EXPLORATION OF SEROTONIN DISTRIBUTION AND ACTIVITY WITHIN THE PAVEMENT ANT BRAIN (*TETRAMORIUM CAESPITUM*)

by

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Thesis directed by Associate Professor Michael Greene and Professor John Swallow

ABSTRACT

Ants have miniaturized brains, yet they exhibit surprisingly complex behaviors. In social insect colonies, individuals gather information, integrate it, and compare that information to an inherent set of rules to make behavioral decisions. Each individual decision can lead to complex, self-organized behaviors. However, little is known about the proximate mechanisms behind these collective behaviors. The study of neurotransmitter monoamines, such as serotonin, provide a possible explanation for such complex behaviors. Serotonin is associated with reproductive dominance, colony foundation, aggression, trophallaxis, behavioral development, division of labor, repertoire expansion, and nestmate recognition in ants. This study used pavement ants (*Tetramorium caespitum)* as a model species to explore the distribution and activity of serotonin within the neural architecture of the ant brain. Ants were exposed to a variety of contexts: social interaction, aggression, food excitement, and antenectomy. After exposure whole brains were dissected and underwent immunohistochemistry (IHC) to stain for the monoamine serotonin and the genetic marker for neuronal activity, c-Fos. Serotonin immunoreactivity was found in the antennal lobes (AL), subesophageal ganglion (SOG), optic lobes (OL), mushroom bodies (MB), and the MB calyces. Serotonergic processes were seen in the MB calyces, terminating in the lip of the calyx. Neuronal symmetry was observed in the AL, SOG, and OL. A maximum estimate of 78 serotonergic neurons were stained. This study provides further information for the serotonergic architecture within the ant brain. Fos colocalization with serotonin was seen in the AL, SOG, OL, and MB calyces in an ant engaged in aggression. This suggest that serotonin is

active in these locations during this behavior. However, only one brain showed positive staining for Fos. The location and activation of serotonergic neurons in the AL and MB calyces suggest that serotonin is released from the AL and shuttled to the MB calyx's lip where it is processed and a decision to aggress is made. If the Fos technique provided in this study can be replicated, it offers a new method to better understand the underlying mechanisms of behavior in ants.

The form and content of this abstract are approved. I recommend publication.

Approved by:

Michael Greene

John Swallow

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CHAPTER 1

INTRODUCTION

Ant colonies are regulated as non-hierarchal, distributed system where individual decisions drive colony behavior. The queen does not "command" her workers. Rather, individuals asses local informational cues, integrate them in their simple, miniaturized brains, and compare that information to an inherent set of rules. Often these cues are based on the interaction rates they have with their nestmates (Greene and Gordon 2007, Davidson and Gordon 2017, Hoover *et al*. 2016, Bubak *et al*. 2016, Pratt 2005), the density of pheromone trails (Sumpter 2005, Muscedere *et al*. 2012), and other olfactory information like cuticular hydrocarbons (Hu *et al*. 2017, Sano *et al*. 2018). Ants share this information locally, and interact with a small, limited network of nestmates. The network is limited to a small fraction of the colony because one ant could not possibly interact with every other individual in the colony. However, these small networks extend to other small networks causing information to spread and a collective behavior to be expressed Therefore, individuals make decisions given their limited information (Sasaki and Pratt 2013, Edwards and Pratt 2009). The many decisions made by individuals lead to the emergence of colony behaviors including foraging, nest construction, brood care, colony defense, and colony maintenance. The colony's survival is dependent on these decisions made by individuals (Pruitt and Riechert 2011). Out of the repeated interactions of individuals a system emerges: self-organization to a collective decision.

Complex emergent behavior can arise from self-organization stemming from a simple set of rules (Sumpter 2005). These collective behaviors are frequently made via consensus from individuals and these collective decisions shape the fitness of the colony (Cronin 2015). Consensus decisions provide a way for individuals to coordinate information and, once a

threshold is reached, elicit a group-level behavior (Cronin 2015). The colony is, in essence, a society that functions as an information processing unit distributing its cognitive tasks to individual ants (Pratt *et al*. 2002). Just as an individual's decision is made from complex interactions between a network of neurons so too does an ant colony's decision emerge from a network of interactions among individual ants (Marshall *et al*. 2009, Edwards and Pratt 2009).

Ants have miniaturized brains, less than 1/1000 the size of a honey bee, yet collectively they exhibit amazingly complex behaviors including farming fungus, raising aphids for honeydew, stealing non-nestmate workers (Alloway 1979), building structures with their own bodies (Lioni and Deneubourg 2004), and warfare (Hoover *et al*. 2016). Relative to their brain volume, they possess enlarged antennal lobes (odor processing) and mushroom bodies (cognition, learning, and memory) (Fahrbach 2006). Using these enlarged structures, individual ants interpret local stimulus using intrinsic rules to make complex behavioral decisions. These decisions lead to the collective behavior of the colony.

Within Hymenoptera (wasps, bees, ants, and sawflies), we see that brain structures associated sensory collection and integration are enlarged. *Apis mellifera* have large optic lobes (OL) that improve vision which allows them to among other things, discriminate colors (von Frisch 1914) and even human faces (Dyer *et al*. 2005). However, most ants see a reduction in the size of their optic lobes because they mainly rely on olfaction (Gronenberg 1996). Since ant's primary sense organ is their antennae, the ant brain has antennal lobes (AL) that are larger, relative to body and brain size, and more complex than in other social Hymentoptera (Gronenberg 1996). Honey bee's brains contain approximately 166 AL glomeruli while ants possess an estimated 200 some of which are larger and more complex than in other insects. Glomeruli are associated with odor detection and larger and more complex glomeruli suggest the

importance of olfaction for social behavior in ants (Gronenberg 1996). Lastly, most social Hymenoptera have enlarged mushroom bodies (MB) a region responsible for higher order processing like sensory integration, learning, memory, and spatial orientation, all functions important to the social life of ants and bees (Gronenberg 1996). Within ants we see larger MB when compared to other insects and other Hymentopterans (Gronenberg 1996). In ants and bees, the mushroom bodies are divided into two calyces: the medial and lateral calyx. The role of the MB calyces in bees and ants is different from other insects as well. While other insects primarily use the MB calyces for odor processing, bee and ant MB calyces are a center for multimodal sensory integration (Rossler and Groh 2012). For example, the MB in *A. mellifera* work in tandem with the AL and the subesopheagel ganglion (SOG) during olfactory learning (Gauthier and Grunewald 2012). These three regions appear to play important roles within social insects and their behavior.

Social insects, like ants and other Hymenoptera, have relatively simple brains yet they can exhibit extremely complex behaviors. Hoyle (1985) established the orchestration hypothesis as a means to explain the coordination of complex behaviors by neurotransmitters. He discovered that direct manipulation of monoamines could elicit specific behaviors and that monoamines worked on the neural circuitry of organisms. With the development of the orchestration hypothesis, researchers had a new possible mechanism to study, monoamines. Octopamine (OA), dopamine (DA), and serotonin (5-HT) are commonly studied to determine the animergic effects on behavior (Kamhi and Traniello 2013, Hoyer *et al*. 2005, Tsuji *et al*. 2007, Muscedere *et al*. 2012, Muscedere *et al*. 2012, Muscedere *et al*. 2016, Bubak *et al*. 2016, Hoover *et al*. 2015). Ant researchers began to study these monoamines to understand the mechanisms underlying social behaviors (Kamhi and Traniello 2013). Exploring monoamines offers novel explanations for the

modulation of complex social behaviors. By examining neurochemistry and neural architecture, we can determine the underpinnings of social behavior, colony-level division of labor, and collective intelligence (Kamhi and Traniello 2013).

