. Therefore, the concentration of dopamine does appear to influence its modulatory effects on the LG neuron.

### *Temperature affects dopaminergic modulation in the LG neuron*

There was a correlation between temperature and dopaminergic modulation in these experiments. At 20° C dopamine consistently depressed EPSPs in the LG neuron by as much as 20% (Figure 2-2B). However, below 17° C there was more variation in the effects of dopamine both in magnitude and sign of the effect. In one experiment EPSPs were depressed 80%, and in six experiments facilitation occurred (Figure 2-4).



**Figure 2-3.** Dopaminergic modulation of the LG neuron at different concentrations. Applications of 1  $\mu\text{M}$  dopamine (A:  $n = 3$ ) or 50  $\mu\text{M}$  dopamine (B:  $n = 5$ ) did not consistently depress sensory evoked EPSPs in the LG neuron. The 1  $\mu\text{M}$  dopamine application appears to enhance EPSPs.

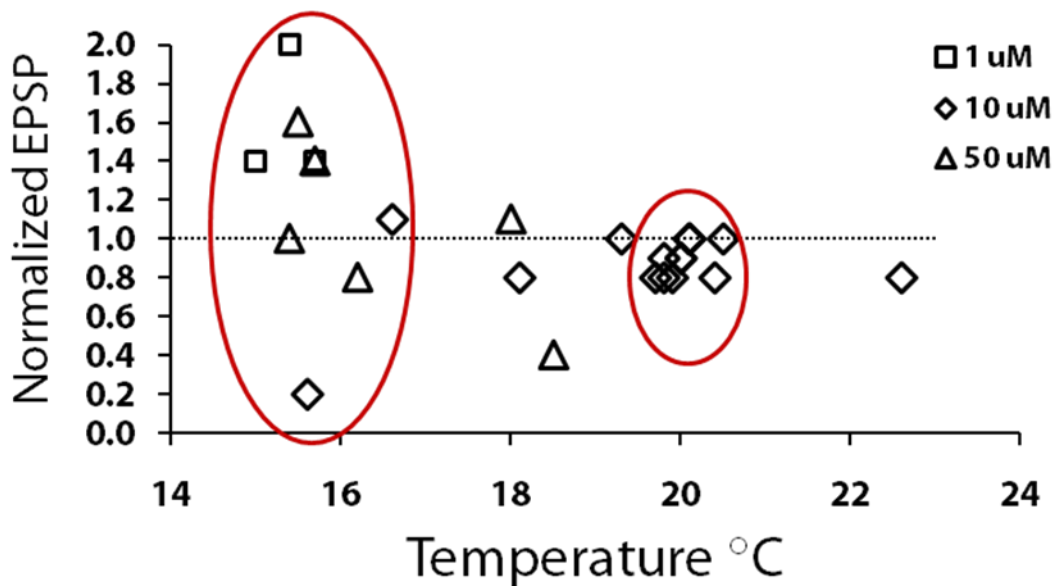
## Discussion

The data in this chapter show that a 10  $\mu\text{M}$  application of dopamine has a depressive effect on sensory evoked  $\beta$  EPSPs in the LG circuit at 20°C. Changes in temperature and/or concentration can make the effect of dopamine facilitatory.

### *Dopaminergic depression is persistent*

Dopaminergic depression continues at least an hour after the end of treatment. This persistence means that the effects are mediated through second messenger cascades, either by covalent modifications to signaling enzymes and ion channels, altering translation of those proteins, or altering transcription of those proteins. The persistence of dopaminergic modulation suggests that gene expression is affected, though gene expression does not account for the changes seen immediately after the amine is applied.

Antonsen (2008) showed that the depressive effects of dopamine on the LG neuron are completely blocked by pre-injecting the cell with ethylene glycol tetraacetic acid (EGTA), a calcium chelating agent. This calcium could be released from intracellular stores by activation of the phospholipase C (PLC) pathway. A mammalian  $D_1$ -like receptor called the PI-linked DA receptor has recently been shown to couple to the PLC/ $\text{IP}_3$  pathway (Liu et al., 2009). Moreover the  $D_{1\alpha\text{Pan}}$  receptor in the lobster stomatogastric nervous system couples with  $G_q$  that also activates the PLC pathway (Clark et al., 2008). The source of calcium could also be extracellular, as dopamine has been shown to modulate calcium currents in the lobster (Johnson et al., 2003).



**Figure 2-4.** Dopaminergic modulation at different temperatures. Shown is the normalized EPSP amplitude at the end of dopamine superfusion plotted against the bath temperature during that experiment. At 20°C the range is of dopaminergic effects is 20%. Below 17°C, the modulatory effects were more variable at each concentration that was tested.

It is also possible that dopaminergic modulation is mediated by chloride channels. Increases in membrane conductance seen during serotonergic depression are associated with a depolarization in membrane potential (Vu and Krasne, 1993), which is likely due to the opening of chloride channels (Lee et al., 2008). In *C. elegans* there are at least four ligand-gated chloride channels that can be activated by biogenic amines (Ringstad et al., 2009). The dopamine receptor LGC-53 has a high affinity for dopamine, and the dopamine GPCR antagonists risperidone, haloperidol, and spiperone all block dopamine-evoked whole-cell currents (Ringstad et al., 2009). Moreover an intracellular calcium increase produced by activation of the PLC pathway could cause a calcium-activated chloride channel to open. This hypothesis could be tested by redoing the experiments in low chloride saline.

Serotonin can also depress sensory evoked EPSPs in the LG. Though the mechanisms of serotonergic depression are also unknown, Lee et al. (2008) were able to eliminate two possibilities. A cAMP analog did not cause depression, and inhibitors of PKA and PKC failed to block serotonergic depression (Lee et al., 2008).

*Dopaminergic and serotonergic depression have similar strength, but different rate of onset*

The net decrease of  $\beta$  EPSPs in dopaminergic depression is 15-20%, a change in amplitude that is comparable to serotonergic depression (Teshiba et al., 2001; Lee et al., 2008). Dopaminergic depression of EPSPs occurs gradually, taking 30 minutes to reach maximum decrease in EPSP size. Generally serotonergic depression has a much quicker onset. It reaches its maximum modulatory change almost instantly. The variation in rate of onset could occur at the level of coupling between the ligands, their receptors, and the



respective G proteins. The G protein and downstream messengers could react at different rates while still involving the same reactants.

#### *Dopaminergic modulation of the LG varies at different temperatures*

Ectotherms have little to no intrinsic control over their internal body temperature. The temperature of aquatic ecosystems can vary by as much as 5°C on a given day, and 20°C throughout the year; thus, it is critical that neural networks be able to adapt to these changes. The goldfish startle response circuit is sensitive to changes in temperature of this magnitude. In response to a hammer repeatedly striking their tank, cold fish (acclimated to 5°C) initiated their stereotypical escape response 75% of the time, whereas the warm fish (acclimated to 25°C) responded to 100% of the strikes (Szabo et al., 2008). The difference in excitable behavior correlated with changes to intrinsic cellular properties in the underlying neural circuit. Action potentials were broader and input resistance was greater in the cold fish's Mauthner neuron, a command cell that initiates the escape response (Szabo et al., 2008). Input resistance in the LG neuron is also higher at cold temperatures (Heitler and Edwards, 1998), which would explain why facilitatory effects were seen at 16°C.

One role of neuromodulators is to broaden the functional temperature of neural circuits. Serotonergic modulation has been shown to vary in the lobster *Homarus americanus*, which has to adapt to a wide temperature range in the North Atlantic Ocean. Resting muscle tension, membrane potential, and input resistance are all modulated by serotonin, and the effects change with temperature (Hamilton et al., 2007). Increasing temperature also has an effect on neurotransmission in the mammalian brain. Field EPSPs

in mouse hippocampal slices decrease significantly when temperature is raised 6°C and quickly return to baseline when temperature decreases (Masino and Dunwiddie, 1999). Similar effects have been seen in the hamster brain, where temperature fluctuates as the animal goes into hibernation. The enhancing effect of serotonin on hippocampal slice activity is more pronounced at 15°C and 35°C than at 25°C (Horrigan and Horowitz, 1990).

The effect of dopamine was more variable below 17°C than at 20°C. Experiments over a broader temperature range in individual specimens are necessary to determine precisely how temperature affects dopaminergic modulation.

The results in Figure 2-3 should be interpreted with caution for two reasons. First, the sample sizes are small. Second, EPSP amplitudes increased gradually during the baseline, making it unclear whether facilitation was caused by dopamine treatment or an unstable preparation. Nevertheless, at these concentrations, dopamine did not cause depression. Facilitation that occurred during 1 µM dopamine treatment was 60% greater than baseline, and it was persistent through a 30 minute wash. Facilitation was observed in each of the 1 µM treatments (n = 3). The 50 µM treatment did not produce a statistically relevant change. Facilitation occurred twice, depression twice, and there was no change in the other experiment. Dopaminergic facilitation has also been shown in the rat striatum (Arias-Montaña et al., 2007).

The finding that 10 µM dopamine depresses sensory evoked EPSPs in the LG is consistent with preliminary data (Antonsen, 2008). Dopamine and serotonin have distinct

effects on the LG's responsiveness. The next chapter addresses how the LG's responsiveness is affected by both of the amines at the same time.

### **Chapter 3- Combined modulatory effects of dopamine and serotonin on the lateral giant neuron**

#### ABSTRACT

The individual modulatory effects of dopamine and serotonin have been described in many different neural circuits. They are known to have opposite effects on how the lateral giant neuron (LG) responds to sensory input. That they tend to have opposing effects and modulate many of the same circuits are compelling reasons to address whether the presence of one influences the other.

The electrophysiological protocol described in the previous chapter was again used to determine how mechanosensory integration is modulated by the monoamines. Dopamine and serotonin were combined in 500 mL of saline at concentrations that normally produce dopaminergic depression and serotonergic facilitation of transmission in the LG escape circuit.

Surprisingly, the combined (metamodulatory) effect resembled facilitation, i.e., excitatory post synaptic potentials (EPSPs) increased 20-80%. The facilitatory effects persisted for at least an hour longer than application of the drugs. In several experiments EPSPs grew above threshold and the lateral giant neuron (LG) fired action potentials. The LG is a command neuron in the circuit, meaning that an action potential in the LG is sufficient to evoke a tail flip. These results show that together these amines have a synergistic effect.

## Introduction

Serotonin and dopamine modulation have been studied in simple and complex neural circuits, and their effects are generally believed to be complementary and distinct (Kemnitz, 2005). Svensson et al. (2001) point out that the simultaneous release of neuromodulators gives them an opportunity to interact. Due to the plethora of modulatory substances (Kupfermann, 1991), and the contextual nature of modulatory effects, learning the result of every combination is impractical. However, by investigating metamodulation in neural circuits that have shown sensitivity to multiple substances, a fundamental understanding of the phenomenon is attainable.

