Calcium-Mediated Excitation and Plasticity in Primary Olfactory Pathways of the Honey Bee Antennal Lobe

by

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Doctor of Philosophy

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ABSTRACT

Spatiotemporal processing in the mammalian olfactory bulb (OB), and its analog, the invertebrate antennal lobe (AL), is subject to plasticity driven by biogenic amines. I study plasticity using honey bees, which have been extensively studied with respect to nonassociative and associative based olfactory learning and memory. Octopamine (OA) release in the AL is the functional analog to epinephrine in the OB. Blockade of OA receptors in the AL blocks plasticity induced changes in behavior. I have now begun to test specific hypotheses related to how this biogenic amine might be involved in plasticity in neural circuits within the AL. OA acts via different receptor subtypes, AmOA1, which gates calcium release from intracellular stores, and AmOAβ, which results in an increase of cAMP. Calcium also enters AL interneurons via nicotinic acetylcholine receptors, which are driven by acetylcholine release from sensory neuron terminals, as well as through voltage-gated calcium channels. I employ 2-photon excitation (2PE) microscopy using fluorescent calcium indicators to investigate potential sources of plasticity as revealed by calcium fluctuations in AL projection neuron (PN) dendrites *in vivo*. PNs are analogous to mitral cells in the OB and have dendritic processes that show calcium increases in response to odor stimulation. These calcium signals frequently change after association of odor with appetitive reinforcement. However, it is unclear whether the reported plasticity in calcium signals are due to changes intrinsic to the PNs or to changes in other neural components of the network. My studies were aimed toward understanding the role of OA for establishing associative plasticity in the AL network. Accordingly, I developed a treatment that isolates intact, functioning PNs *in vivo*. A second study revealed that cAMP is a likely component of plasticity in the AL, thus implicating the

AmOAβ receptors. Finally, I developed a method for loading calcium indicators into neural components of the AL that have yet to be studied in detail. These manipulations are now revealing the molecular mechanisms contributing to associative plasticity in the AL. These studies will allow for a greater understanding of plasticity in several neural components of the honey bee AL and mammalian OB.

DEDICATION

I would like to dedicate this work to my grandparents for their endless support throughout my life. A special thanks to my brother for his ceaseless encouragement and support throughout my graduate career. I appreciate all Devin Taylor has done for me and all of the wonderful support he has provided throughout the final years of my graduate career and especially through the writing of this work.

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LIST OF ABBREVIATIONS

Abbreviation/Symbol:	Definition:
$\Delta F/F_0$	Change in fluorescence over the initial fluorescence
2PE	2-photon excitation
α-BTX	α-bungarotoxin
ACh	Acetylcholine
AL	Antennal lobe
AmOA1	*Apis mellifera* OA receptor (type-1)
AmOAβ	*Apis mellifera* OA receptor (beta-adrenergic type)
ANOVA	Analysis of variance
cAMP	3'-5'-cyclic adenosine monophosphate
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
$CdCl_2$	Cadmium chloride
CS	Conditioned stimulus
DmOA1	*Drosophila melanogaster* OA receptor (type 1)
DmOAβ	*Drosophila melanogaster* OA receptor (beta-adrenergic type)
ER	Endoplasmic reticulum
fs	femtosecond
GABA	γ-aminobutyric acid
Hz	Hertz
ITI	Inter-trial interval
KCl	Potassium chloride

Abbreviation/Symbol:	Definition:
KOH	Potassium hydroxide
LIN	Local interneurons
M	Molar
MB	Mushroom body
MEC	Mecamylamine
$MgCl_2$	Magnesium chloride
μM	Micromolar
mM	Millimolar
MW	Molecular weight
NA	Numerical aperture
Na^+	Sodium
nACh	Nicotinic acetylcholine
NaCl	Sodium chloride
OA	OA
OB	Olfactory bulb
OCT	OA
OER	Odor-evoked response
ORN	Olfactory receptor neuron
PBS	Phosphate buffered saline
PER	Proboscis extension reflex
pL	Picoliter
PMT	Photomultiplier tube
PN	Projection neuron

Abbreviation/Symbol:	Definition:
PSTH	Peri-stimulus time histogram
RP-8-Br-cAMPS	8-Bromoadenosine-3',5'-cyclic monophosphorothioate, RP-isomer
RyR	Ryanodine receptor
SEM	Standard error of the mean
SMB	Sodium metabisulfite
TTX	Tetrodotoxin
US	Unconditioned stimulus
VGCC	Voltage-gated calcium channel
VUM_{mx1}	Ventral unpaired median neuron maxillare 1
W	Watt

CHAPTER 1

INTRODUCTION

Olfaction is relevant for many mammalian and invertebrate behaviors, including mate selection, food localization, and regulating emotional states (Wilson, Fletcher, & Sullivan, 2004). Odors elicit powerful behavioral responses in both vertebrates and invertebrates, which have independently evolved functionally similar olfactory structures (Kaupp, 2010). In the periphery, there is a large family of odorant receptors expressed on dendrites of olfactory receptor neurons (ORNs) (Bargmann, 2006; Kaupp, 2010). With respect to a certain panel of odors, some receptors are broadly tuned and some are finely tuned, displaying cross-fiber coding properties with spatial and temporal components (Bargmann, 2006; Galizia, Sachse, Rappert, & Menzel, 1999). Despite the similar coding properties at the neural network level, the olfactory receptors of mammals and insects function very differently on a molecular level, in that they involve different transduction pathways. The mammalian olfactory receptors are G-protein coupled while insects have a co-receptor for every unique odorant receptor, and together they function ionotropically (Ache & Young, 2005; Kaupp, 2010; Sato et al., 2008). The unique odorant receptor gives rise to the specificity (Ache & Young, 2005; Bargmann, 2006; Kaupp, 2010). Aside from the sensory code, another commonality between vertebrates and invertebrates is that the ORNs that express the same receptor converge to the same area – called a 'glomerulus' - within the mammalian olfactory bulb (OB) and invertebrate antennal lobe (AL) (Ache & Young, 2005; Bargmann, 2006). Both vertebrates and invertebrates have three distinct types of neurons involved at the glomerular level. The OB has dendrites of mitral/tufted cells postsynaptic to the ORNs. These cells are similar to the post-synaptic

projection neurons (PNs) found in the AL. In the OB, inhibitory interneurons or mitral/tufted cells, interconnect glomeruli, which are similar to the function of local interneurons (LINs) in the AL (Strausfeld & Hildebrand, 1999).