Octopamine (OA), DA, and 5-HT have been implicated in a wide range of ant behaviors including colony foundation, interspecies and predatory aggression, learning, development, trophallaxis, and nestmate recognition (Kamhi and Taniello 2013, Table 1). Kamhi *et al*. (2015) showed that OA is instrumental in aggression in the Australian weaver ant, *Oecophylla smaragdina.* This species has different worker castes; large majors engage in aggressive territorial defense while minors care for brood and collect food. For example, Kamhi *et al*. (2015) found that majors contained higher brain titer levels of OA. To see if OA influenced aggression they manipulated OA levels within minors and saw an increase in aggressive behaviors. Smith *et al*. (2013) found that in the ant, *Achromyrmex echinatior*, DA, OA, and 5-HT brain levels differed based on castes suggesting that these monoamines influence worker specialization. In a recent study, Wada-Katsumata *et al*. (2011) show that DA and OA are linked to the social behaviors of grooming and trophallaxis in *Formica japonica*. In this study ants where starved while DA and OA levels were monitored. Starving ants displayed low levels of DA, which was rescued after trophallaxis. Ants were also isolated from social interaction. This increased OA levels. When introduced to nestmates these ants increased their trophallaxis duration, allogrooming, and self-grooming behaviors suggesting that high levels of OA influences social behaviors. Queens from the species, *Veromessor pergandei*, have varying colony foundation strategies, founding a colony singly, or working cooperatively with other queens. Muscedere *et al*. (2016) examined the role of DA, OA, and 5-HT in these different strategies. Queens that found colonies together often engage in aggressive conflict with each

other to establish dominance after worker eclosion. Early in colony foundation, young queens that founded singly had heightened brain levels of 5-HT and queens that founded together had heightened levels after workers eclosed. This suggests that 5-HT modulates aggression during colony foundation (Muscedere *et al*. 2016). Serotonin was found to influence foraging behaviors in *Pheidole dentata* (Muscedere *et al*. 2012). This study pharmacologically decreased serotonin in individuals. These individuals were less likely to follow pheromone trails and if oriented to trails only followed for short distances compared to control ants. These studies illustrate just how many behaviors are modulated by monoamines.

Although, monoamines have been shown to have many important functions in regulating social insect behavior, little is known about the underlying mechanisms that differentiate how monoamines modulate these complex behaviors. Most of our current methods for studying monoaminergic effects on behavior rely on manipulations of whole brain levels which offer much insight into how levels of monoamines can affect behaviors, but lack resolution at the level of brain architecture (Muscedere *et al*. 2016, Bubak *et al*. 2016, Penick *et al*. 2014, Smith *et al*. 2013, Kamhi *et al*. 2015, Muscedere *et al*. 2012, Wada-Katsumata *et al*. 2011). These studies typically employ High Powered Liquid Chromatography (HPLC) to measure the monoamine content within a whole brain. They also incorporate pharmacological manipulations to see how increases and decreases in whole brain levels of monoamines affect behaviors. These studies are limited in how they explore the possible mechanisms behind a given behavior. Pharmacological manipulations flood the brain with monoamines, higher levels than those found naturally. This blunt approach makes it easy to elicit a behavioral response and correlate a monoamine with a given behavior. However, this does little to show how these monoamines work within the brain to generate the behavior. If a deluge of monoamines floods the brain, how can you determine

what, where, when, and how these molecules modulate behavior. One can only make a general assumption that having high levels of monoamines causes a change in behavior. Unfortunately, these types of studies only look at the whole brain titers of the monoamines rather than looking at the neural architecture or the location of the monoamine within the brain. How then do we differentiate the specific effects of each monoamine? Do these monoamines work in concert or are they antagonistic? Does one modulate the others? While we have determined that these monoamines influence these specific behaviors, it remains unclear how exactly monoamines modulate behaviors (Hoyer *et al*. 2005).

As stated above there are limitations to overriding the brain with monoamines. To gain better insight into the relevant cells and structures involved in behavior, I used immunohistochemistry (IHC). This tool allows for the exploration of possible monoaminergic architecture of the brain to provide insights into the mechanisms that contribute to the generation of behavior (Giraldo *et al*. 2013, Hoyer *et al*. 2005, Smith *et al*. 2013, Kamhi *et al*. 2015). IHC is used commonly to explore social insect brain architecture (Hoyer *et al*. 2005, Galizia *et al*. 2012, Gronenberg 1996, Giraldo *et al*. 2013). Research often focuses on monoaminergic systems within the brain (Hoyer *et al*. 2005, Tsuji *et al*. 2007, Giraldo *et al*. 2013). By understanding the architecture within the brain, these studies link the systems within the brain and its architecture to specific behaviors including aggression (Hoyer *et al*. 2005), division of labor and repitoire expansion (Giraldo *et al*. 2013, Muscedere and Traniello 2012, Galizia *et al*. 2012), and sensory integration (Galizia *et al*. 2012).

Immunohistochemistry is a widely used technique to study the distribution and activity of monoamines. It involves detection of specific epitopes in the cells or tissues of interest using antibodies that bind to those specific epitopes. Additionally, fluorescence proteins are added for

better resolution of the target molecules. Studies that use this technique can pinpoint neurons and neuronal pathways associated with specific behaviors such as how 5-HT regulates division of labor in *Pheidole dentata* (Giraldo *et al*. 2013), or the role DA and 5-HT play in intraspecific aggression in *Harpegnathos saltator* (Hoyer *et al*. 2005). Giraldo *et al*. (2013) used IHC to determine structural differences between castes as they age as a possible explanation for behavioral repertoire expansion. Using IHC they were able to identify serotonergic neurons and their arborization within the mushroom bodies for all castes. Giraldo *et* al. (2013) discovered that only the major castes displayed structural changes as they matured as well as the majors had significantly more complex arborization within the mushroom bodies. They concluded that these structural differences were a possible explanation for the differences in caste behavior. Hoyer *et al*. (2005) investigated the serotonergic and dopaminergic neuronal systems as they relate to intraspecies aggression and to determine any differences in neural anatomy between castes and sex by employing dual staining IHC. They hoped to find structural differences between these monoaminergic systems during aggression and between castes and sex. The study was able to describe the serotonergic and dopaminergic systems within the brain, but they could not find any anatomical changes during aggression. However, they did find that males had smaller brain volumes proportionally to females that resulted in reduced size of the mushroom bodies. Additionally, the two female castes possessed more serotonergic processes while the males displayed higher dopaminergic processes. These differences could explain the differences in behavioral repertoire between caste and sex (Hoyer *et al*. 2005). Again, this study was able to describe monoaminergic neural anatomy to better understand behavior. These studies use this technique to gives us a better picture about the mechanisms of ant behaviors (Hoyer *et al*. 2005,

Giraldo *et al*. 2013). Using this technique can give a detailed picture of the origin, location, and mechanism of a behavior.