In the leech, serotonin and dopamine both modulate the swimming network. Serotonin induces spontaneous swimming (Friesen and Kristan, 2007), and dopamine blocks swimming (Crisp and Mesce, 2004). Under the influence of both amines, dopamine trumps serotonin and swimming is blocked (Mesce and Pierce-Shimomura, 2010).

In the swamp crayfish *P. Clarkii*, serotonin and dopamine have opposing effects on sensory evoked EPSPs in the LG neuron. Dopamine depresses EPSPs 20% (Chapter 2), and serotonin facilitates EPSPs by 20-40% (Teshiba et al., 2001; Antonsen et al., 2008). Clearly the LG has the biochemical systems necessary to generate a response to dopamine and serotonin. This chapter addresses the question of how the responsiveness of the LG neuron changes when both systems are activated at the same time.

## Methods

### *Specimens and dissection*

Juvenile crayfish, *P. Clarkii*, were isolated at least two weeks prior to experimentation. Preparation of the specimens for experiments in this chapter was identical to preparation in Chapter 2. The abdomen was isolated from the rest of the body and pinned to Sylgard in the recording chamber. A suction electrode was filled with saline and attached to the second nerve in the last abdominal ganglion. A microelectrode was filled with 3 M KCl and inserted into the LG neuron on the same side. The suction electrode was then used to send an afferent volley to the LG every three minutes for the length of the experiment. Fresh oxygenated saline and/or the amines flowed through the recording chamber at 0.35 mL/min, comparable to the SLOW applications of Lee et al., (2008).

### *Amine solutions*

5-hydroxytryptamine (creatinine sulfate, Sigma) and dopamine (hydrochloride: Sigma) were individually weighed and combined in physiological saline. The final concentrations for serotonin and dopamine were 50  $\mu$ M and 10  $\mu$ M respectively.

### *Immunohistochemistry*

After dissection, specimens were stored overnight in 4% paraformaldehyde fix and crayfish saline. Nerve cords were removed, rinsed in water, then washed 6 x 50 minutes in PBTX (.5M sodium phosphate + 2% Triton X). The tissue was stored in PBTX solution overnight on a shaker at 4°C with either rabbit anti-serotonin or mouse anti-tyrosine hydroxylase antibody. PBTX washes were repeated the next day, and nerve cords were

stored overnight in PBTX with Alexafluor 568 goat anti-rabbit and Alexafluor 488 goat anti-mouse secondary antibody (Molecular Probes). The next day PBTX washes were repeated. Nerve cords were dehydrated with a series of 8-minute ethanol washes (10%, 30%, 50%, 70%, 90%, 95% and 100%), and mounted in methyl salicylate (J.T. Baker, Phillipsburg, NJ).

## **Results**

### *The effects of dopamine are concealed by serotonin*

Dopamine and serotonin bias the LG's excitability in opposite directions and to different extents. Their combined effect resembles serotonergic facilitation. The data in Figure 3-1 show that, as the extracellular concentration of both modulators increases, their net effect on sensory evoked EPSPs increases. The  $\beta$  component of the EPSP amplitude grew an average of 20% during treatment and stayed above threshold as the amines were washed out of the recording chamber.

The decrease in EPSP amplitude at the beginning of wash was an anomaly. In two experiments, the EPSP amplitude decreased during the wash but quickly recovered. The decrease was more than 40%, which was enough to have an effect on the mean. EPSP amplitudes increased by at least 15% in 7 of 10 experiments (Figure 3-2). That the EPSP amplitude increased and persisted through washout is consistent with preliminary data (Antonsen, 2008).

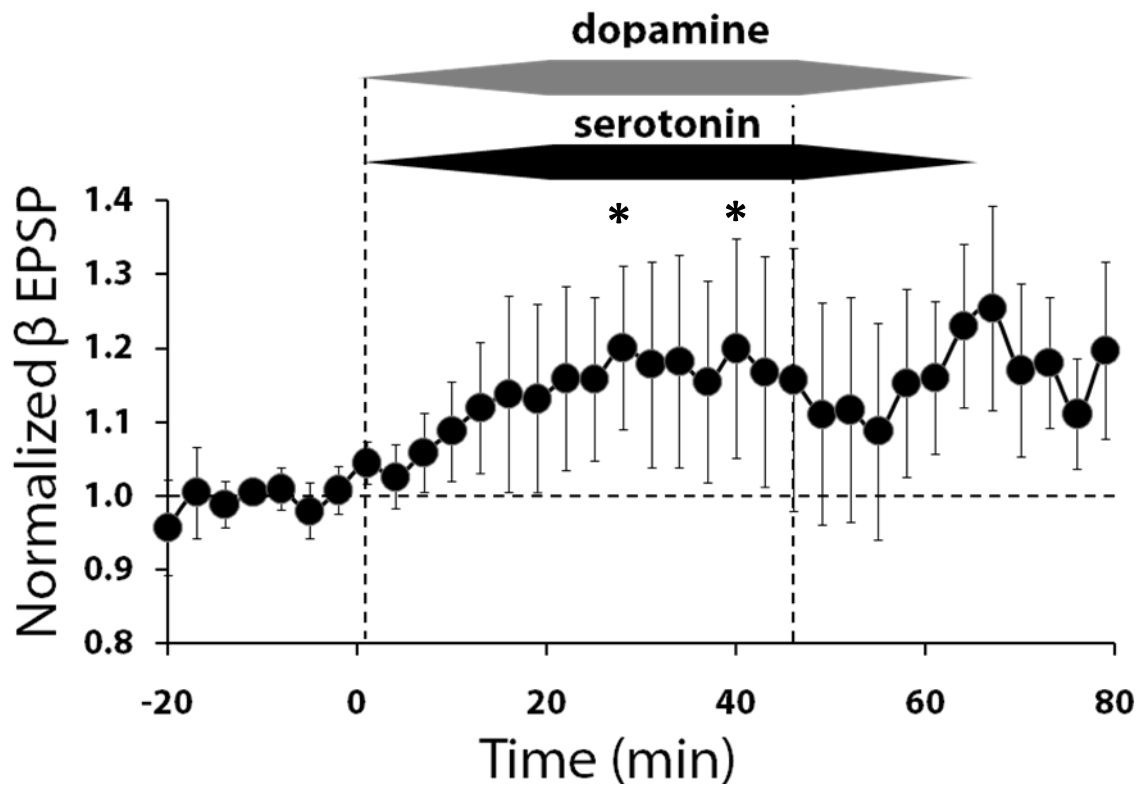
*Dopamine and serotonin increase EPSPs above threshold*

In two trials the LG neuron fired. On the first occasion, the cell reached threshold just minutes after the amines were applied. Amplitude of the  $\beta$  EPSP peak grew from 8 mV to 14 mV before firing. The cell then fired on 18 of the next 19 stimulations. Traces of two action potentials (APs) are shown in Figure 3-3. The arrow points to the first AP, which has a spike threshold potential just below 14 mV that occurs at the  $\beta$  peak. The other AP, evoked 12 trials later, has a spike threshold below 10 mV that is initiated at the  $\alpha$  peak. This has previously been seen in only in smaller animals (Edwards et al., 1994A,B).

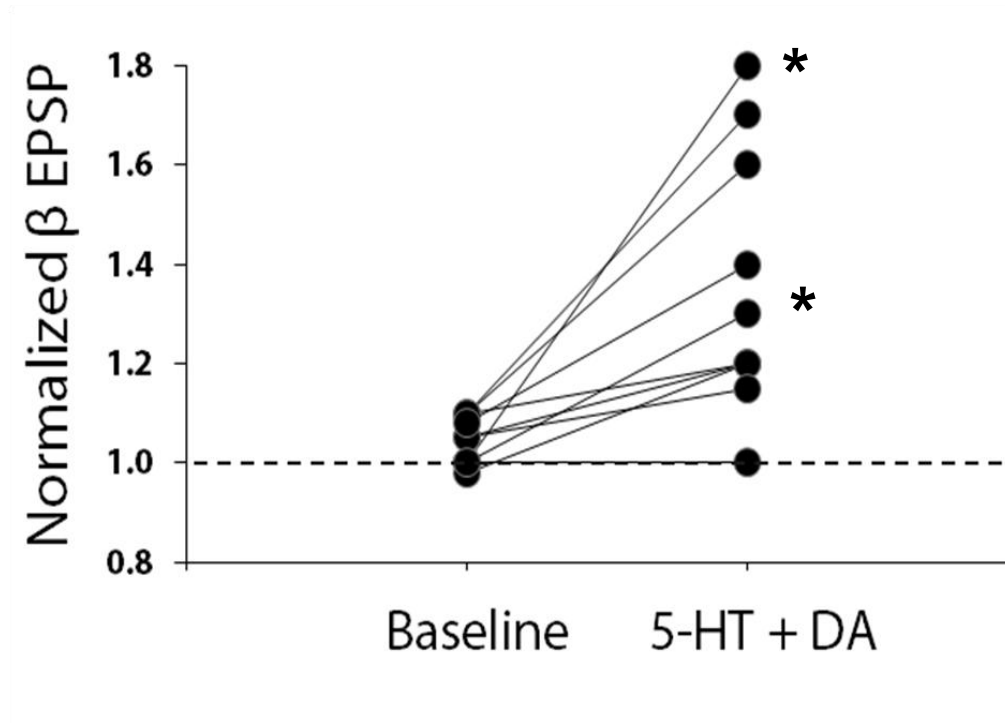
*Serotonergic and dopaminergic neurons are located in the terminal ganglion*

Nerve cords from many of the specimens were fixed and labeled for serotonin or dopamine (tyrosine hydroxylase) to confirm the existence of both amines in the last abdominal ganglion. Indeed there were neural processes and cell bodies displaying immunoreactivity for both amines in A6 (Figure 3-4). The majority of spinal boutons containing the amines are located in the medial aspect of the ganglion.