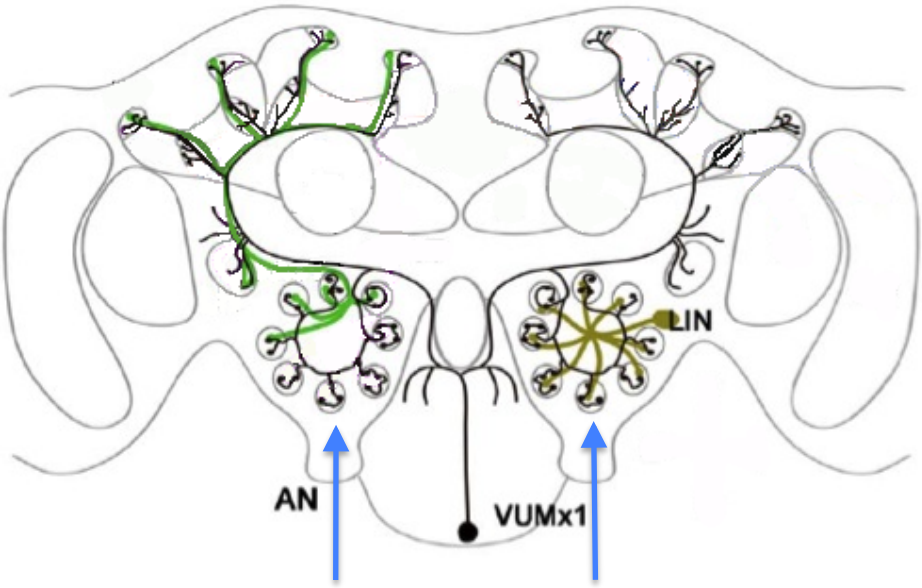

FIGURE 1: The figure shows the circuitry in the honey bee brain. The blue arrows indicate olfactory input from olfactory receptor neurons. These olfactory receptor neurons synapse onto both the projection neurons (green) as well as the local interneurons (yellow) within the primary sensory region in the honey bee brain, the antennal lobe. The local interneurons provide local connections within the glomeruli in the antennal lobe while the projection neurons project to higher brain regions - the mushroom bodies. The ventral unpaired median neuron (VUM_{mx1}, black) has a cell body located in the subesophogeal ganglion with dendritic arborizations in areas important for learning and memory, including the antennal lobes and mushroom bodies. Adapted from (Sinakevitch, Mustard, & Smith, 2011).

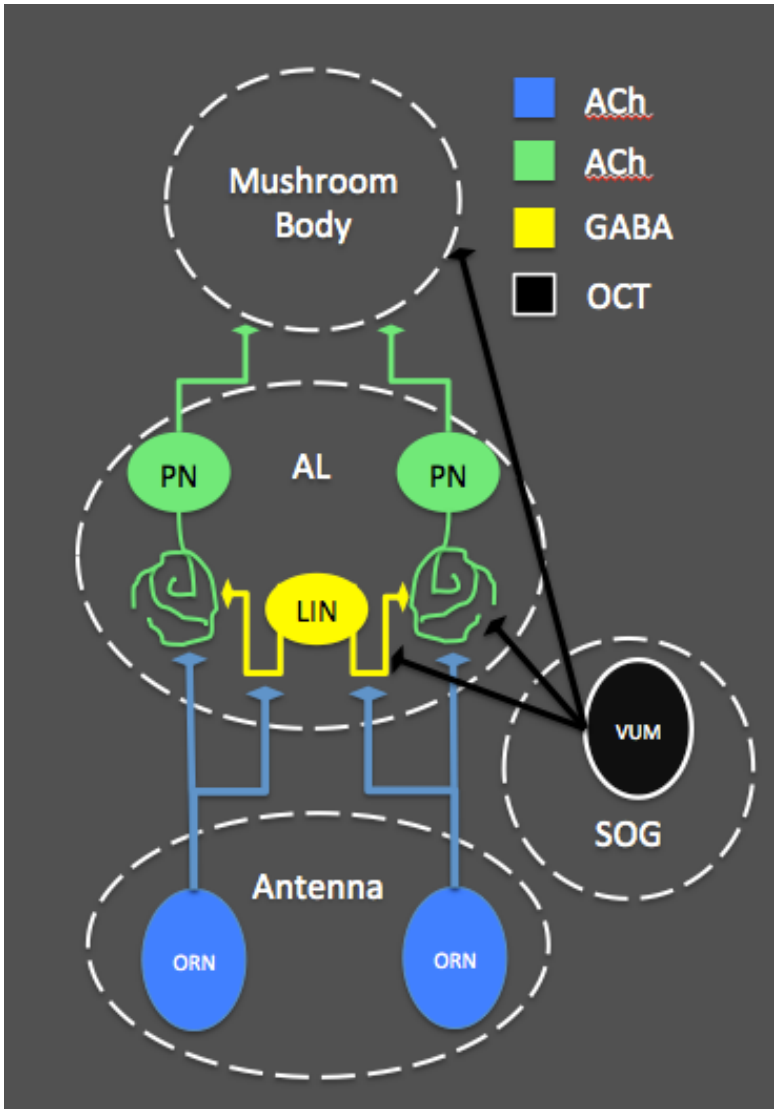

FIGURE 2: Circuit diagram for neuronal network in the honey bee brain. The olfactory receptor neurons (ORNs, blue) are cholinergic and synapse onto both local interneurons (LINs, yellow) and projections neurons (PNs, green) in the antennal lobe. The LINs provide connections among glomeruli in the AL and are primarily GABA-ergic. The PNs are cholinergic and project to the mushroom bodies. The ventral unpaired median neuron (VUM_{mx1}, black) is located in the subesophogeal ganglion and releases octopamine in the AL and MBs.