Since IHC has a variety of applications that extend to neuronal tissues, it becomes an excellent tool when trying to determine the mechanism behind specific behaviors (Hoyer *et al*. 2005, Tsuji *et al*. 2007). This technique allows you to visualize the tissue of interest to locate particular molecules of interest, in this case monoamines. Hoyer *et al*. (2005) used IHC to determine if the distribution of DA and 5-HT was different between castes and sexes in *H. saltator* since brain morphology did not change among these groups. The researchers found that there was deep innervation of serotoninergic neurons within mushroom bodies (responsible for cognition, learning, and memory) with serotonin neurons making connections between the mushroom bodies and antennal lobes (responsible for olfaction). Additionally, they were able to show that sterile workers and males had different distributions of serotonergic processes as compared to other castes and sexes. Sterile workers' dopaminergic processes were also different from other castes. Hoyer *et al*. (2005) showed that different brain architecture exists in ants based on behavioral repertoire. They illustrated that monoamines must work differently within castes and sex. This is a possible explanation for the difference of behaviors among different castes of workers and between sexes. Tsuji et al. (2007) was able to provide the complete serotonergic network found in the antennal lobes of *C. japonicas*. This network could be used to determine how serotonin can orchestrate the social behaviors of *C.japonicas*. In both these studies, IHC provided a detailed map of the location of these monoamines in the brain. Knowing the location of these monoamines informs us of its function. If monoamines are in a brain region responsible for olfaction, they are likely responsible for modulating behaviors requiring olfaction

and chemosensation. Since each region of the brain is responsible for different functions, this technique shows the possible mechanism that monoamines employ to elicit behavior.

IHC provides a map showing regions where monoamines are present. The information gathered from these maps can help us determine how these monoamines work in the brain and what kind of behaviors they may modulate (Hoyer *et al*. 2005, Tsuji *et al*. 2007). IHC gives the exact location of these monoamines during specific behaviors (Hoyer *et al*. 2005). Since the function of each brain region is known, we can use the location of these monoamines within those regions to explain the mechanisms behind behaviors like aggression and social interaction. But the method is limited since behavior relies on the firing of action potentials within neurons, which is difficult to detect with IHC.

To address these concerns with IHC, the methods proposed in this paper will incorporate the early activator gene, c-Fos. c-Fos can shows us the neuronal activity within the brain and help to pinpoint areas that are active during a specific behavior. This can illuminate possible mechanisms including which serotonergic neurons are active during specific behavioral contexts. c-Fos is an early activator gene that is expressed when an action potential fires and has become a marker for neuronal activity (Day *et al*. 2008, Dragunow and Faull 1989). c-Fos is visualized in IHC by targeting the proteins expressed by c-Fos. Dragunow and Robertson (1988) found that c-Fos was expressed in recently activated neurons in the rat brain and began using it as a high resolution marker for synaptic pathways in the mammalian brain (Dragunow and Faull 1989). C-Fos became used in vertebrates to understand the neural mechanisms and pathways behind behaviors (Guzowski *et al*. 2001, Shu 2002, Neumaier *et al*. 2001, Bastle *et al*. 2016). Bastle et al. (2016) used c-Fos to determine an interaction between the social and nicotine reward system in the brains of adult male rats. Additionally, Shu (2002) used cFos to explain the mechanism of

learning in rats. C-Fos allowed Neumairer *et al*. (2001) to visualize the location 5-HT7 receptors in rat brains which provided insight into their role in the circadian rhythm and potential enhancement of serotonergic pharmaceuticals. As these studies show, c-Fos is a powerful tool to explain neural mechanisms in animals.

c-Fos has been important tool for the understanding of neural mechanisms of behavior. Rats learning to locate platforms submerged under water saw increased expression of c-Fos RNA in the hippocampus, entorhinal cortex, and visual cortex when compared to controls. Additionally, the act of learning the trail increased the level of c-Fos RNA expression in the dorsal hippocampi when compared to controls. These data suggest that Fos is expressed during spatial learning and that the regions showing Fos activity are responsible for the behavior (Guzowksi *et al*. 2001). To determine the efficacy of a specific neuropeptide thought to regulate hunger, NMUR2, c-Fos was used to show regions of Fos expression when mice were given a NMUR2 agonist. Fos was measured after the agonist, NMU-7005, was given to obese and fasted mice. Obese mice saw increased Fos expression in the lateral part of the hypothalamus when given the agonist when compared to controls given a saline solution. While fasted mice showed increased expression in the medial part of the hypothalamus compared to obese mice. Finally, obese mice given the agonist had increased Fos expression in the medulla oblongata. Again, this study shows where this drug is active providing a possible mechanism for the agonist (Kaisho *et al* 2017). c-Fos provides insight into the mechanism for a given behavior. It pinpoints the regions that express it which highlights where the behavior is likely occurring.

While this technique is widely used with vertebrate models, we rarely see it used in invertebrates (Renucci *et al*. 2000, Ghosal *et al*. 2010). There is little reason not to use c-Fos since studies have shown that invertebrates express c-Fos and c-Fos related antigens (Ghosal *et*

al. 2010, Renucci *et al*. 2000). Ghosal *et al*. (2010) measured the immunoreactivity for c-Fos and Fos related antigens (FRA's) in male crickets using IHC. They identified specific brain regions that expressed c-Fos and FRA proteins during aggression. They found FRA and Fos immunoreactivity in the ventromedial region of the brain, the deutocerebrum, and among the Kenyon cells of the cortex of the MB. Additionally, Fos was located in the nuclei of brain cells. Renucci *et* al. (2000) also measured the immunoreactivity of Fos and FRA's using IHC with the cricket brain. They saw staining within the nuclei of neurons of female crickets confirming the presence of Fos and FRA's in the insect brain. Still many invertebrate studies do not take advantage of this tool to understand the neural mechanisms of behavior. c-Fos can bring a higher resolution of the neural pathways behind behaviors to insects especially ants. As shown above c-Fos has aided in understanding complex behaviors in rats. We can use this tool to understand the complex, collective behaviors of ants.

As mentioned previously, monoamines are of growing interest as a possible explanation for ant collective behavior (Kamhi and Traniello 2013). This is especially relevant since these neurotransmitters are highly conserved across taxa (Iyer et al. 2004, Kang et al. 2009). Marshall *et al*. (2009) points out that neural mechanisms behind primate decision making bear a striking similarity to the self- organized decisions made by ant colonies. If ant collective behavior can inform how we understand how our neurons behave during the decision-making process, then perhaps learning how monoamines drive social behaviors in ants could explain role these monoamines play our own social collective behavior. In this way we could create a foundation that develops Hoyle's orchestration hypothesis as the basis for complex social behavior. Studies investigation these monoamines typically look at serotonin, dopamine, and octopamine when

exploring the possible neural mechanisms behind collective behavior (Kamhi and Traniello 2013). For ease of analysis and technique this study focused on a single monoamine, serotonin. (5-HT).

Behavior	Monoamine
	5-HT
Reproductive dominance and colony foundation	*S. invicta* [Boulay et al., 2001]; *H. saltator* [Hoyer et al., 2005]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006]; *F. japonica* [Aonuma and Watanabe, 2012a]
Subcaste-related division of labor	*P. dentata* [Giraldo et al., 2013; Giraldo and Traniello, unpubl. obs.]; *A. echinatior* [Smith et al., 2013]
Worker behavioral development, repertoire expansion, and temporal polyethism	*P. dentata* [Seid and Traniello, 2005; Seid et al., 2008; Muscedere et al., 2012; Giraldo et al., 2013]; *S. peetersi* [Cuvillier-Hot and Lenoir, 2006]; *F. polyctena* [Wnuk et al., 2010]
Social food flow (trophallaxis)	*C. mus* [Falibene et al., 2012]; *P. dentata* [Muscedere et al., 2013]
Aggression	F. japonica [Aonuma and Watanabe, 2012]; *P. dentata* [Giraldo et al., unpubl. obs.] *T. caespitum* [Hoover et al., 2015; Bubak et al. 2016]
Nestmate recognition	*C. fellah* [Boulay et al., 2000]; *O. smaragdina* [Kamhi and Traniello, unpubl. obs.] *T. caespitum* [Hoover et al., 2015; Bubak et al. 2016]

Table 1. Serotonin and the social behaviors associated with serotonin*

*Table modified from Kamhi and Traniello 2013.