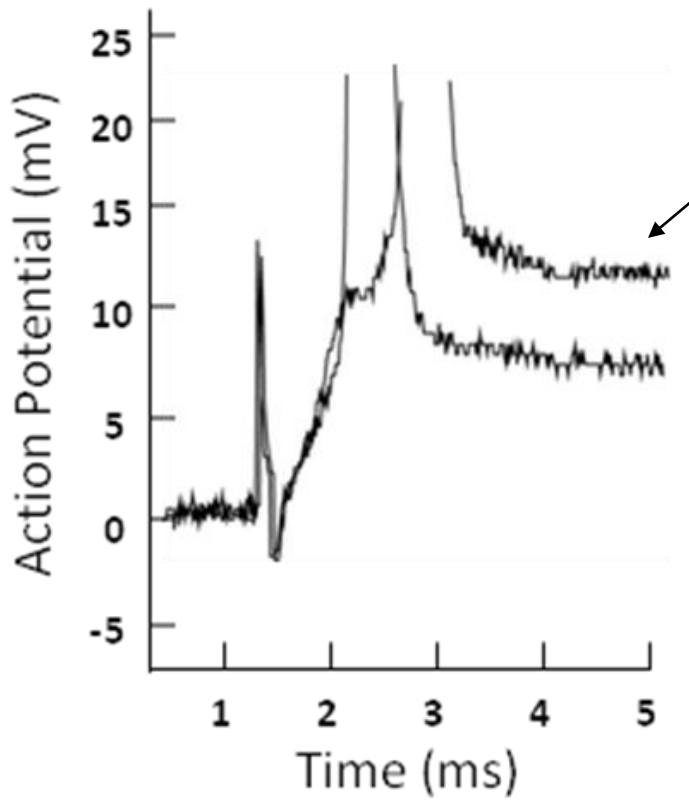




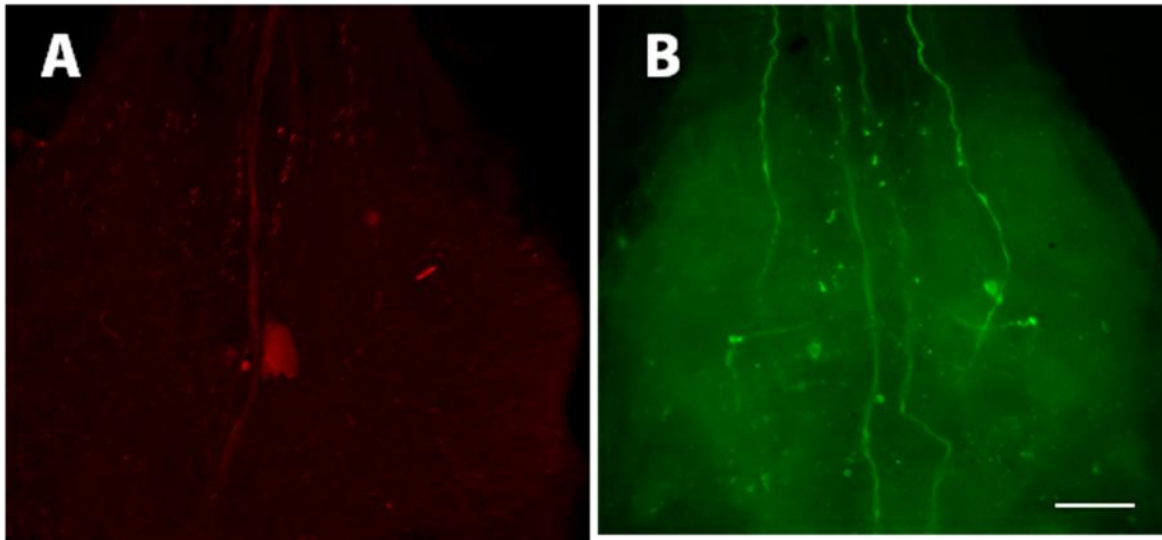
**Figure 3-1.** The LG escape circuit “chooses” facilitation over depression. Asterisks indicate that the LG fired action potentials in two experiments (mean  $\pm$  SEM; n = 10).



**Figure 3-2.** Change in  $\beta$  EPSP amplitude caused by combining dopamine and serotonin. *A*-EPSPs grew above threshold in two experiments. *B*- Combined effects of dopamine and serotonin from each experiment (line) at the end of baseline and amine treatment (filled circles). Asterisks indicate experiments where the LG fired.



**Figure 3-3.** Spike threshold was modulated by dopamine and serotonin. These traces were from the same experiment during dopamine and serotonin treatment, top trace came first (arrow). Clearly there is a difference in threshold potential and timing in the later spike.



**Figure 3-4.** Distribution of serotonin and dopamine in the 6<sup>th</sup> abdominal ganglion of crayfish. Change in  $\beta$  EPSP amplitude caused by combining dopamine and serotonin A and B are both stacks of photomicrographs viewed from the dorsal side. *Red*- immunoreactivity of serotonin; *Green*- immunoreactivity of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis (scale bar = 100  $\mu$ m). B was prepared and imaged by Amber Inman.

## Discussion

Dopamine decreases evoked EPSPs in the LG by 15% (Chapter 2). Serotonin increases the LG EPSPs by 40% (Teshiba et al., 2001; Antonsen and Edwards, 2007). Here it was shown that facilitation is favored when both modulators are present. The mean combined effect for the ten trials was less pronounced than the data presented in Figure 1-3A. More importantly the sign was the same and action potentials were initiated. Thus, facilitation prevails over depression when both forms of modulation are activated simultaneously in the LG.

The calcium chelator EGTA prevents serotonergic facilitation, dopaminergic depression, and combined facilitation (Antonsen, 2008), indicating that intracellular signaling pathways specific to calcium are mediating metamodulation at some level. How is a serotonergic bias chosen over the dopaminergic bias? Serotonergic facilitation is produced by three mechanisms: increased conductance in gap junctions between primary afferents and the LG, early increase in input resistance in the LG dendrites, and a later increase in input resistance near the initial segment of LG (Antonsen and Edwards, 2007). These parameters have not been investigated for dopaminergic depression, but mechanisms of serotonergic depression have been demonstrated in the LG circuit. Serotonin depresses EPSPs by decreasing the input resistance in LG dendrites but does not change the input resistance near the initial segment (Vu et al., 1993). In general, if facilitatory mechanisms outweigh the possible inhibitory mechanisms, then facilitation would be expected if the two forms of modulation were produced simultaneously (Lee et al., 2008).

### *Behavioral relevance of metamodulation*

The data in this chapter indicate that dopamine and serotonin act as synergists in neuromodulation of the LG neuron. The LG is a command neuron (Olson and Krasne, 1981), meaning it alone can evoke the tail flip escape behavior by firing an action potential. The presence of dopamine and serotonin causes a behavioral response to a stimulus that the crayfish would otherwise not respond to. Following are examples of when levels of the amines would be expected to change.

Levels of serotonin and dopamine are altered by exercise, age, diet, light, stress, and drugs in humans and animal models (for serotonin see: Mosko et al., 1974, Wilson et al., 1996, and Young, 2007; for dopamine see: Wenk et al., 1989; Dishman, 1997). In crustaceans the monoamines are associated with agonistic behavior. Sneddon et al. (2000) determined a link between fighting ability in crabs and relative levels of dopamine, serotonin, and octopamine (a putative crustacean analogue to norepinephrine). They found that the winning fighters had higher levels of all three amines in their hemolymph after fighting, suggesting that the behavior causes an increase in levels of the amines. If this occurs in crayfish, fighting could increase dopamine and serotonin in the hemolymph at the same time, making the experiments in this chapter applicable to a real physiological context.

There are also behaviors and stimuli that specifically elevate dopamine or serotonin in the nervous system. Would an extracellular increase in serotonin have the same modulatory effect following activation of the dopamine system? If so, how long does

dopamine interfere with serotonin's modulatory effect? These questions are addressed in the next chapter.

## **Chapter 4- Dopamine modulates serotonergic facilitation in the LG neuron.**

### **ABSTRACT**

The lateral giant neuron (LG) and neurons in many other animals are modulated by dopamine and serotonin. Though individually the amines have opposite effects on the LG, the previous chapter showed that serotonergic facilitation is “chosen” by the cell when both modulators are present at the same time. Preliminary data showed that serotonin conceals dopaminergic depression when serotonin is applied prior to dopamine. However, when the dopamine system is activated first, the effect of dopamine on serotonergic facilitation in the LG seemed to depend on how long the dopamine system was activated prior to an application of serotonin.

Using the same crayfish nerve cord preparation and electrophysiological methods, this chapter aimed to understand these temporal differences in the interaction between the two modulatory effects. It was found that long doses of dopamine blocked serotonergic facilitation. Short doses of dopamine given prior to serotonin application produced the strong facilitatory effect observed when the amine systems were activated simultaneously, as indicated by the occurrence of superthreshold EPSPs that caused the LG to fire action potentials. These results show that dopamine has two distinct effects on serotonergic facilitation that depends on how long dopaminergic pathways have been activated.



## Introduction

Specific timing in modulation is based on the notion that the internal milieu of cells and molecules is dynamic. Sleep cycle, seasonal temperature changes, mating cycle, and feeding are only a few of the situations that alter an animal's biochemistry. The biogenic amines serotonin and dopamine are known to play a role in adapting those changes. Serotonergic and dopaminergic cells release the amines in response to environmental stimuli. Dopamine levels in the rat brain are increased by rewarding behaviors such as copulation and feeding (Melis et al., 2003). Serotonin levels in the rat brain are increased by stressors such as forced swimming or exposure to a cat (Rueter and Jacobs, 1997). Neural circuits are modulated when extracellular concentrations of the amines increase. The functionality of the circuit then becomes biased, and an animal's response to future stimuli changes. Does the modulatory effect of dopamine interfere with serotonergic modulation, and how long does the first modulatory event have to be to interfere with the action of future modulatory events? Those questions are addressed with the experiments in this chapter.

Serotonin alone has complex modulatory effects in the LG circuit that depend on time. LONG application of the modulator produces a persistent (long lasting) modulatory effect; SHORT application produces a transient (short lasting) effect (Lee et al., 2008). LONG application of dopamine also produces a persistent modulatory effect in the LG. The hypothesis tested in this chapter was that the persistent effect of dopamine blocks

serotonergic facilitation of sensory evoked EPSPs. It was also hypothesized that SHORT application of dopamine would not block serotonergic facilitation.

## **Methods**

### *Specimens and experimental design resemble previous chapters*

The experimental protocol, including animals, dissection, electrophysiology, and data collection, was the same here as in previous chapters. A prepared specimen was placed in a recording chamber with a constant saline flow rate of 0.35 mL/min. One glass electrode was partially filled with saline and attached to the second nerve of the 6<sup>th</sup> ganglion to apply a stimulus. Another electrode was filled with 3M KCl and inserted into the LG axon on the same side to record membrane potential. An EPSP was induced every three minutes throughout the experiment.

### *Amines were applied separately at different intervals*

The experiments in this chapter deviated from previous experiments with respect to how the amines were applied. First, after a 30 minute baseline period, 10  $\mu$ M dopamine was applied for 15 minutes, followed immediately by a 45 minute application of 50  $\mu$ M serotonin. This regimen is referred to as SHORT. In the second protocol, after a 30 minute baseline period, 10  $\mu$ M dopamine was applied for 45 minutes. Then, saline was applied for 15 minutes, followed by 45 minutes of 50  $\mu$ M serotonin. This regimen is referred to as LONG. There were 10 trials of each experiment.