The honey bee is an excellent model organism for studying olfactory plasticity. Forager honey bees must form memories based on many different environmental stimuli associated with floral food sources in order for a colony to survive. Floral scents help bees to locate and forage on the nectar and pollen a flower offers in exchange for pollination (B.H. Smith, Wright, & Daly, 2006). As in mammals, and in insects like the honey bee, information about food (e.g. what flowers are in bloom) will change very rapidly within an animal's lifetime. Since these food sources change from hour to hour, day to day, and week to week, a great deal of plasticity is exhibited in honey bee behavior, which maps to plasticity in the brain. With fewer than a million neurons, the honey bee brain is easily accessible and simpler in structure relative to a mammalian brain (Figures 1 and 2) (Galizia & Rossler, 2010; Witthöft, 1967). Under controlled laboratory conditions, honey bees can perform associative conditioning tasks identical to those performed under natural conditions. Behavior can be easily assayed using a procedure called Proboscis Extension Reflex (PER) conditioning. In this conditioning paradigm, the odor is the conditioned stimulus (CS) and the food reward (a sucrose droplet) is the unconditioned stimulus (US). Timing of the delivery of the US in relation to the CS is crucial (Figure 3). The CS needs to overlap with the US and either be presented slightly before or simultaneously with the US (M. Hammer & Menzel, 1995). If there is no overlap of the CS and US, the normal increase in PER in response to odor is greatly diminished or absent. Several different control procedures have been used to show that the change in behavior with proper CS-US pairing arises from associative conditioning and not sensitization (Bitterman, Menzel, Fietz, & Schäfer, 1983).

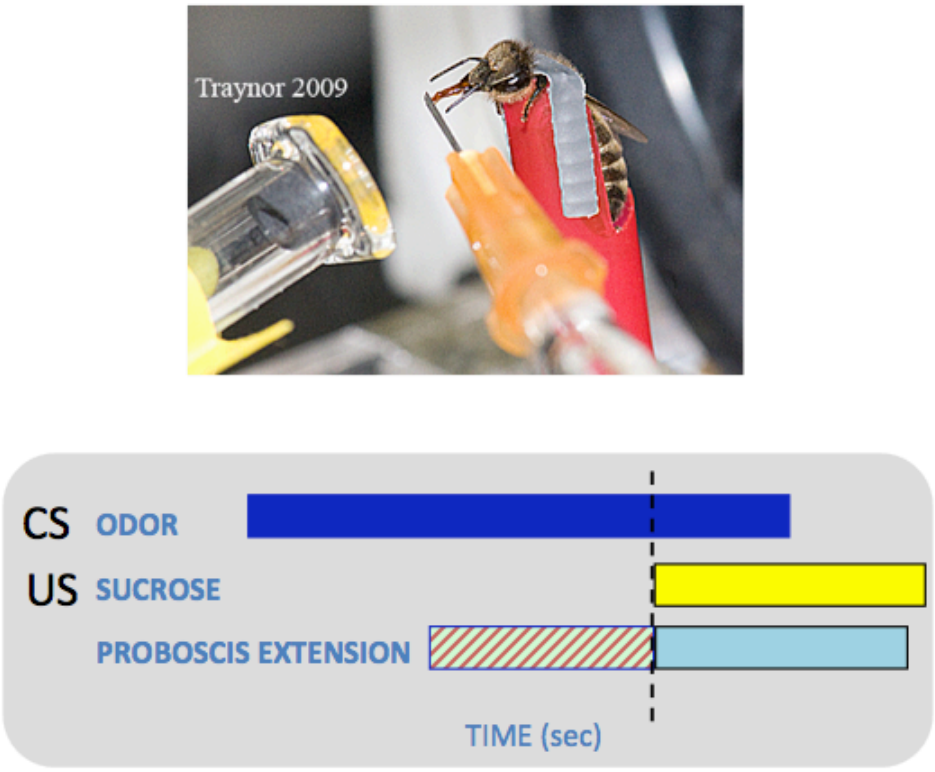

FIGURE 3: Proboscis extension reflex. In this conditioning paradigm, the odor stimulus is the conditioned stimulus (CS) and the food reward (a sucrose droplet) is the unconditioned stimulus (US). Timing of the delivery of the US in relation to the CS is crucial. The CS needs to precede the US and if there is no overlap of the CS and US, the normal increase in PER in response to odor is greatly diminished or absent.

Processing the insect AL and the vertebrate OB can be modulated by similar mechanisms (Davis, 2004). Processing pathways for the CS (odor) and the US (sucrose) converge first in the AL. In particular, one small group of neurons (e.g. VUM_{mx1}) receives input from sucrose-sensitive taste receptors and releases a biogenic amine, octopamine (OA), into the AL (Figures 1, 2 and 4). OA is a key neuromodulator in areas important for learning and memory in the honey bee brain. Coincidence of calcium influx from acetylcholine (ACh) channels, via activation from cholinergic ORNs (Figures 1, 2, and 5), with activation of an OA receptor, may contribute to long-term modifications of the AL network.

Olfactory associative and nonassociative learning paradigms modify odor-evoked activity in the OB of mammals (Fletcher & Wilson, 2002; Salcedo, Zhang, Kronberg, & Restrepo, 2005) and the AL of invertebrates (Fernandez, Locatelli, Person-Rennell, Deleo, & Smith, 2009; Locatelli et al., 2013). Olfactory associative conditioning has been shown to induce synaptic plasticity in the AL (Faber, Joerges, & Menzel, 1999; Grunbaum & Muller, 1998), as evidenced behaviorally by increased PER to an odor as well as through changes in odor-evoked calcium signals in imaging studies. Our lab has shown that this synaptic plasticity persists for at least 24 hours after conditioning and serves to increase the separation between odors with different meaning in regard to food (Fernandez et al., 2009). Electrophysiological studies done on the AL of the moth (*Manduca sexta)* have reveled several types of learning dependent on different reinforcement contexts. In forward pairing and differential conditioning, there was always a net increase in the number of responsive units, although this results from a loss of some units and a gain of others (Daly, Christensen, Lei, Smith, & Hildebrand, 2004).

Collectively, this research has conclusively shown that AL circuitry is not fixed and is subject to experience-dependent plasticity.