Serotonin is an intracellular signaling molecule that can be found in all organisms with a central nervous system (Vleugels *et al*. 2015). This molecule is so widely conserved across taxa that it is found in plants and fungi (Kang et al. 2009). 5-HT is derived from tryptophan and is associated with the regulation of aggression, feeding/appetite, learning, memory, and social behaviors in insects (Vleugels et al. 2015). In ants, this monoamine has a wide variety of effects on their social behaviors. Studies have shown that 5-HT plays a role in colony foundation, interspecies and predatory aggression, learning, development, trophallaxis, and nestmate recognition (Kamhi and Traniello 2013, Table 1). Table 1 displays the species and papers that show a link between 5-HT and a specific social behavior. While this research shows that 5-HT is responsible for a long list of behaviors, we do not yet know how 5-HT modulates each of these behaviors. Thus, we need techniques like IHC to determine the mechanisms by which 5-HT influences specific behavior. This study will attempt to use IHC to establish 5-HT's role in modulating aggression in pavement ants.

Pavement ants (*Tetramorium caespitum*) are a ubiquitous invasive species found throughout the urban environment. They prefer to live under slabs, or flat rocks, hence the name pavement ants. These qualities make them an easy subject to obtain and study. In addition, pavement ants display a very conspicuous collective behavior, war. Pavement ants engage in conflict with other species of ants as well as conspecifics. Wars with other species are fought to the death, but not when pavement ants encounter non-nestmate conspecifics. Pavement ant war is ritualized with little to no causalities incurred by both colonies. Individual ants will use their mandibles to lock onto an opponent and contest in a "tug-o-war" battle to push the other colony out of their territory. Wars will last for hours until a victor is decided. Recent interactions with

nestmates and the frequency of those interactions influence an individual to decide to fight when encountering a non-nestmate (Hoover *et al*. 2016, Bubak *et al*. 2016). These interactions between nest-mates cause an increase in 5-HT and OA levels which prime the brain for aggressive confrontation with non-nestmates (Bubak *et al*. 2016, Hoover *et al*. 2016). Each interaction spikes OA and 5-HT levels in the brain after which the levels of monoamines start to decline. So repeated interactions are necessary to sustain monoamines at their threshold to encourage a decision to fight (Hoover *et al*. 2016). It is unclear what levels of monoamines are necessary for a decision to fight nor what the rate of decline of monoamines is. The methods of this paper were developed to better understand the mechanisms behind this decision to fight.

The methods in this paper were developed to further our understanding of the neural architecture and to create a technique in which we capture the neural activity of pavements ant based on different behavioral concepts. With these methods I will describe the serotoninergic architecture in pavement ants, show how this method can be used to measure neuronal activity, and provide an example on how these methods can be employed to make behavioral comparisons

CHAPTER 2

METHODS

Materials

Animals: Pavement ants were collected in Denver, CO by aspirating individuals from bait trails. Ants were baited with peanut butter. Ants were housed in aspirator tubes with grass to provide a level of humidity. Ants were housed in these containers for 24hrs prior to experimentation and sacrifice.

Special Equipment:

The following lists contains all the equipment used to conduct the experiment. The specificity of the list is to account for all possible contributions to the images generated by IHC.

- Horizontal shaker (Belly Dancer, Stovall Life Science, Greensboro, NC USA).
- Cold room horizontal shaker (2314FS Lab Rotator Stirrer Shaker, Fisher Scientific, Waltham, MA USA).
- Sterilized 48 well cell culture plates (Greiner Bio-One, Kremsmünster, Austria)
- Cold room (Environmental Growth Chambers, Chagrin Falls, OH USA)
- For dissections, cold plate (CaterWare Round Aluminum Cooling Plate 11-inch x 1 3/4 inch, TableCraft, Gurnee, IL USA)
- Brains were imaged using Ziess LSM 700 confocal microscope.
- Some images were imaged on a 3I Marianas inverted Spinning Disk confocal microscope

Chemicals

The following list contains all chemicals used in the immohistochemical reactions. I provide the specific chemicals used to ensure that the procedure laid out in this section can be replicated accurately. The specificity of the list accounts for all possible contributions to the success of the IHC.

- 4% Formaldehyde in phosphate buffered saline (pH 7.4±0.2) (BM-154, Lot# E1RF25R Boston BioProducts Ashland, MA USA)

- Gultaraldehyde, 50% aqueous solution (LOT: P27A045, Alfa Aesar, Ward Hill, MA USA)
- Phosphate buffered saline (pH 7.4, 1X, Gibco-Thermo Fisher Scientific, Waltham, MA USA).
- Normal goat serum (S26-100ML, Lot#2855574, EMD Millipore, Temecula, CA USA)
- Primary anti-5HT rabbit polyclonal IgG antibody (20080, Lot#1650001 Immunostar Hudson, WI USA)
- Primary anti-Fos mouse monoclonal IgG antibody (E-8, sc-166940, Santa Cruz Biotechnology, Dallas, TX USA)
- Secondary goat anti-rabbit IgG Alexa Flour® 594 (A21121, Lot#1889303, Thermo Fisher Scientific, Waltham, MA USA). Secondary goat anti-mouse IgG Alexa Flour® 488 (A11012, Lot#1745478, Thermo Fisher Scientific, Waltham, MA USA).
- Triton X-100 (Lot# SLBJ0812V, Sigma Life Science, St. Louis, MO)
- Vectashield mounting medium for fluorescence with DAPI (H-1200, Vector Laboratories, Burlingame, CA USA)

Detailed Procedure

Behavioral trails: Prior to dissections ant were placed into different behavioral contexts. This was to determine their brain states under differing conditions for comparison.

Aggression: Ants from two different colonies were collected. 50 ants from each colony were placed in 11cm X 11cm plastic container. Ants were allowed to acclimate for 15 minutes. After 15min ants that engaged in aggressive behavior (defined as biting, locking mandibles) were selected and sacrificed.

Social (control): Ants from the same colony were placed in 11cm X 11cm plastic container. Ants were allowed to acclimate for 15 minutes. After the acclimation period ants engaged in social behavior (defined as attenuating another individual) were sampled and sacrificed.

Dissection: All ants were dissected on a cold plate kept stored at -20°C. Prior to dissection the cold plate was allowed to warm for 20 minutes. Once ants were selected they were placed on ice for 15 secs to anesthetize them. Once anesthetized they were transferred to the cold plate for

dissection. Using micro dissection tools ant heads were removed. The ant heads had their mandibles removed to allow fixative into the cranial cavity.

Immunohistochemsitry: All wash and incubation steps used a horizontal shaker. All steps were performed at room temperature (21°C) except fixation and primary antibodies incubations.