## *Analysis*

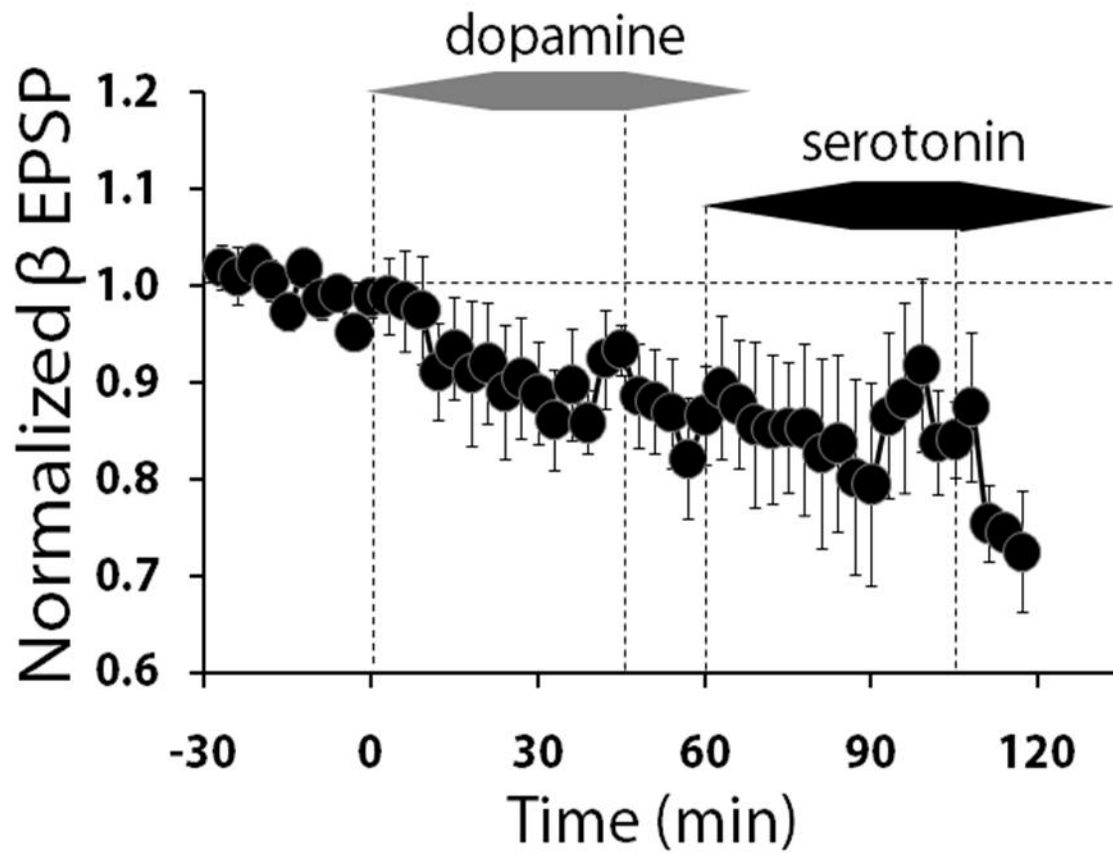
Statistical tests were used to determine whether the length of dopamine treatment had an effect on subsequent serotonin treatments. The average amplitude (normalized EPSP value) from the baseline, dopamine treatment, and serotonin treatment from each experiment was compared using Friedman's test with Dunn's post test (MedCalc).

## **Results**

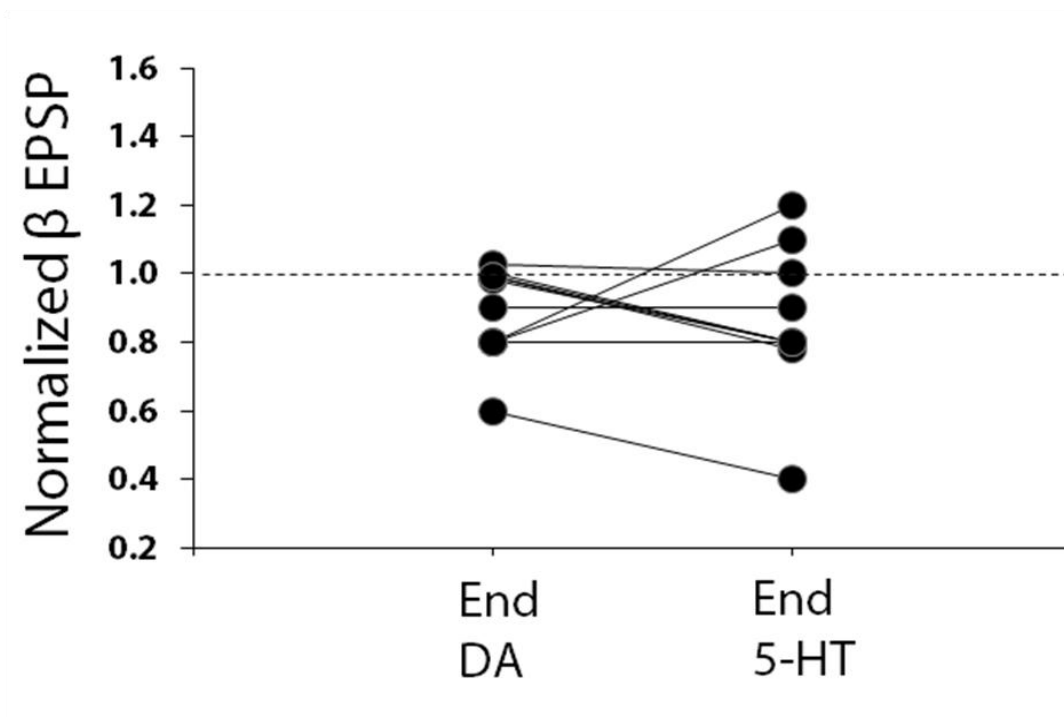
### *Serotonergic facilitation is blocked by LONG application of dopamine*

The LG response to sensory input is facilitated by 50  $\mu$ M serotonin applied SLOW (Teshiba et al, 2001; Antonsen and Edwards, 2007). Here, serotonergic facilitation was blocked when the nerve cord was bathed in 50  $\mu$ M dopamine for 60 minutes prior to serotonin application.

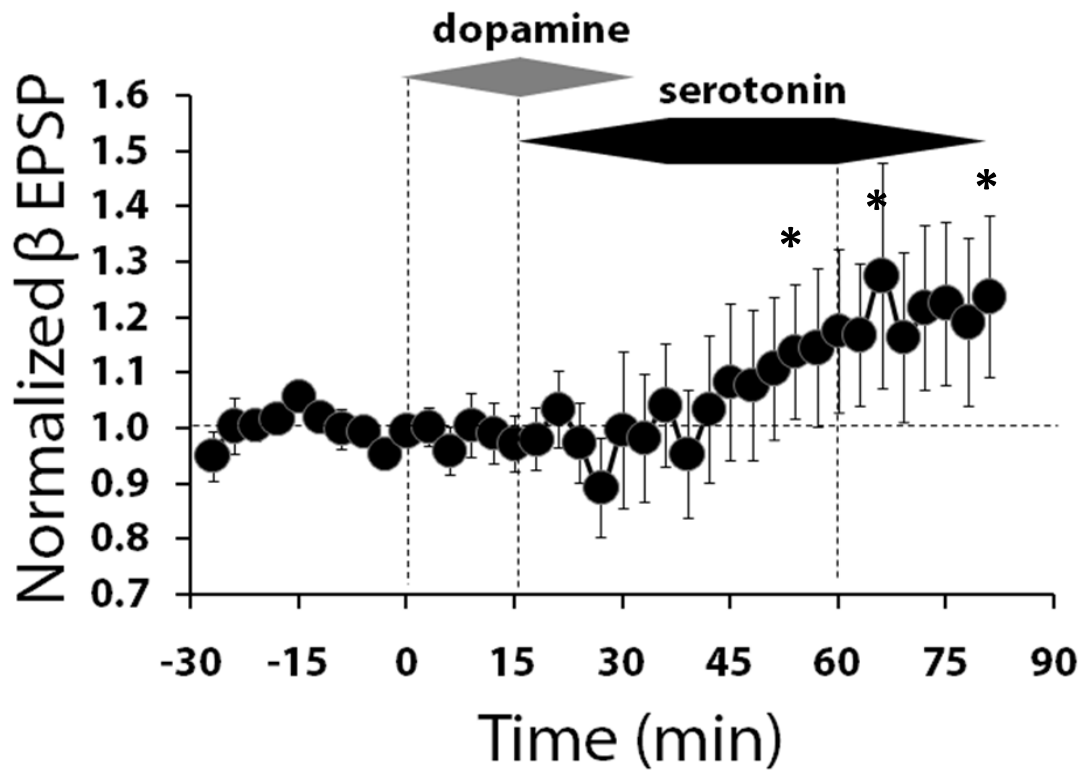
On average, dopamine application reduced the EPSP amplitude by 15% (Figure 4-1). The EPSP amplitude continued to decrease as dopamine was washed out. Onset of dopaminergic depression was slow, which is consistent with data from preliminary experiments and Chapter 2. The mean normalized  $\beta$  EPSP amplitude at the end of dopamine application, and at the end of serotonin application for each trial are shown in Figure 4-2. Facilitation was blocked in eight of the ten trials. Dopamine typically depressed EPSPs, but facilitation was blocked even if depression did not occur. Three trials were lost at 90 minutes and two were lost at 105 minutes because of electrode displacement. That is the cause for fluctuations in the trend at those two time points.