Biogenic amines are found in both vertebrates and invertebrates, have a major role in plasticity, and significantly influence learning and memory (Blenau & Baumann, 2001). Norepinephrine and epinephrine are preferentially synthesized in vertebrates while their functional analogs, tyramine and OA respectively, are synthesized in invertebrates (Blenau & Baumann, 2001). OA has been implicated in reinforcement processing and associative conditioning in the honey bee (Blenau & Baumann, 2001; T. Farooqui, 2007; M. Hammer & Menzel, 1998). As described above, OA is released from the ventral unpaired median neurons when the honey bee's mouthparts are stimulated by sucrose (Figure 5); the best studied is VUM_{mx1} (Figure 4). Both the ALs and the mushroom bodies – a multimodal integration center that comprises about a third of the entire brain - receive input from the VUM_{mx1} neuron (Martin Hammer, 1993). OA can trigger two different signaling cascades via two classes of OA receptors (Figure 5). The first type of OA receptor (AmOA1 and its fruit fly ortholog DmOA1) drives release of calcium from intracellular stores via PLC/IP3 signaling (Balfanz, Strunker, Frings, & Baumann, 2005; Evans & Robb, 1993; T. Farooqui, 2007). The second class of OA receptors (AmOAβ and DmOAβ), which includes potentially several subtypes, acts via an increase in cAMP (Evans & Robb, 1993; T. Farooqui, 2007; Roeder, 1999).

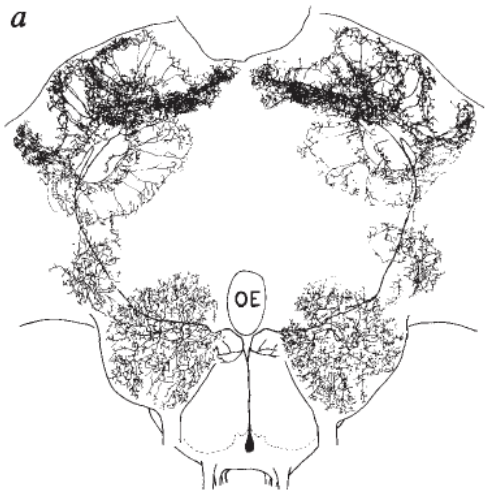

FIGURE 4: The ventral unpaired median (VUM_{mx1}) neuron which arborizes extensively, and releases OA, in areas important for learning and memory within the honey bee brain including the ALs and the MBs. Figure from (Martin Hammer, 1993).

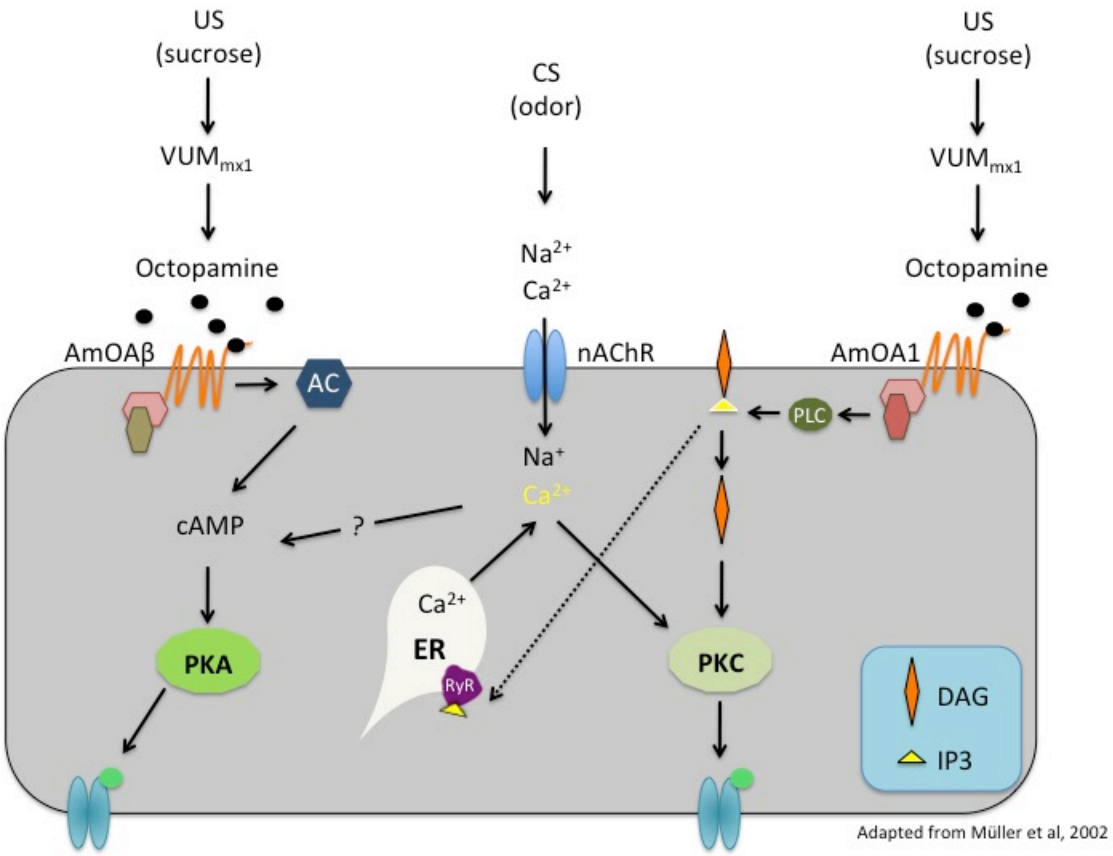

FIGURE 5: Potential signaling cascades elicited by activation of AmOA1 or AmOAβ by its agonist ligand OA are shown. Activation of the AmOA1 receptor by OA results in activation of ryanodine receptors downstream, which leads to release of intracellular calcium from the endoplasmic reticulum. This then activates PKC and leads to phosphorylation of ion channels. Activation of the AmOAβ receptor activates adenylate cyclase and leads to an increase in intracellular cAMP. This then activates PKA, which phosphorylates targets such as ion channels. What is phosphorylated following PKA and/or PKC is not yet known. Figure adapted from (Müller, 2002).

Muller et al. (2000) studied regulation of cAMP levels in the AL and central brain. They hypothesized that, due to the widespread arborizations of the VUM_{mx1} neuron throughout the honey bee brain, the sucrose induced OA release is a potential modulator of cAMP. This modulation leads to changes in PKA and other components of the intracellular signaling cascade in LINs (Müller, 2002). Accordingly, odor and mechanical stimulation of the antennae alone do not induce PKA in the antennal lobes (Hildebrandt & Müller, 1995). In the same study, it was proposed that this modulation of cAMP occurs across all glomeruli but that the odor induced activation occurs in a glomerulus specific manner. This produces the correlation of pre- and post-synaptic activity in specific glomeruli needed to drive associative plasticity in the brain. Indeed, blocking PKA during associative conditioning training will result in a loss of long term memory formation (Müller, 2000). However, it is still unclear what proteins are being phosphorylated (Müller, 2002). The timing of PKA activation in associative conditioning tasks is dependent on the temporal pairing of the odor conditioned and sucrose unconditioned stimulus as well as on the number of pairings that occur (Müller, 2000). In another study, PKA was found to be mostly in the LINs in the AL (Müller, 1997). Müller et al (2000) tried to imitate PKA activation by photolyzing caged cAMP in the AL. Photolyzed cAMP without odor or a conditioning trial had no effect on the conditioned behavior of the honey bees. A single conditioning trial without the photolyzed cAMP was not sufficient to produce long-term memory. However, when photolyzed cAMP was combined with a single conditioning trial, long-term memory formation was induced (Müller, 2000).