Dissected ant heads were fixed in 4% formaldehyde with 0.1% Gluteralaldehyde for 5 hours. After 5 hours the brains were removed from the skull exoskeleton. The brains were then washed in PBS (3 X 10min) Following fixation ant brains were placed in 0.3% Triton X-100 PBS for 2 hours at to permeabilize the tissue. To reduce non-specific bindings brains were incubated in 10% Normal Goat Serum (NGS) in PBS for 1 hour. Then each brain underwent standard secondary IHC staining for serotonin and c-Fos. Serotonin antibody used was ImmunoStar rabbit anti-5-HT. c-Fos antibody used Santa Cruz mouse anti-Fos. Ant brains were incubated in a dilution of rabbit anti-5-HT primary antibody (1:500, 0.5% NGS in PBS) and mouse anti-Fos primary antibody (1:50, 0.5% NGS in PBS) for 3 days at 4°C. After the incubation period in primary antibodies the brains were washed in PBS (3 X 10min). Following the washes, the tissue was incubated in secondary antibodies, goat anti-rabbit IgG Alexa Flour® 594 for serotonin and goat anti-mouse IgG Alexa Flour® 488 for c-Fos (1:500 dilution in a solution of PBS). This was followed by a wash in PBS (3 X 10min). Finally, the brains were mounted on positively charged slides using Vectashield anti-fading solution with DAPI. The slides were prepared using a piece of black tape with a window cut into it as the mounting field.

Confocal Microscopy. Images created from IHC were generated using Ziess LSM 700 confocal microscope. All images were created using the 20X objective lens. Red and green lasers were used at 594nm and 488nm. The red channel laser's power was set to 1.9% and the green channel laser's power was set to 3.0% for each image. Gain was left at default setting. Each image was

generated using z-stack. Imaging was set to the optimal slice thickness by using the built-in Ziess imaging software "Optimal" feature.

Image processing. Images were processed using FIJI. Images are 3d max projections of z-stack images. All images were compared to an atlas created by Tsuji *et al*. (2007) to identify morphological structures.

Controls. Controls were run on the secondary antibodies to ensure lack of nonspecific binding. These brains underwent the entire protocol, but without primary antibodies.

Due to issues with the technique's ability replicate c-Fos staining several different experiments were run to generate Fos expression.

Sucrose excitement. Ants were starved for 24 hours. After the starvation period, ants were given a solution of 10% sucrose prior to dissection. Dissection was performed the same as above. Ants were dissected at three different time points: immediately after sucrose ingestions, 15min after sucrose ingestion, and 1hr after ingestion.

Antennal removal experiment: Ants were divided into three groups: controls, right antenna removed, or both antenna removed. Dissections were performed as described above, however prior to dissection each group was placed on ice for 15 secs, then placed on the cold plate where their antennae were removed. Controls underwent the same process, but did not have their antennae cut. All antennae were cut using micro dissection scissors. Ants from each group were sacrificed at three different time points: 5min, 6hrs, and 24hrs after antennal removal.

Analysis. The brains analyzed in the results are the best selected from each experimental group. Thus, all brains used in results are pooled from all the experimental contexts.

CHAPTER 3

RESULTS

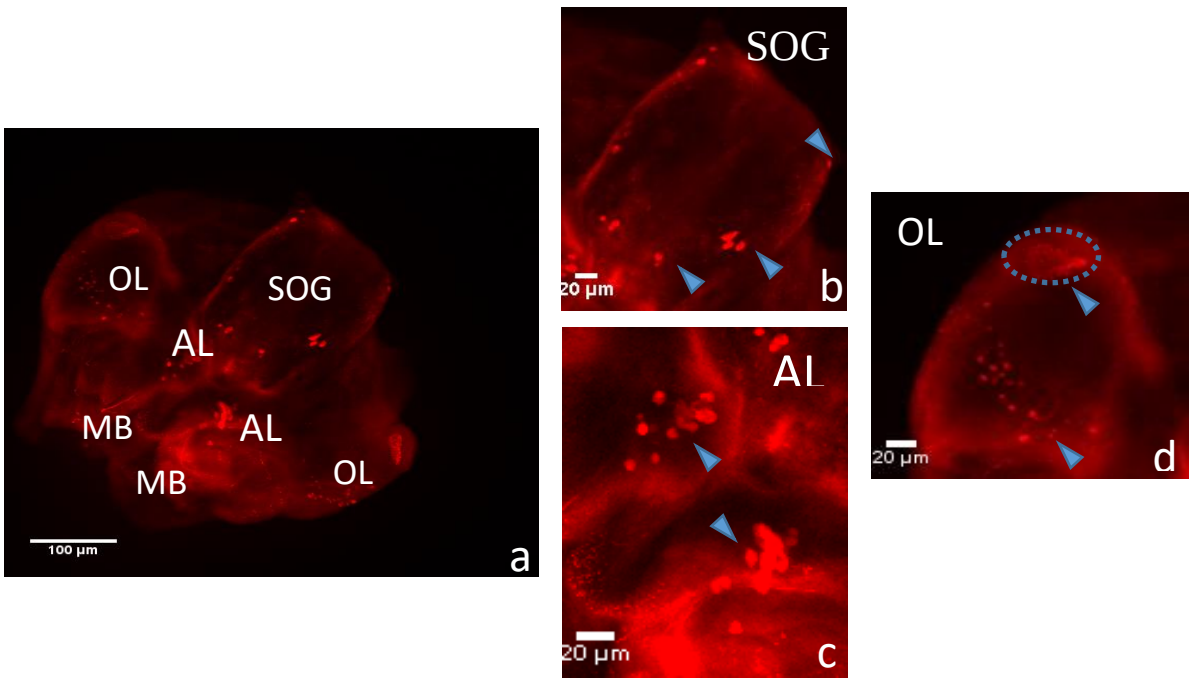

Figure 1. Images of a whole *T. caespitum* brain stained for serotonin including ROI (regions of interest) and serotonin (5-HT) positive neurons. The figure is made up the best representations of each ROI. 5-HT is stained in red. a) Serotonin (5-HT) staining in a whole *T.caespitum* brain. Imaged at 20x magnification. AL = antennal lobes, MB = mushroom bodies, OL = optic lobe, and SOG = subesophageal ganglion. Brain in ventral orientation. b) SOG shows three pairs of serotonergic neuronal clusters highlighted by the blue arrows. c) A zoomed view of the serotonergic neuronal clusters in the antennal lobes. d) Highlighted serotonergic neurons within the optic lobe. 5-HT saturated medulla cells are present in the enclosed within the circle.