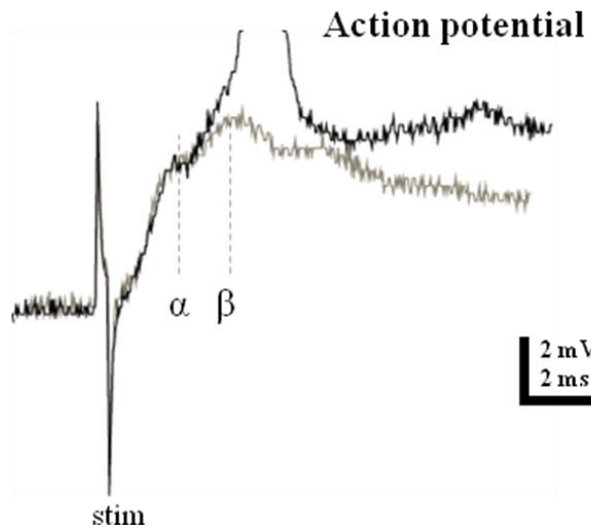
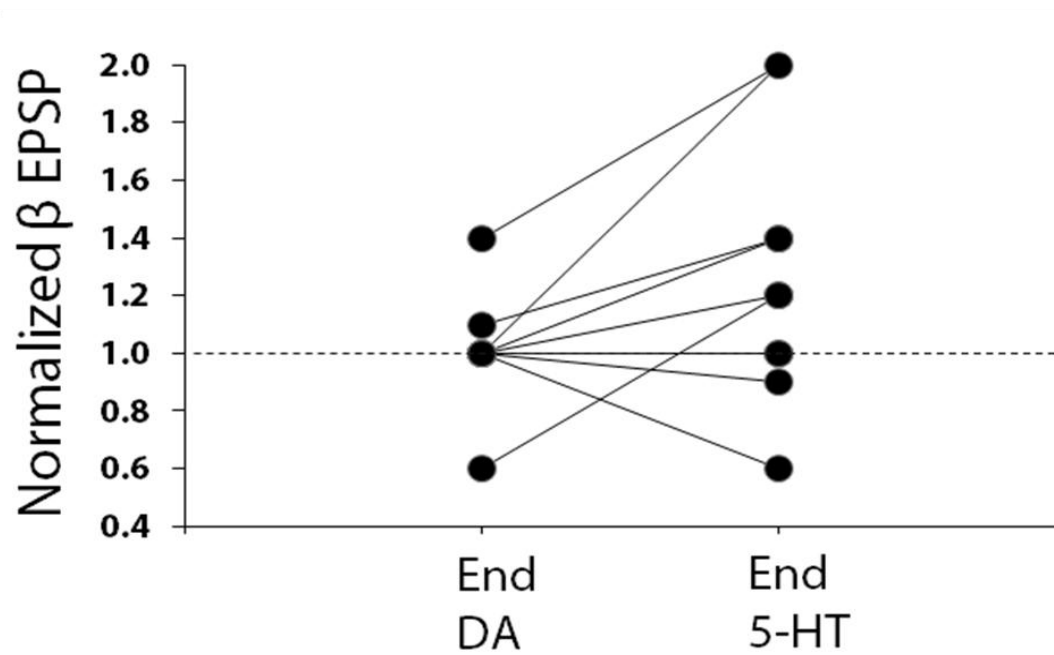
**Figure 4-1.** LONG dopamine superfusion blocks serotonergic facilitation. *B*- 10  $\mu$ M DA applied for 45 mins depressed sensory evoked EPSPs in the LG. Dopaminergic depression persisted through washout and blocked serotonergic facilitation (mean  $\pm$  SEM,  $n = 10$ ).



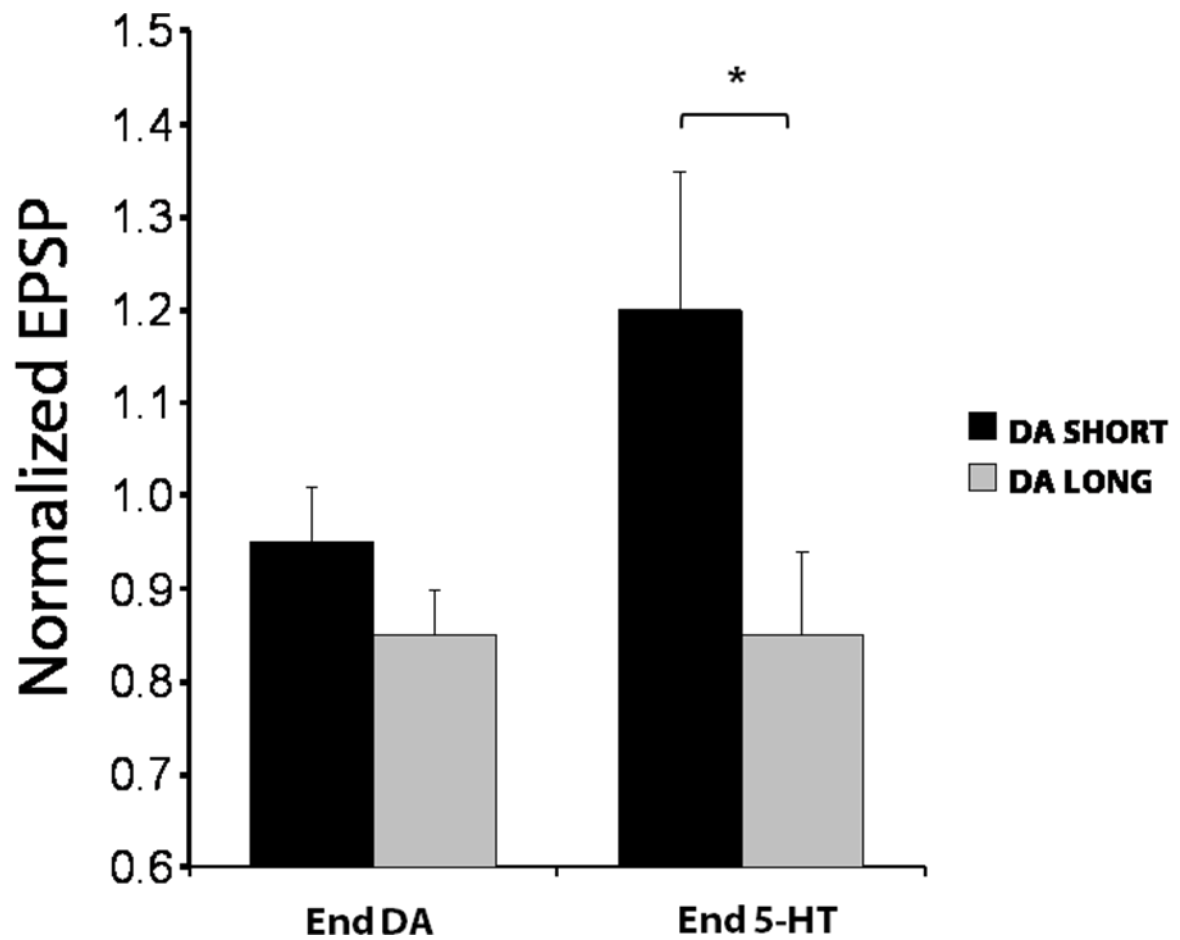
**Figure 4-2.** Serotonergic facilitation is blocked by dopamine. This graph shows the size of EPSPs relative to baseline after 45 minutes of dopamine treatment, and at the end of subsequent serotonin treatment. Serotonergic facilitation was blocked in eight of the ten trials.



**Figure 4-3.** Serotonergic facilitation after SHORT dopamine treatment. Dopaminergic depression begins to occur after the fifteen minute application. As serotonin replaces dopamine, depression reverses to facilitation (mean  $\pm$  SEM, n = 10). Asterisks indicate when the LG fired action potentials.

**A****B**

**Figure 4-4.** Serotonergic modulation of EPSP size after SHORT dopamine treatment. A- Traces from an experiment where EPSPs grew above threshold and fired an action potential. The change in EPSP amplitude at the end of dopamine and serotonin treatments are shown (dark circles), each line represents a different trial ( $n = 10$ ).



**Figure 4-5.** Dopamine's effect on serotonergic facilitation depends on the duration of treatment. LONG dopamine treatment (gray bars) depressed EPSPs more than SHORT treatment (black bars), and blocked serotonergic facilitation (Friedman's test,  $p < 0.05$ ,  $n=10$ , mean  $\pm$  SEM).



### *SHORT application of dopamine does not block serotonergic facilitation*

The second experiment tested the effect of a SHORT application of dopamine on serotonergic facilitation. This 15-minute application was enough to initiate depression but did not reach full concentration (10  $\mu$ M) or maximum depression. As dopamine was replaced with serotonin, EPSP size began to increase (Figure 4-3). The mean increase in  $\beta$  EPSP size from the end of dopamine application to the end of serotonin application was 30%.

In one trial, the EPSP grew above threshold and the LG fired an action potential (Figure 4-4A). In two trials, the LG fired multiple action potentials. Serotonergic facilitation was only blocked in two of the ten trials (Figure 4-4B). At the end of serotonin application, the average EPSP size was 40% greater in the group that was preceded by SHORT dopamine applications (Figure 4-5).

### **Discussion**

These experiments have demonstrated that the LG escape circuit is sensitive to temporal patterns of dopamine and serotonin exposure. Serotonergic facilitation occurs after a SHORT dopamine exposure but is blocked by a LONG application of dopamine, even after the amine is washed out.

### *Proposed biochemical explanation*

Preliminary data showed that serotonergic facilitation was blocked when dopamine was added 45 minutes earlier than serotonin (Antonsen, 2008). In that study, dopamine application continued while serotonin was applied, so the dopamine concentration was

constant. Here, dopamine application was discontinued, and saline was circulated between doses to decrease dopamine concentration in the bath. This experiment showed that the modulatory effect persists, despite a decrease in ligand availability.

This general dissociation equation describes the kinetics of an interaction between a ligand and its receptor:

$$K_d = \frac{[P][L]}{[C]}$$

where  $K_d$  is the dissociation constant, [P] equals protein (DA receptor) concentration, [L] equals ligand concentration, and [C] equals the concentration of ligand-receptor complexes.

Given that ligand concentration decreased, the dissociation constant would also decrease, and equilibrium would favor a ligand-receptor complex. The empirical findings are consistent with this relation.

#### *Dopaminergic modulation switches from transient to persistent*

Another explanation for the difference in serotonergic effects when preceded by LONG and SHORT applications of dopamine is that there could be different forms of dopamine depression, one that is transient and one that is persistent. This is indeed the case for serotonergic modulation. SHORT application (15 minutes) of serotonin induces transient modulatory effects, and LONG application (45 minutes) induces persistent effects (Lee et al., 2008). The difference could be the result of a switch from purely second messenger mediated effects to the addition of translation-dependent effects. A diagram of the timing in that process is shown in Figure 4-6.