Studies from Muller et al. (Müller, 2000, 2002, 2008; Müller & Hildebrandt, 2002) have reveled much about the cAMP/PKA pathway in the honey bee and conversion of sucrose coupled to odor into long term memory. In fact, different studies have implied that each of the AmOA receptor subtypes is involved in plasticity in the AL (Tahira Farooqui, 2012; Müller, 2002). However, it remains unclear which cell types these OA receptor subtypes are expressed on different neurons within the AL neural network and how they are involved in the plasticity that has been identified. Existing studies of plasticity of PNs cannot clearly show whether the plasticity results from intrinsic properties of the PNs or from plasticity in other cells that are connected to the PNs. So it is important to develop methods for recording from PNs, as well as from other components of the network, when they are isolated *in vivo* from synaptic inputs. Therefore,, my goal was to study plasticity in PN dendrites in an AL in which synaptic connectivity is intact and to develop a method to study plasticity in which synaptic connectivity to PNs has been eliminated in an *in vivo* system. I also attempted to extend these types of studies to investigate intrinsic properties of LINs in the honey bee AL.

CHAPTER 2

OCTOPAMINE MODULATION OF THE ODOR RESPONSE

INTRODUCTION

The insect AL and the vertebrate OB are the major areas of the brain that receive afferent input from sensory receptors located on the antenna or olfactory epithelium (Bargmann, 2006; Kaupp, 2010). Both the AL and OB exhibit a similar organization for processing sensory input. All of the olfactory sensory neurons that express the same receptor (mammals), or pair of receptors (insects), converge onto the same area for synaptic interaction with local and projection interneurons. The AL/OB networks then process the sensory pattern across the subset of ORNs activated by an odor and transmit the pattern to the central brain (Bargmann, 2006; Kaupp, 2010).

It has been well established that this early processing can be modulated by association of an odor with reinforcement (Davis, 2004). Processing pathways for the CS (odor) and the US (sucrose) converge first in the AL. In particular, the ventral unpaired median neuron receives input from sucrose-sensitive taste receptors and releases a biogenic amine, OA, into the AL (Martin Hammer, 1993). OA is a key neuromodulator in areas important for learning and memory in the honey bee brain (M. Hammer & Menzel, 1995; Martin Hammer, 1993). Coincidence of calcium influx from ACh channels, via activation from cholinergic olfactory receptor neurons, with activation of an OA receptor may contribute to long-term modifications of the AL network. Olfactory associative and nonassociative learning paradigms modify odor-evoked activity in the OB of mammals (Fletcher & Wilson, 2002; Salcedo et al., 2005) and the AL of invertebrates (Fernandez et

al., 2009; Locatelli et al., 2013). Olfactory associative conditioning has been shown to induce synaptic plasticity in the AL (Faber et al., 1999; Grunbaum & Muller, 1998), as evidenced behaviorally by increased PER to an odor as well as through changes in odor-evoked calcium signals in imaging studies. Our lab has shown that synaptic plasticity in the AL persists for at least 24 hours after conditioning and serves to increase the separation between odors with different meaning in regard to food (Fernandez et al., 2009). Electrophysiological studies done on the AL of the moth (*Manduca sexta)* has reveled several types of learning dependent on differential types of reinforcement contexts (Daly et al., 2004). In forward pairing and differential conditioning paradigms, there was always a net increase in the number of responsive units, although this results from a loss of some units and a gain of others (Daly et al., 2004). Collectively, this research has conclusively shown that AL circuitry is not fixed, but instead is subject to experience-dependent plasticity.

Biogenic amines are found in both vertebrates and invertebrates, have a major role in plasticity, and significantly influence learning and memory (Blenau & Baumann, 2001). Norepinephrine and epinephrine are preferentially synthesized in vertebrates while their functional analogs, tyramine and OA respectively, are synthesized in invertebrates (Blenau & Baumann, 2001). OA has been implicated in reinforcement processing and associative conditioning in the honey bee (Blenau & Baumann, 2001; T. Farooqui, 2007; M. Hammer & Menzel, 1998). OA is released from the ventral unpaired median neurons (e.g. VUM_{mx1}) when the honey bee's mouthparts are stimulated by sucrose. Both the AL and the mushroom bodies are involved in processing olfactory sensory information and receive input from the VUM_{mx1} neuron (Martin Hammer, 1993). OA can trigger two

different signaling cascades via two classes of OA receptors. The first type of OA receptor (AmOA1 and its fruit fly ortholog DmOA1) drives release of calcium from intracellular stores via PLC/IP3 signaling (Balfanz et al., 2005; Evans & Robb, 1993; T. Farooqui, 2007). The second class of OA receptors (AmOAβ and DmOAβ) are beta-adrenergic and activation results in an increase in cAMP (Evans & Robb, 1993; T. Farooqui, 2007; Müller, 2002; Roeder, 1999).

It is unclear how these OA receptor subtypes are involved in the different cell types of the AL neural network to drive the plasticity that has been identified in PNs. Presumably, coincidence of depolarization of interneurons and projection neurons in the AL with activation of one or more types of octopamine receptors leads to long-term modifications of the AL network. However, it is unclear which of the octopamine receptor subtypes are involved in the LINs and PNs to drive the plasticity that has been identified in bioimaging studies. The objective of our studies was to use standard imaging of PNs backfilled with the calcium indicator fura-2-dextran. We coupled the imaging with use of octopamine, as well as with blockers of intracellular signaling, to establish which of the octopamine receptor subtypes could be involved in driving the plasticity reported in our earlier studies (Fernandez et al., 2009).