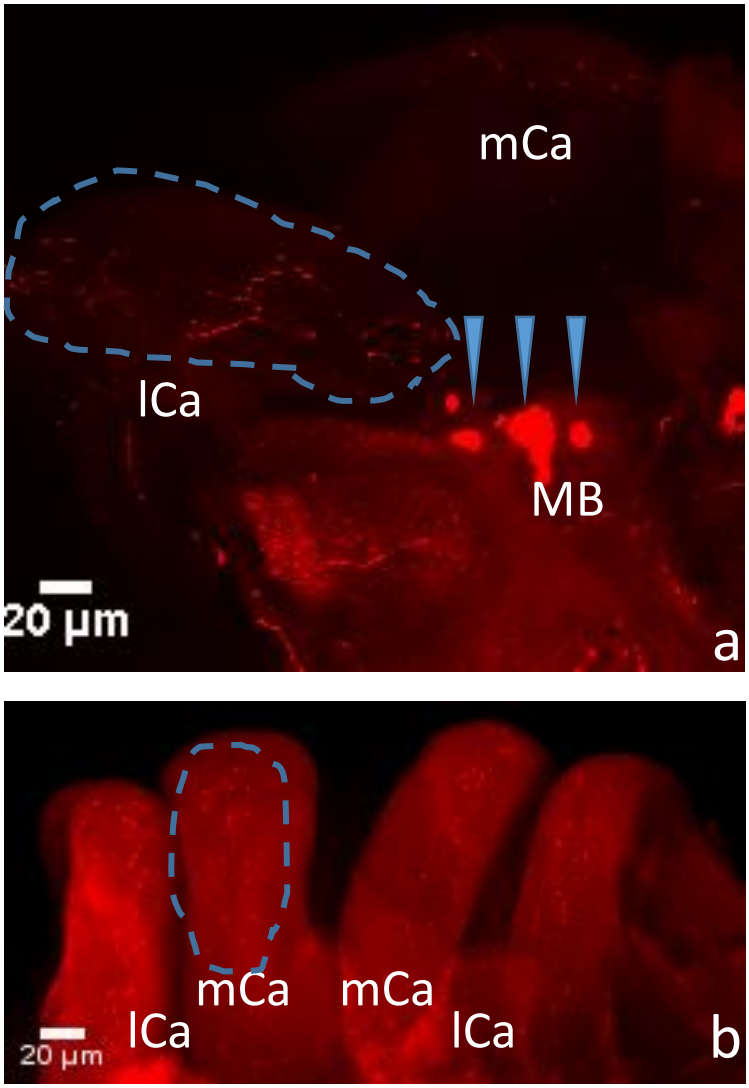

Figure 2. Images of the mushroom body (MB) structure in *T. caespitum* brains. The figure is made of the best representation of the MB. Serotonin (5-HT) is stained in red. Images produced at 20X magnification. MB = mushroom body, lCa = lateral calyx, and mCa = medial calyx. a) Zoomed image showing a MB. The arrows point to neurons in the MB and the dashed circle highlights serotonergic processes. b) Image showing the different calyces in the MB. The dashed circle highlights serotonergic processes.

Serotonin anatomy in T.caespitum brain. Our protocol showed excellent staining for serotonin (5-HT) (figure 1.a). In figure 1 we saw strong presence of serotonergic neurons in the antennal lobes (AL), optic lobes (OL), and the subesophagael ganglion (SOG). There appears to be bilateral symmetry in number of neurons in those the regions (figure 1.a). The AL showed an estimate of 10 neurons in each lobe (figure1.c). OL contained an estimate of 22 neurons per lobe.

The OL medulla appears saturated with 5-HT (figure 1.d). Within the SOG there appears to be three clusters of neurons. A pair of clusters located in the anterior, toward the AL (n = 3 per cluster), a pair on lateral side (n = 3 per cluster), and a pair located laterally in the posterior (n = 3 per cluster) (figure 1.b). The mushroom bodies (MB) also showed presence of serotonergic neurons (Figure 2.a). There is an estimate of 9 neurons in the MB (figure 2.b). The calyces of the MB have serotonergic processes suggesting that 5-HT is shuttled into the calyces from neurons elsewhere in the brain. The processes appear to terminate at the lip of each calyx.

Neuron count. Neurons were counted in images in which morphology could be identified (n=32). The neuron count represents an estimate of the number of neurons within in a *T. caespitum* brain. Due to the limitations of the technique, the numbers displayed the distribution in Figure 3 represent our best estimates of whole brain 5-HT neurons. Our max total count for 5-HT neurons was 78, our minimum was 1. The mean for the total number of neurons is 23.29. The median values for each region in the brain are close with their means very close to the median value

except for the OL. These neurons counted were the only neurons that were detected by our technique, meaning we can only provide an estimate on count.

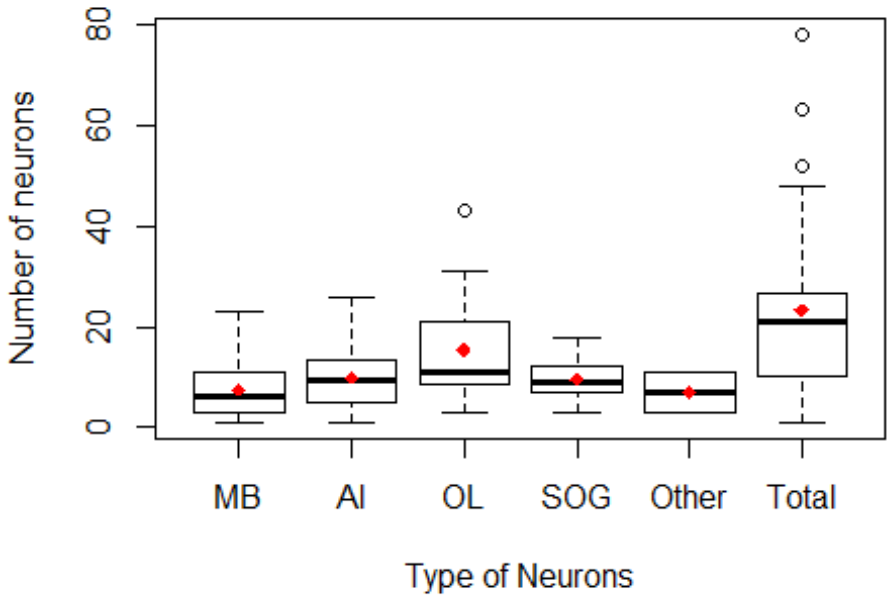

Figure 3. Boxplot showing the distribution of 5-HT neurons within *T.caespitum*. The black bar represents the median and the red diamond is the mean. Open circles are outlying points. AL = antennal lobes, MB = mushroom bodies, OL = optic lobe, and SOG = subesophageal ganglion.

Other represents any neurons that were present, but could not be placed within a specific morphological structure.

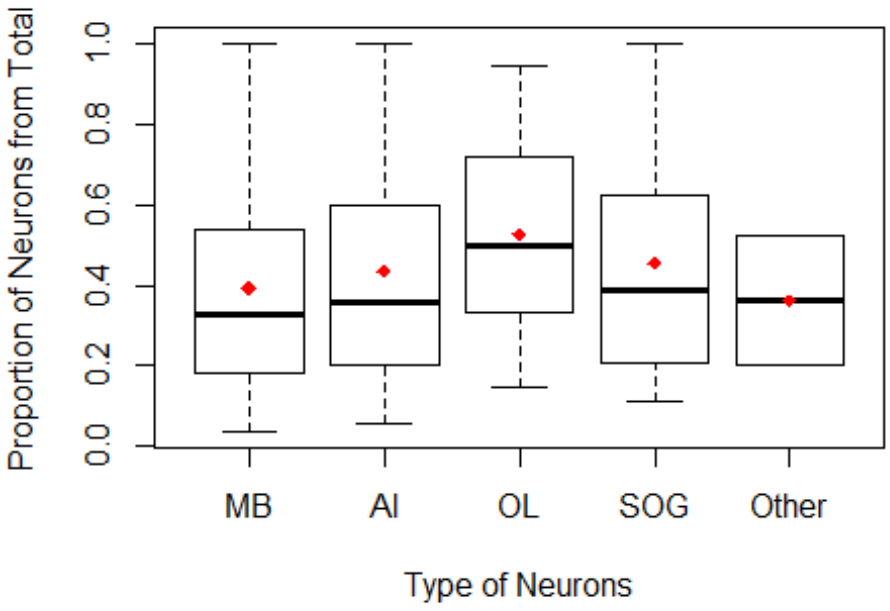

Figure 4. Boxplot showing the distribution of proportions of the total neuron count that are in each morphological structure. The proportions of each region were calculated using the total number of neurons within each imaged brain. The black bar represents the median and the red diamond is the mean. AL = antennal lobes, MB = mushroom bodies, OL = optic lobe, and SOG = subesophageal ganglion.