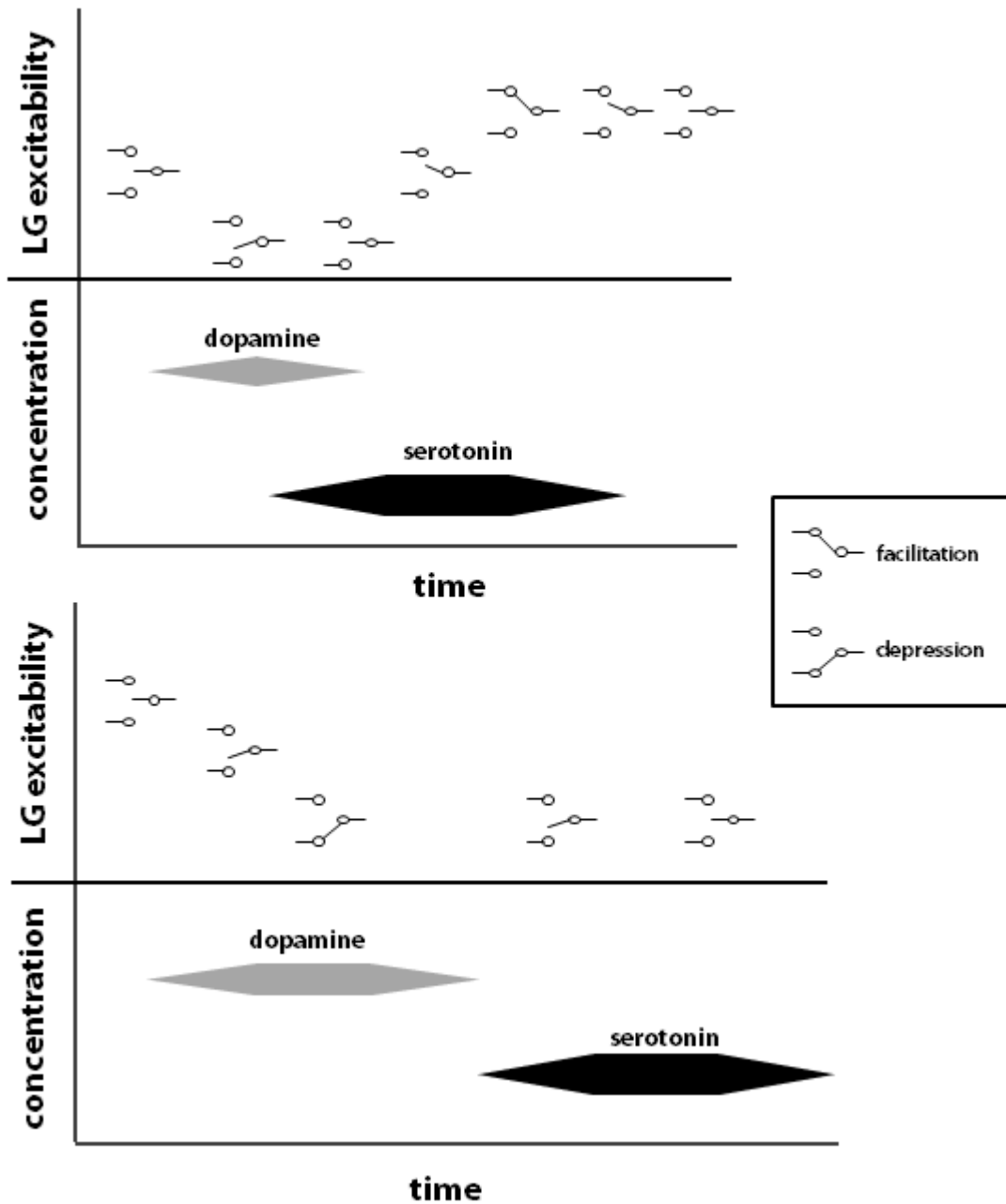


Figure 4-6. Illustrative summary of dopamine's distinct effects on serotonergic modulation. Switches are shown for each time point of the experiment, from baseline to wash. Their height relative to baseline represents change in LG excitability. Short dopamine application, *top*, does not throw the switch, and depression can be reversed to facilitation. Long application of dopamine, *bottom*, completely throws the switch and facilitation is blocked. The switch represents activation of additional mechanisms of modulation, such as translation-dependent effects or receptor shuffling, that make depression persistent.

These data show that metamodulation, like metaplasticity, enables the integration of synaptic events across time (Mockett and Hulme, 2008). Whether or not the proposed mechanism is correct, this is still a novel finding. It shows that priming a circuit with one form of modulation interferes with another form of modulation. Such priming is also known to occur in metaplasticity. High frequency stimulation inhibits the induction of long-term depression in the rat dentate gyrus (Rush et al., 2002). Prior activation of metabotropic glutamate receptors prolongs long-term potentiation (Raymond et al., 2000). Dark rearing and stress protocols also influence long-term potentiation (Mockett and Hulme, 2008). So, priming can originate from behavior, in addition to pharmacological and activity-derived priming.

Behavioral priming was controlled in this study by only using specimens that were isolated at least 14 days prior to experimentation. Activity-derived priming is a variable that is difficult to control. Series of stimuli were delivered in the beginning of each trial to identify the cell. Long term potentiation (LTP) of the LG does occur (Tsai et al., 2005), so this is a potential source of variability. It also generates another interesting question, i.e., what priming effects would dopamine and serotonin have on LTP and *vice versa*?

A limitation of the experiments in this chapter was that the LONG dopamine application was washed out prior to serotonin application whereas the SHORT dopamine application was washed out as serotonin was applied. In either case, dopamine was present when serotonin was applied. The LONG application of dopamine was not completely washed out in fifteen minutes, and the SHORT application did not reach maximum

concentration. Therefore the bath concentration of dopamine when serotonin was applied was similar in the two experiments.

### *Conclusion*

Time is of the essence. The data collected for this thesis, which are concurrent with preliminary findings and related studies in the literature, indicate that timing matters in neuromodulation. The modulatory effect of dopamine plus serotonin is not equal to the effect of dopamine plus 60 minutes plus serotonin. Cross talk between dopamine and serotonin pathways is not intuitive but could be if 1) each receptor type expressed by the LG was known, and if 2) fluctuations of their respective second messengers could be measured as the amines were applied. Until the mechanisms of metamodulation and the behavioral contexts in which it occurs are determined, its benefits to society will not be realized.

## **Chapter 5- General Discussion**

### *A novel type of metamodulation in the crayfish LG escape circuit*

Edwards et al. (2002) define metamodulation as neuromodulation that is itself modulated. The rate of serotonin application and the social experience of the animal each affect serotonergic modulation (Yeh et al., 1996, 1997). The application regimen affects serotonergic modulation through feature detectors that are sensitive to subtle changes in concentration (Edwards et al., 2002). The second mechanism of metamodulation is a change in receptor populations. Receptor shuffling mediates modulatory effects over an extended period of time (Katz and Edwards, 1999).

Seven classes of serotonin receptor and three classes of dopamine receptor are known in crustaceans (Scheiner et al., 2006). The crustacean serotonin receptors resemble their mammalian homologs in base sequence and second messenger coupling (Sosa et al., 2004). Araki et al. (2005) showed that serotonin's modulatory effects on the LG were mediated through an increase in cAMP. They also blocked facilitation by inhibiting adenylyl cyclase with SG22536, which prevents an increase in cAMP concentration inside the cell. H-89 (a PKA inhibitor) also blocks serotonergic facilitation of the LG (Lee et al., 2008).

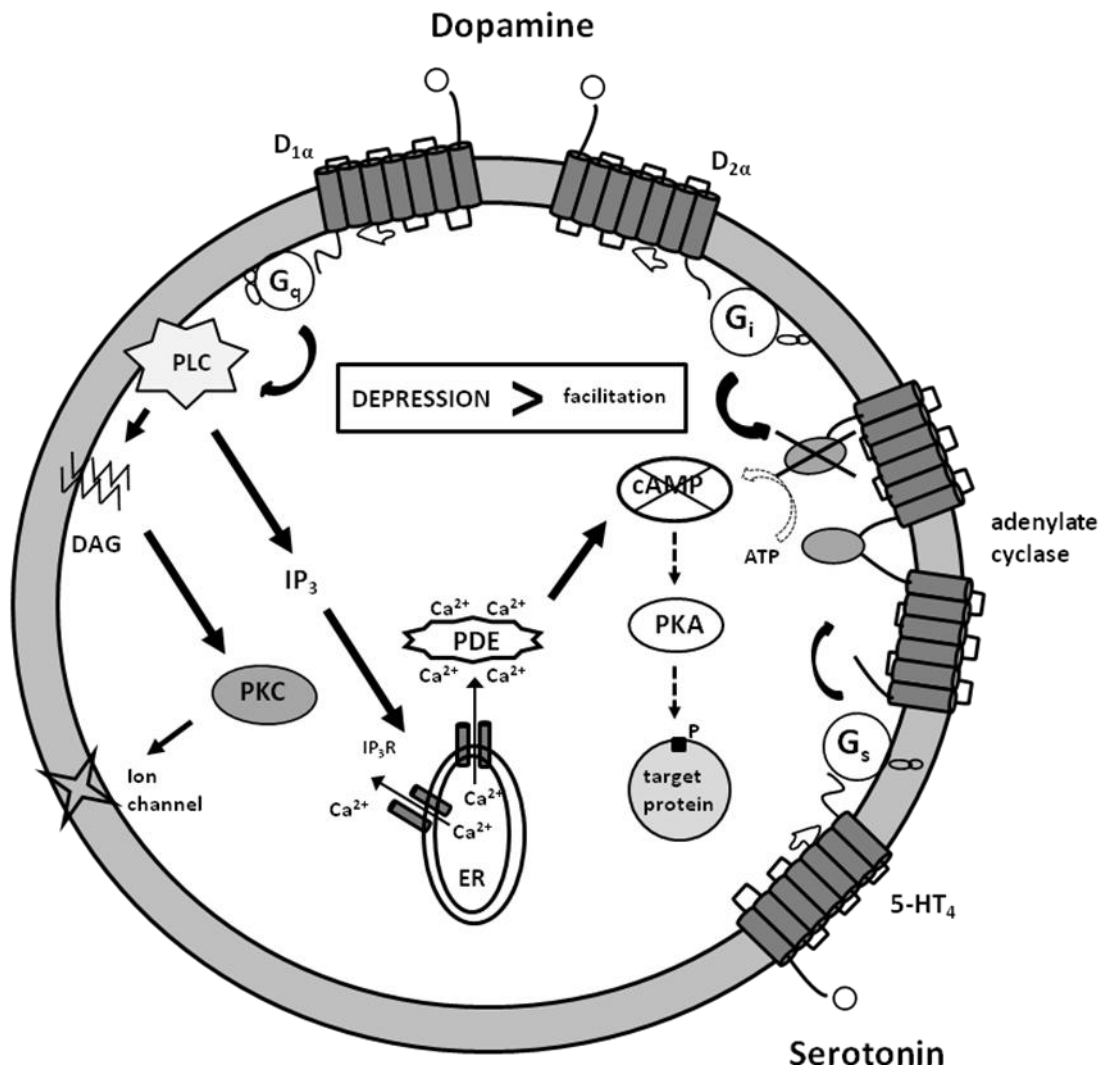
The two dopamine receptor classes in mammals are metabotropic receptors that either stimulate or inhibit the production of adenylyl cyclase, an enzyme in the cyclic-AMP second messenger pathway (Mustard et al., 2005). The  $D_{1\alpha Pan}$  receptor couples to  $G_q$ , a G protein that activates the PLC pathway (Clark et al., 2008). Dopamine could block serotonergic facilitation through this pathway. Calcium ions are second messengers

downstream of PLC. An increase in intracellular calcium could activate phosphodiesterases (PDEs), which are enzymes that degrade cAMP. Without cAMP serotonergic facilitation does not occur (Figure 5-1). The exact mechanisms are not yet known, but Teshiba et al. (2001) elegantly describe how one form of modulation prevents the formation of another.