MATERIALS AND METHODS

SPECIMEN PREPARATION AND DYE LOADING

For each experiment below, a separate set of foraging worker honey bees were prepared for imaging following a modified protocol of (Galizia, Joerges, Kuttner, Faber, & Menzel, 1997). Forager honey bees (*Apis melifera*) were collected at the entrance to

the hive the day preceding the imaging session and immediately placed on ice to briefly anesthetize them. The bees were then mounted in plexiglass frames with wax (Figures 6A and 6B) and fed 1M sucrose to satiation. The bees were kept in a humid chamber until 12 hours before the imaging session at which time a rectangular window in the cuticle was made from compound eye to compound eye and from antennae to medial ocellus (Figure 6C). The glands and trachea were carefully pushed aside until the alpha lobe could be visualized, for use as a landmark (Figure 6C). A bolus of fura-2-dextran dye on the tip of a quartz glass microelectrode was used to create a lesion along the medial and lateral antennocerebral tracts at a point close to where they enter the mushroom bodies (Figure 6D). The piece of the head capsule that was removed was placed back on the bee to close the window, and then it was covered with wax. The bee was fed 1M sucrose and placed back in the humid chamber for 12 hours to allow the dye to travel along the tracts and backfill the projection neuron dendrites.

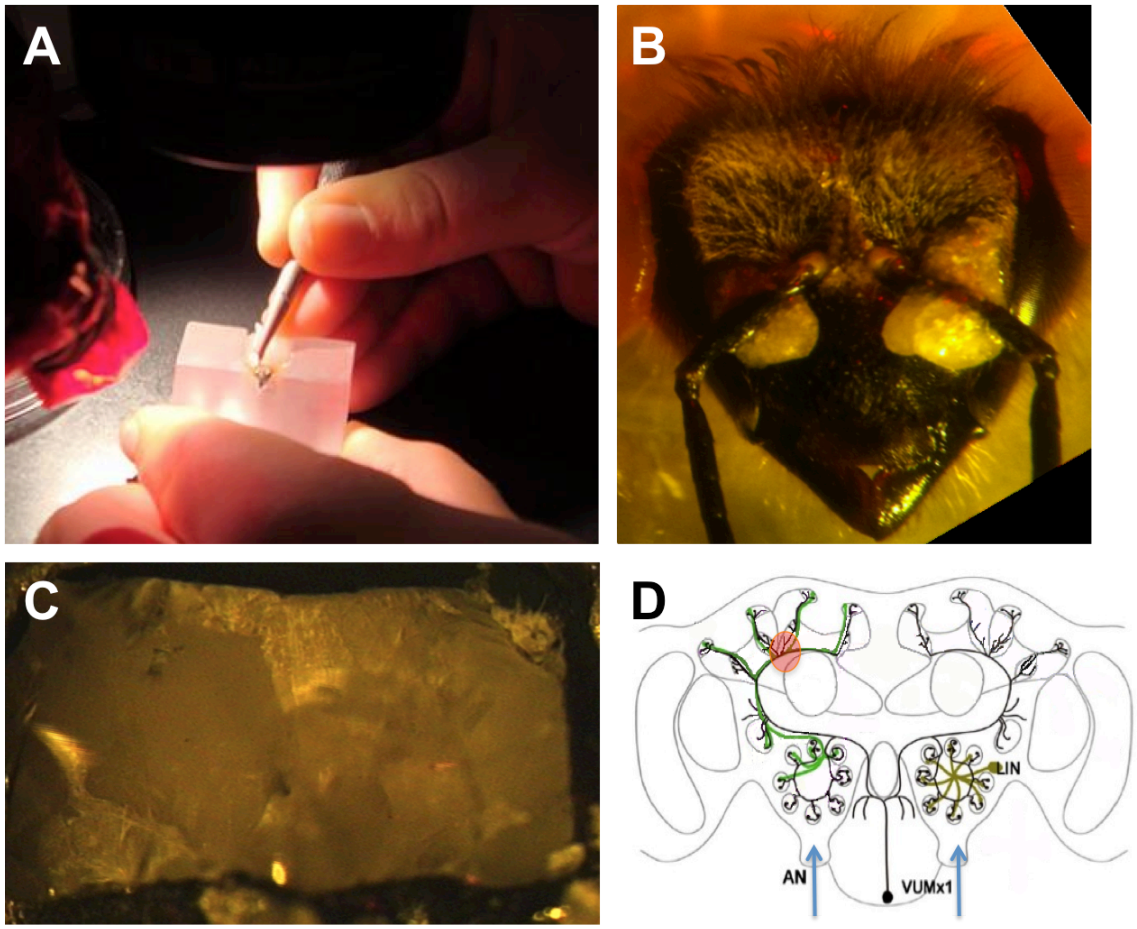

FIGURE 6: Bee preparations for imaging. 6A) Bee mounted in plexiglass imaging holder being prepared for imaging. 6B) Bee's head fixed in place with a soft wax (white) and dental wax (pink). Bee's antennae fixed in place with low temp wax (eicosane, white). 6C) A rectangular window in the head capsule was opened and trachea removed in order to reveal the bee's brain. 6D) Schematic of honey bee brain circuitry. Injection spot of the fura-2-dextran is shown in red, along the medial and lateral antennocerebral tracts at a point close to where they enter the mushroom bodies.

PHARMACOLOGICAL AGENTS

All pharmacological agents were dissolved in saline solution (130mM NaCl, 7mM $CaCl_2$, 6mM KCl, 2mM $MgCl_2$, 160mM Sucrose, 25mM Glucose, 10mM Hepes, 0.02M Ascorbic Acid). OA (*Sigma-Aldrich*), an agonist of AmOA1 and AmOAβ, receptors was pressure injected (0.04pL,10psi; Picospritzer III, Parker Instruments) directly above the AL neuropil at differing time points according to the experimental protocol used, at a low dose (1mM) and at a high dose (10mM) in different bees. The nACh receptor antagonists, mecamylamine (MEC) and α-bungarotoxin (α-BTX), were bath applied (1mM and 100μM respectively) in the head capsule for 20 minutes prior to imaging in one experimental protocol at the doses described by a previous study (Cano Lozano, Bonnard, Gauthier, & Richard, 1996; Gauthier et al., 2006).
(-)-Nicotine hydrogen tartrate salt (Sigma-Aldrich) was pressure injected (1mM, 0.04pL, 10psi; Picospritzer III, Parker Instruments) directly next to the honey bee AL neuropil in the cholinergic system studies at a dose described in a previous study (Thany & Gauthier, 2005). The cAMP blocker, RP-8-Br-cAMPS (500μM), was bath applied for 20 minutes prior to odor-octopamine pairing according to a previous study performed on honey bees (Müller, 2000). Saline solution was used for all controls.