We see a wide variability in each region of how many neurons are contained within that region (Figure 4) Mean MB = 39%, Mean AL = 43%, Mean OL = 52%, Mean SOG = 45%, Mean Other = 36%. We see the greatest proportion of neurons within the OL, mean proportion = 52%. The other regions are relatively similar in their proportion of the total.

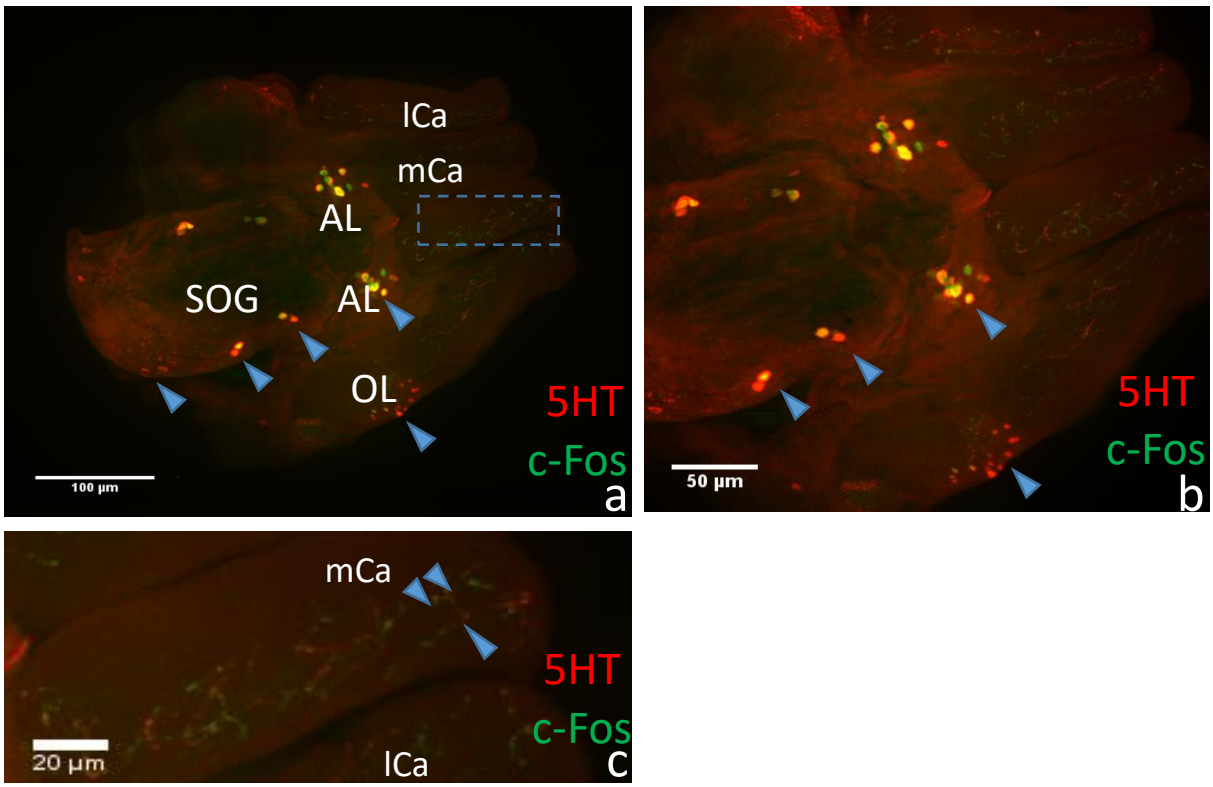

Figure 5. Images of a whole *T. caespitum* brain stained for serotonin and c-Fos including ROI (regions of interest), serotonin (5-HT), c-Fos positive neurons. 5-HT is in red and c-Fos is in green. Yellow indicates co-localization of Fos and 5-HT. AL = antennal lobes, lCa = lateral calyx, and mCa = medial calyx, OL = optic lobe, and SOG = subesophageal ganglion. Brain in ventral orientation. Brain is in ventral orientation. All images are from the same brain. a) This image illustrates the whole brain with dual staining for Fos and 5-HT. The yellow staining shows serotonergic neurons that are active. Activity is located in the SOG, OL, and AL. Arrows point to neuronal clusters. The dashed box highlights both serotonergic and Fos processes, a closer view is represented in figure 3x.c Image is at 20x magnification. b) A closer view of the clusters to illustrate co-localization. Yellow indicates active serotonergic neurons. Arrows point to active neuronal clusters. Image is at 40x magnification. c) Zoomed view of Fos and 5-HT processes within the mushroom body calyces. Arrows highlight points of co-localization. Yellow (co-localization) indicates active 5-HT processes. Image is at 20x magnification. Ant brain dissected from an ant during aggression.

c-Fos staining. Using the method laid out previously, we were able to see distinct c-Fos staining (Figure 5). We see similar neuronal clusters and bilateral symmetry as in Figure 1, however there are distinct Fos neurons stained green (Figure 5). Those neurons stained red show presence of 5-HT and those colored yellow indicate co-localization of c-Fos and 5-HT. Co-localization with Fos indicates activity within those neurons, specifically serotonergic activity (Figure 5). We see that there is 5-HT activity within the AL, OL, SOG, and MB calyces. Within the AL we see unique 5-HT and Fos neurons. Again, we see processes within the mushroom body calyces, but now we see Fos activation as well (Figure 5.c). These processes also have co-localization at points indicating serotonergic activity in the calyces (Figure 5.c).

Looking at Table 2, of the 51 neurons present the majority are serotonergic (91.49%). 50% of the neurons show the presence of c-Fos and 44.68% show co-localization. At the time of the staining 44.68% of the serotonergic neurons within this individual were active. All areas of the brain showed 5-HT activation (62.50% in the AL, 25% in the OL, and 46.67% in the SOG). Most of the serotonergic activity was in the AL and SOG (62.50% and 46.67% of neurons showing co-localization respectively). 100% of the neurons within the OL and SOG are serotonergic, but only 25% and 46.67% showed activity. Only the AL showed presence of Fos only neurons (Table 2).

Table 2. Number, type, and proportion of neurons within co-stained brain. All counts are based on Figure 3X. Neurons were counted as co-localized if the neuron contained any amount of yellow. Numbers were rounded to two decimal places.