*Growth and decay rates can help predict metamodulatory outcomes*

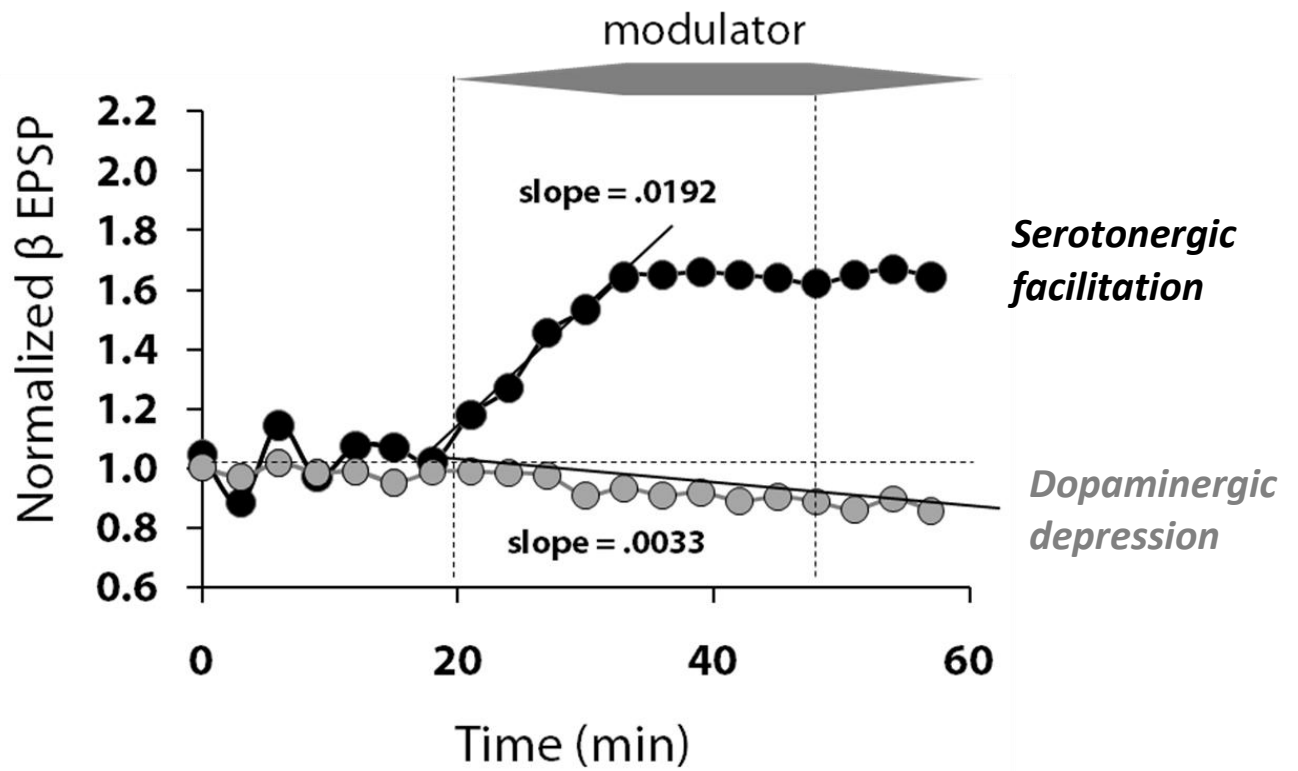
Two forms of modulation can coexist in the LG circuit. Lee et al. (2008) showed that serotonergic facilitation and depression could be activated simultaneously. They posed a theory called *reciprocal stimulation of decay*, stating that when facilitation and depression are both active, they decay under stimulation from the other until only one form remains. Serotonergic depression has a faster growth rate and slower decay rate than depression, so it is expressed if both pathways are active. This theory can also explain why serotonergic facilitation was expressed instead of dopaminergic depression in Chapter 2.

Growth of serotonergic facilitation is five times faster than growth of dopaminergic depression. Figure 5-2 shows the typical change in EPSP size over time for dopaminergic depression and serotonergic facilitation of the LG neuron. The rate of change for the serotonin experiment was five times faster than dopaminergic depression, and the peak change is reached in half the time, even though the modulators were applied at the same rate. If the two modulators use the same second messenger or G protein, the ligand that initiates the cascade faster will convey its effects. Alternatively the position of serotonin receptors in the cell membrane of the LG could be closer to adenylate cyclase than dopamine receptors.



**Figure 5-1.** Second messenger pathways that mediate dopamine and serotonin modulation. Dopamine and serotonin activate GPCRs, which in turn activate either the PLC or adenylyl cyclase pathway. The schematic posits a mechanism by which dopamine blocks serotonergic facilitation. The PLC pathway liberates intracellular calcium ions that activate a phosphodiesterase (PDE). PDE breaks down cAMP, which is needed to elicit facilitation.





**Figure 5-2.** The onset of serotonergic facilitation is five times faster than dopaminergic depression.

### *Biomedical relevance of metamodulation*

One reason the indolamine and catecholamine systems are studied is because they are known to modulate neural circuits of important function in humans. The raphe nuclei in the brainstem project axons to the forebrain and spinal cord, forming classical synapses and affecting many targets by volume transmission, including structures that regulate motor, limbic, and somatosensory systems (Hornung, 2003). Dopaminergic neurons project from ventral tegmental area and the nigrostriatum to innervate the forebrain and spinal cord (Baskerville and Douglas, 2010). Thus, the main argument in this thesis, that metamodulation is not a simple sum of modulatory interactions, has implications for cells in those circuits with similar morphology and biochemical makeup.

Dopamine is associated with symptoms of behavioral and movement disorders like addiction, Parkinson's disease, and restless legs syndrome (Arias-Carrión and Pöppel, 2007; Han et al., 2007; Clemens et al., 2006). The common neuropathological problem is that dopamine systems within an underlying neural circuit are not functioning properly. Ironically, the source of dysfunction could be serotonergic systems. Cooperativity between the converging modulatory effects of dopamine and serotonin on a single circuit are not uncommon. Serotonin modulates dopamine transmission in three major dopaminergic pathways of the human brain (Alex and Pehek, 2007), which are important in motor activation, motivation and reward (Esposito, 2006). The interaction between serotonin and dopamine systems in the prefrontal cortex is said to play a role in impulsive aggression (Seo

et al., 2008). Many neurological disorders are likely caused by deficiencies in more than one neuromodulatory system.

### *Implications and future directions*

These data support the hypothesis that dopamine has distinct effects on serotonergic facilitation. Because both modulators have their own different effects and the circuit has a specific way of switching between modulatory states, these situations likely arise naturally and serve a physiological purpose. This specificity is adaptive for the animal as a way to attribute priority to different situations. A stimulus that evokes the simultaneous release of dopamine and serotonin, say a slow moving predator or another crayfish, puts the crayfish's LG reflex in an excitable state. A stimulus that continuously evokes only dopamine release suppresses the reflex. This persistent state allows the animal to continue pursuing the food, mating opportunity, or whatever stimulus evoked the dopamine release.

In other simple organisms, the effects of serotonin and dopamine have been demonstrated within the same circuit. In *Aplysia*, buccal motor programs are modulated by both amines. Bite-like movements are increased by dopamine, whereas swallow-like movements are increased by serotonin (Kabotyanski et al., 2000). Modulation experiments involving bath-applied serotonin or dopamine have also been done in *Drosophila* and *C. elegans* (Dasari and Cooper, 2004; Sawin et al., 2000). One question that should be asked is how the modulatory effects of dopamine and serotonin interact at the single cell level in these and other identified neural circuits. Problems pertaining to intrinsic neural circuit

function and correlating neuromodulation to a specific behavior or development pattern are more likely to be solved in a simple system. Nematodes, crayfish, lobsters, honey bees, and fruit flies are now being used to better understand connections between neuromodulators and behavior (Kravitz and Huber, 2003; Christyakova, 1990). The current findings accentuate a need to study metamodulation and learn how each type of neuron responds to combinations of neuromodulators.

## **Appendix**

### *Thermal considerations*

Adapting to temperature change is a major challenge for 95% of the animals on this planet. Populations of those animals will serve as indices of climate change in the future. Because the nervous system is the animal's functional interface with the environment, determining how neurophysiology fluctuates with temperature will help us understand why some organisms perish while others flourish as the earth gets warmer. Given that dopamine and serotonin are involved in protective behaviors like escape reflexes, and the data from this report showing that dopaminergic modulation of the LG neuron is correlated with temperature, one might infer that, if crayfish perish, it would be due in part to maladaptive neurophysiology. Conjecture aside, these data indicate that it is important to control temperature when studying neuromodulation in ectothermic organisms.

*P. clarkii* used in this study hail from the rice patties near the Atchafalaya River in Raceland, LA. The average temperature there according to National Oceanic and Atmospheric Administration is  $20^{\circ}\text{C} \pm 7^{\circ}\text{C}$ . In the labs at Marshall University, temperature ranges from  $15\text{-}22^{\circ}\text{C}$ . When this study began, it was assumed that water temperature in the animal's tank would be consistent with the temperature in the physiological flow chamber, so temperature was recorded but not controlled. When the variance of dopamine experiments was less than desirable, I plotted the results against temperature. Indeed Figure 2-4 shows that the dopamine experiments near  $20^{\circ}\text{C}$  are more consistent than data collected at  $16^{\circ}\text{C}$ .