ODORS AND ODOR DELIVERY SYSTEM

The odors were delivered using a custom built olfactometer using parts from an autom8 pressurized perfusion system (AutoMate Scientific Inc., Berkeley, CA, USA). The odor stimulus was either nonaldehyde, or a 1:1 mixture of 2-octanone:1-hexanol, (Sigma-Aldrich). The odors were prepared to a final volume of 0.02M in mineral oil

(Sigma-Aldrich). The odor delivery was presented by loading the odor cartridges with filter paper soaked with 20 μL of the odor mixture of 1:1 2-octanone and 1-hexanol. Filter papers were loaded in the custom built olfactometer. Odors were injected in a filtered constant air stream and delivered to the antennae of the honey bee. Odor blanks of mineral oil were used for controls. Each odor stimulus was 0.5 seconds in length. This odor preparation was similar to other honey bee odor stimulation protocols (Fernandez et al., 2009; Locatelli et al., 2013). Each odor presentation for the pre-test and post-test involved 3 successive odor stimulations at 3 min inter-trial intervals (ITIs). For all figures the three individual stimulations are shown in black and the average is shown in red.

DATA COLLECTION AND PROCESSING

A Prairie View Ultima IV was used for imaging and was equipped with 6mm galvanometer scanners. A Ti:Sapphire laser (830 nm, 80 MHz, <100 fs pulse width; Mai Tai HP, Spectra Physics, Mountain View, California) pumped by an integrated 14W Millenia® pump laser (Spectra Physics) was used for 2PE through A 20X water-immersion objective (Olympus XLUMPLFL20XW/IR-SP, 0.95 NA, 1.9 mm working distance). PrairieView software was utilized to control scan and filter settings, laser wavelength and power, photomultiplier tube (PMT) gain, and pre-session wavelength spectra optimization. Emission light was collected using multi-alkali PMTs (Hamamatsu, Bridgewater, New Jersey). The ImageJ MBF "ImageJ for Microscopy" collection was used for fluorescence over time image analysis.

QUANTIFYING CALCIUM CHANGES

The change in fluorescence was imaged at a rate of 24-25Hz, which allows for the detection of fast calcium transients. The first 15 frames of each time-series preceding the stimulus onset were averaged as a baseline for the initial fluorescence (F_0). The observed changes in calcium were quantified as $\Delta F/F_0$ across time. The calculated $\Delta F/F_0$ is proportional to intracellular calcium concentrations. A significant calcium signal was defined as a change in F greater than 5% and is 1.5 standard deviations away from the background. The threshold for statistical calcium signals is shown as a dotted orange line in figures.

STATISTICAL ANALYSIS

T-tests were used to test for significant differences of peak amplitudes of $\Delta F/F_0$ between treatment groups (1mM or 10mM) upon octopamine application. Repeated measures ANOVAs were used to test for significant differences between responding and non-responding glomeruli before and after pharmacological conditioning treatments in saline and RP-8-Br-cAMPS or saline and nACh receptor antagonists. The repeated measures were the 3 trials pre-OA conditioning or post-OA-conditioning. The total areas of odor responses during either the odor or nicotine stimuli were analyzed using GraphPad Prism® 5 for experiments in which nicotine was applied pre- and post-odor-octopamine pairing.

EXPERIMENT 1: INCREASED ODOR RESPONSES WHEN OCTOPAMINE IS PHARMACOLOGICALLY PAIRED WITH ODOR STIMULUS

We paired the odor stimulus with octopamine in order to test for OA-driven associative changes in excitability of PN dendrites. To test for baseline responses to odor, we initially delivered the 1:1 mixture of 2-octanone:1-hexanol to the antennae three times at an inter-trial intervals of three minutes. These odor response scans were used as the pre-OA conditioning responses. Stimulations during these tests were completed in a bath of saline. Following the initial odor responses, the micropipette filled with 10mM OA was positioned directly above the honey bee's AL neuropil and directly adjacent to the glomeruli of interest. The pharmacological conditioning trial involved a pressure injection of 10mM OA (0.04pL) in between two odor stimulations of 0.5 seconds each (Figure 8, Top). Following this conditioning trial, the saline solution was removed and replaced with fresh saline. Ten minutes then passed to allow the OA to clear the system. Three odor trials with inter-trial intervals of three minutes were then completed with the 1:1 mixture of 2-octanone:1-hexanol in order to record the post-conditioning odor responses. For offline analysis, the initial odor responses were divided into two categories according to the definition of a significant $\Delta F/F_0$ response above: responding glomeruli and non-responding glomeruli. The pre-conditioning odor-evoked responses of the two classes of glomeruli were then separately compared to their post-conditioning odor-evoked responses

EXPERIMENT 2: OCTOPAMINE APPLIED ALONE

In order to test the excitability of PNs in response to OA when applied without odor stimulation, OA was pressure injected in different preparations at a low dose (1mM) or a high dose (10mM) directly above and adjacent to the glomeruli. Each bee in this experiment received three pressure injections of OA with no odor stimulus. Between each OA presentation, the saline was removed, replaced, and 10 minutes passed before the next OA presentation in order to allow the OA to clear the system.

EXPERIMENT 3: EFFECTS OF A cAMP BLOCKADE ON ODOR-EVOKED RESPONSES

In order to test the hypothesis that the increased excitability of PN dendrites following olfactory conditioning is dependent on cAMP, we used a bath of a the cAMP blocker RP-8-Br-cAMPS, which we applied for 20 minutes prior to imaging of olfactory responses. Honey bees were pre-tested, conditioned to odor associated with OA application, and then post-tested as in experiment 1. Data analysis was also as in experiment 1. Following this conditioning trial, the RP-8-Br-cAMPS bath solution was removed and replaced with fresh RP-8-Br-cAMPS and ten minutes passed in order to allow the OA to clear the system.