Type of Neuron	**Total Number of Neurons**	**Total c-Fos positive neurons**	**Total 5-HT positive Neurons**	**Co-localized neurons**	**% c-Fos**	**% 5-HT**	**% co-localized**
AL	16	13	12	10	81.25	75.00	62.50
OL	16	4	16	4	25.00	100.00	25.00
SOG	15	7	15	7	46.67	100	46.67
Total	47	24	43	21	51.0	91.49	44.68

CHAPTER 4

DISCUSSION

Serotonergic staining. The technique employed in this paper provides an estimate for the number of 5-HT neurons within *T. caespitum* brains. It also shows where these neurons are likely located and the location of their activity. Serotonergic neurons were found in the AL, MB, SOG, and OL. Additionally, serotonergic processes were identified branching into the MB calyces. Both *H. saltator* (Hoyer *et al*. 2005) and *C. japonicus* (Tsuji *et al*. 2007) were found to have similar serotonin immunoreactivity. Both studies showed serotonergic processes and arborization within the MB calyces similar to Figure 2. However, the number of neuronal cells found in the AL (Fig.1, Fig.3) differs from the single AL neuron found previously (Tsuji *et al*. 2007). The processes within the MB calyces appear to terminate in the lip of the calyx (Fig.2). The termination of 5-HT processes in the lip of the MB calyx (Fig. 2) was also found in Tsuji *et al*.'s (2007) results. In ants, the lip of the MB calyces contains afferents from the antennal lobes and is associated with olfactory input (Gronenberg 2001, Fahrbach 2006). Therefore the 5-HT processes highlighted could be those afferents. If so, serotonin may influence higher order processing of olfactory information (Hoyer *et al*. 2005). Muscedere *et al*. (2012) blocked 5-HT in *P. dentata* and found that workers failed to locate pheromone trails. Without 5-HT they struggled to locate and follow olfactory information. This also suggests the importance of 5-HT in sensory integration. Since ants primarily rely on their antennae for sensory information, 5-HT provides a mechanism for how that information is processed within the MB. The SOG is responsible for control over an insect's mouthparts, salivary glands and neck muscles. The presence of serotonin in the SOG suggests 5-HT's role in modulating mandible control. For *T. caespitum* this is quite important in regard to aggression against conspecifics. *T. caespitum* lock

mandibles during conflict with conspecifics so 5-HT is likely active within those neurons during aggression. We also see presence of 5-HT within the optic lobes and medulla of the optic lobes, which confirms other's findings (Seid *et al*. 2008, Hoyer *et al*. 2005, Schafer and Bicker 1986). The serotonergic immunoreactivity in the medulla of the OL (Figure1) is similar to the findings described by Hoyer *et al* (2005). However, most ants do not rely heavily on sight. The presence of 5-HT within the optic lobe could be a remnant of similar architecture found in other hymenopterans (Schafer and Bicker 1986) or the high number of serotonergic neurons is necessary for foragers outside the nest to process visual information to successfully collect food, subdue prey, and defend territory (Seid *et al*. 2008).

c-Fos staining. This technique shows the first example of Fos staining in an ant species. Additionally, it highlights the activity of 5-HT within a *T. caespitum* individual engaged in aggression. 5-HT neurons within the AL, OL, and SOG are shown to be active. We also see active serotonergic processes within the MB calyces. This activity within the antennal lobes and the lip of the MB calyces suggests that 5-HT is sent to the MB from the AL during aggression. Again, this supports 5-HT role in modulating higher order processing of olfactory information (Hoyer *et al*. 2005). We also see serotonergic activation in the SOG. This would correspond with the locking of mandibles that *T. caespitum* engage in during aggression. There was minimal 5-HT activation of the OL. Serotonin may not modulate visual information during aggression. *T.caespitum* primarily relies on olfactory information thus it is not surprising that there is minimal activation in the OL. Our technique helps illustrate how 5-HT is being used during aggression.

Neuron count. We were able to obtain an estimate of 5-HT neurons using our technique. The maximum count of 5-HT neurons was 78. Honeybees have about 75 (Shurmann and Klemm

1984). Other have reported 130 to 200 in *C. japonicus* and *C. herculeanus*, respectively (Tsuji *et al*. 2007, Gronenberg 1996). Given this range our count seems feasible. Brain size is social insects is tied to social organization It is hypothesized that eusocial insect's brains are smaller or miniaturized as social complexity increases. Individual ant's cognition is limited, but as a whole their collective intelligence allows for complex group behaviors (Feinermen and Traniello 2016). The low number of 5-HT neurons helps support a limited cognitive ability within an individual ant. *T.caespitum* is able to perform a wide repertoire of behaviors using a limited number of neuronal cells.

Conceptual Model for 5-HT modulated aggression. Using the data from this technique I would like to propose a conceptual model for 5-HT modulated aggression in *T. caespitum*. Pretend you are an individual ant. You use a random walk to explore your colonies territorial space looking for resources. While walking you encounter other ants. You antentate them to determine their identity by reading their cuticular hydrocarbons and assessing the relative abundance of methyl-branched alkanes and n-alkenes (Sano *et al*. 2018). We know that when individuals interact with their nestmates 5-HT increases (Bubak *et al*. 2016, Hoover *et al*. 2015) so the interactions likely increase 5-HT production in the AL. The large number of 5-HT neurons found in the AL would support this (Fig.3). The 5-HT produced in the AL is sent to the MB lip along the processes found in Figure 5. From there the information is processed and the you recognize the other as nestmate. This memory slowly decays, but as you explore you encounter more nestmates refreshing the 5-HT in your brain. As you explore you begin to encounter other ants. These ant's cuticular hydrocarbons to not match that of your nestmates. Since you have been in constant contact with your nestmates your 5-HT has been constantly refreshed. This constant refreshing of 5-HT by nestmate interaction primes the brain for aggression (Hoover *et al*. 2016). During

aggression we see a rise in brain titer levels of 5-HT (Bubak *et al*. 2016). The rise we see in brain titer levels of 5-HT during aggression could be explained by an increase in production of serotonin from the antennal lobes being shuttled to the MB (Fig.5). Once the 5-HT is integrated within the MB you decide to aggress. You lock mandibles with you opponent. This results in sustained serotonin release in the SOG to keep the mandibles locked with your adversary. (Fig. 5). The ants around you do the same since they share a similar brain state. After some time, a large portion of your colony is with you attacking the enemy. After some monoaminergic threshold is reached the fight ends and you follow the pheromone trail back to your nest.

Limitations. While this study is informative and descriptive of the pavement ant brain, the technique does have limitations. This data collected is based off the best examples from IHC; meaning only the images created that displayed detailed morphology and staining were chosen. Thus, all measurements and interpretations made are estimates. Using whole brains with IHC can make it difficult to achieve uniform orientation so brains in this study had varying orientations. This could have influenced interpretations of morphology and neuron counts. Some neurons tagged by the technique might not have been able to be counted due the orientation of the brain and the limitations of the microscope to penetrate the tissue. This reduces confidence in this techniques ability to establish a true neuron count. Also, the orientations of each brain may lead to inaccurate comparisons between experimental groups. Finally, since the images analyzed were selected as the best examples of the IHC technique, these brains come from a variety of individuals exposed to different behavioral contexts. It would be difficult to draw conclusions to a behavior or experimental setting.

All c-Fos measurements and estimates are based on a single brain. We were unable to replicate c-Fos staining. This limits the conclusions we can draw on the efficacy of our

technique. However, this might be due to the use of Formaldehyde instead of Para-formaldehyde (PFA). The antibodies used were described as working best with PFA as a fixative. Future studies that use this technique should try a variety of fixatives to determine which works best for Fos staining in their subjects.

Future Directions. This study shows that c-Fos can be used within ants to determine potential mechanisms for aggressive behavior in pavement ants. Additionally, once our c-Fos technique is perfected and replicated, we can use this technique to understand other behaviors in other species. The data collected helps build our conceptual model of collective behavior. By looking at an individual's brain we illustrate potential neural mechanisms that influence individual behavior. Those individual behaviors magnified to the whole colony could give insight into how collective decisions are made. This study highlights the proximate mechanism for the beginnings of complex, social behavior.

CHAPTER 5

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