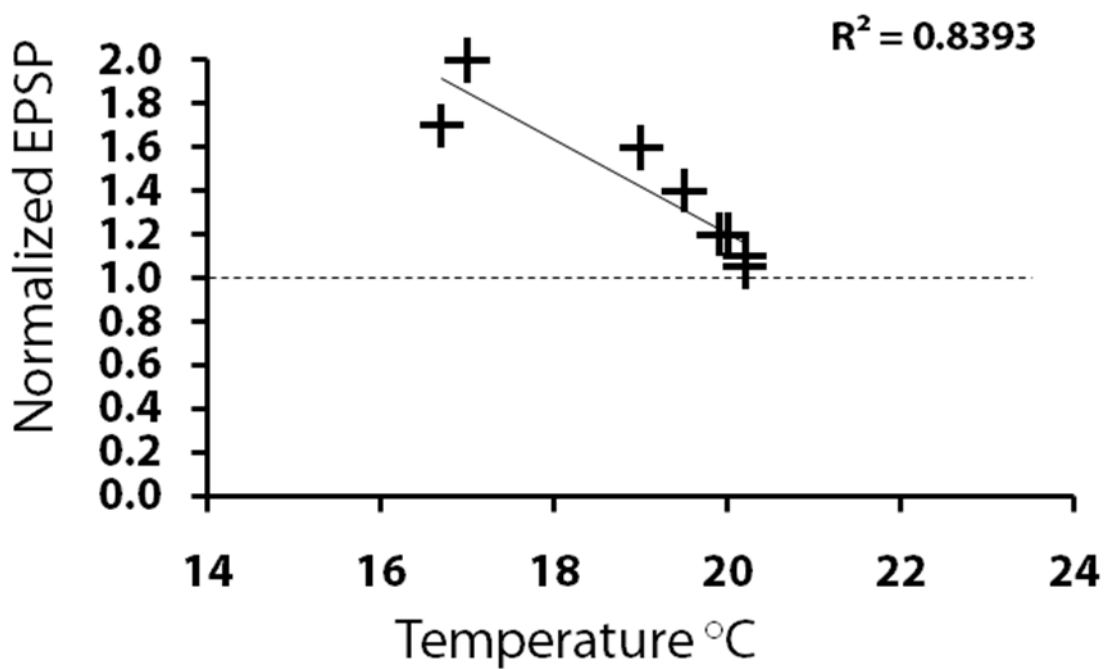
Data in Figures 3-1 and 3-2B are also somewhat variable. Some of the variability is attributable to the fact that each animal is different, but those data were also plotted against temperature. Comparing Figure 3-2B to Figure A-1 strongly suggests that there is a correlation between temperature and the modulatory effects of dopamine on the LG neuron ( $R^2=.84$ ). Facilitation steadily decreased from 16-20°C.

There are very few data points below 20°C in figures A-2 and A-3 because, by that time, temperature had been identified as a variable and a controlled water bath was purchased. That being said, there is still an observable trend in the correlation between the effects of dopamine and temperature. Slope of the trend line is still negative, indicating dopaminergic depression was more consistent at 20°C. The reason sensitivity to temperature is noteworthy is twofold. First, it informs electrophysiologists that temperature is a variable that needs to be controlled when assessing the effects of modulators. Second, crayfish serve as a bioindicator for the health of aquatic ecosystems, which will be a parameter for assessing the impact of climate change. Knowing how an invasive species in the center of a food web adapts to changes in temperature should be useful in analyzing the effects of climate change on the entire system.

### *Cambarus monongalensis*

The final disparate contribution to crayfish biology is the study of the species *Cambarus monongalensis*, the blue crayfish of the Potomac River found in West Virginia and Pennsylvania (Figure A-3A). These specimens were a gift to Dr. Antonsen from Matt Kinsey and our colleagues in Dr. Jones' laboratory. I am told this species spends as much or more

time on land than in water, which would explain why it is recommended that they be kept in shallow tanks. It is also interesting that the LG neurons are present in this species, as a tail flip escape would not be effective on land. Nonetheless, the traces in Figure A-3B are the first examples of intracellular recordings from the LG in *C. monongalensis*. They were obtained using the same protocol described here in previous chapters. Resting membrane potential (-89 mV) and EPSP amplitude (6 mV) were quite similar to recordings from *P. clarkia* and other cambarids. In two subjects, action potentials could not be evoked in the LG, but the bimodal shape of the EPSP indicates that the LG receives heterosynaptic sensory input from the second nerve root.



**Figure A-1.** Combined aminergic modulation varies with temperature. These EPSP values are from the end of treatment with serotonin and dopamine. Facilitation was stronger at colder temperatures, or depression was stronger at higher temperatures. Either way the results are consistent with neurophysiologically studies in the literature showing that input resistance increases as temperature decreases.



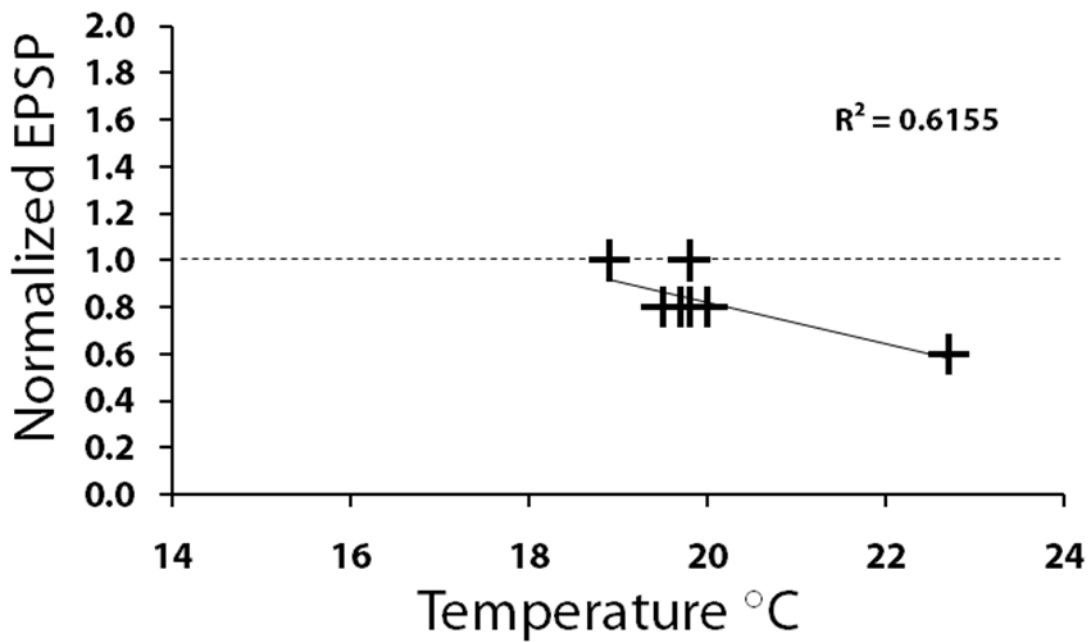
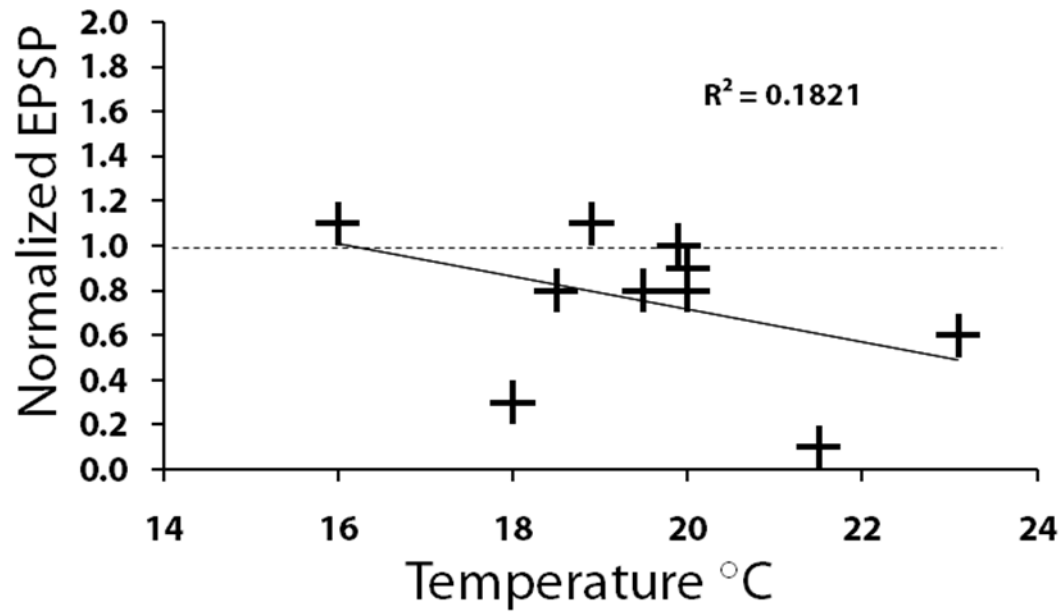


Figure A-2. Effects SHORT and LONG dopamine applications with respect to temperature.

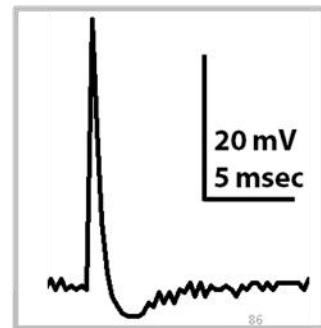
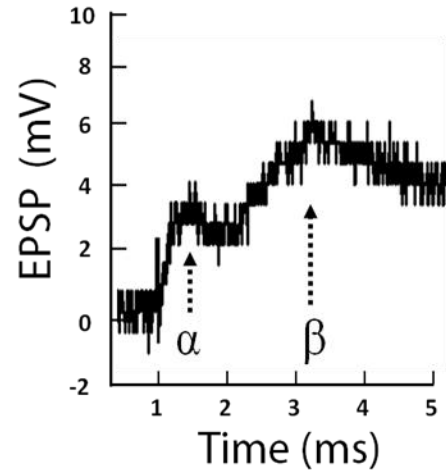
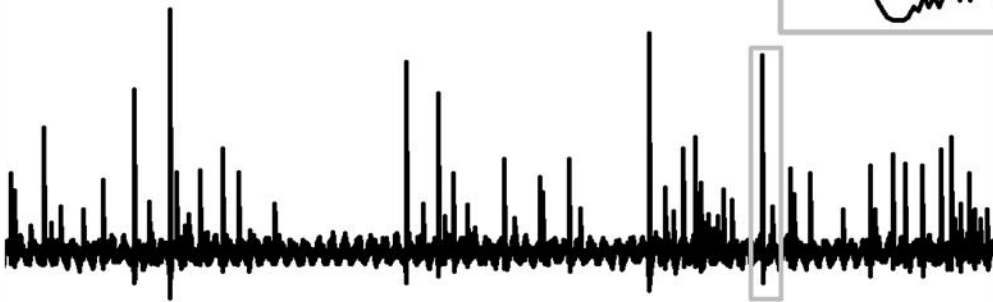
**A***Cambarus monongalensis***B****C**

Figure A-3. Neurophysiological characterization of the LG circuit in *Cambarus monongalensis*. A. The blue crayfish is found near the eastern panhandle of West Virginia. Tapping the tail evokes the stereotypical LG tail flip, so I wanted to see if sensory input to the LG neuron is congruent with other species. Indeed the LG responds to stimulation of the sensory nerve roots with a bimodal EPSP indicative of heterosynaptic input (B). Synchronized spike activity was also observed outside the LG neuron in response to the stimulus (C).

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