EXPERIMENT 4: SPECIFICTIY OF ODOR RESPONSES AFTER OCTOPAMINE PAIRING

Associative conditioning should produce reasonably specific responses to the CS (Mackintosh, 1983). When honey bees are conditioned to an odor, they respond strongly

to the CS (Menzel, Hammer, Braun, Mauelshagen, & Sugawa, 1991; B.H. Smith et al., 2006). They respond less so to a different odor, and the strength of that response depends on the structural similarity of that odor to the CS odor. In order to test for specificity of the odor response in these studies, after conditioning of the mixture associated with octopamine we tested with a dissimilar odor – nonaldehyde – to which honey bees would behaviorally show little generalization after behavioral conditioning to a mixture of a 1:1 mixture of 1-octaonone:2-hexanol (Smith & Menzel 1987). All bees in this experiment received each of the following in a randomized order before coupling the 2-octanone:1-hexanol mixture with 10mM octopamine: mineral oil (blank), 1:1 mixture of 2-octanone:1-hexanol, or nonaldehyde. The ITI between odor presentations was 3 minutes. Following pairing of the pharmacological application of octopamine with the mixture of octanone and hexanol, we waited 10 minutes for the octopamine to clear the system. Following this waiting period, all three odor treatments were presented again in randomized order to test for post-conditioning odor responses and generalization.

EXPERIMENT 5: nACh RECEPTOR BLOCAKDE, BLOCKS ODOR-EVOKED RESPONSES

ACh is the primary neurotransmitter released by sensory axons onto dendrites of interneurons in the AL (Barbara, Grunewald, Paute, Gauthier, & Raymond-Delpech, 2008; J. Dupuis, Louis, Gauthier, & Raymond, 2012; J. P. Dupuis, Gauthier, & Raymond-Delpech, 2011). In order to evaluate changes in response to nACh receptors, we first needed to confirm that blockade of nACh receptors would block odor driven responses. To do that, we used bath applications of nACh receptor blocker antagonists

MEC and α-BTX. MEC has a higher affinity for blocking α2> α7 nACh receptor subunits while α-BTX blocks primarily α7 nACh receptor subunits. Each antagonist was bath applied for 20 minutes prior to imaging of olfactory responses. Honey bees were conditioned as in experiment 1 with the exception that it was performed under nACh blockade with one of the two drugs. Following conditioning, the MEC or α-BTX bath solution was removed and replaced with fresh MEC or α-BTX and ten minutes passed in order to allow the OA to clear the system. As in experiment 1, three odor trials with the 1:1 mixture of 2-octanone:1-hexanol were then presented at ITIs of three minutes in order to record the post-conditioning odor responses. The calcium responses were categorized as non-responding and compared to the saline non-responding group (Figure 13).

EXPERIMENT 6: CHOLINERGIC RESPONSE PROFILE CHANGES FOLLOWING PHARMACOLOGICAL CONDITIONING PARADIGM

In order to test if the cholinergic response changes following a pharmacological conditioning, three pressure injections of nicotine (1mM) in saline were applied directly above and adjacent to the glomeruli of interest at inter-trial intervals of ten minutes. Following each application of nicotine, the saline was removed and replaced and ten minutes passed in order to allow the nicotine to clear the system before the next application of nicotine. Ten minutes following the last pressure injection of nicotine, the same conditioning paradigm as in experiment 1 was used under saline. The initial odor responses were all categorized as responding glomeruli. The pre-conditioning odor-evoked responses were then compared to their post-conditioning odor-evoked responses (Figure 14). The post-pharmacological conditioning cholinergic response profile was then

recorded. Three pressure injections of nicotine (1mM) in saline were applied directly above and adjacent to the same glomeruli at inter-trial intervals of ten minutes. Following each pharmacological application of nicotine, the saline was removed and replaced and ten minutes passed in order to allow the nicotine to clear the system before the next pharmacological application of nicotine. As a confirmation of perfusion of drug across neuropil from a pressure injection, fluorescein was mixed with either 1mM nicotine in saline or saline alone; calcium signals were recorded simultaneously with the perfusion of the solution across the glomerulus being measured (Figure 7).

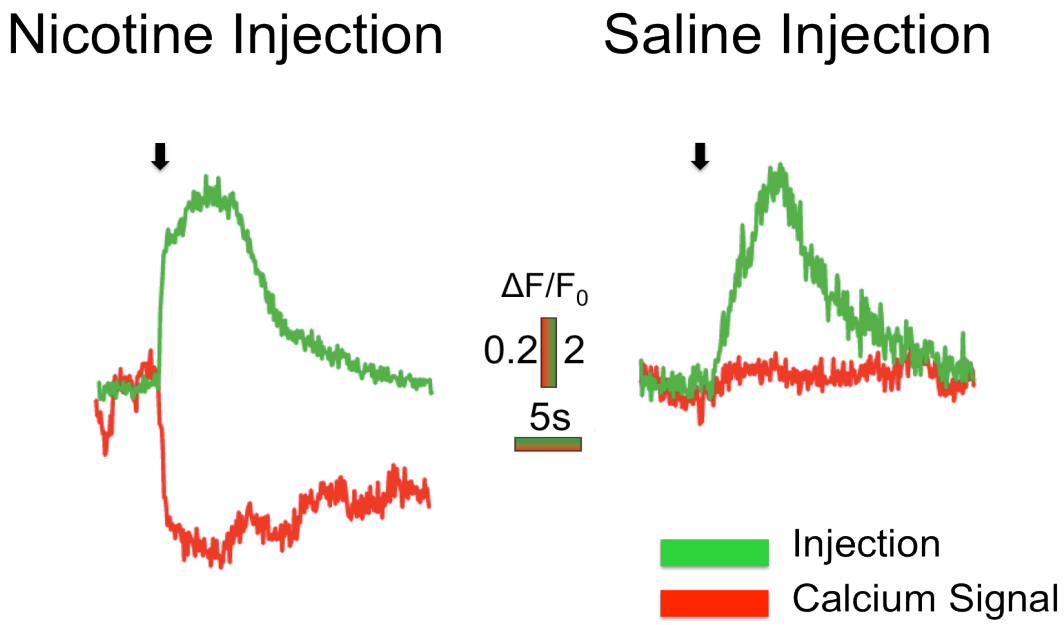

FIGURE 7: Nicotine dissolved in saline and saline solutions were pressure injected with fluorescein mixed with the fluid to allow the calcium signal to be detected simultaneously with the detection of the injection. The perfusion of the pressure injection across the honey bee antennal lobe neuropil is shown in green for both the pressure injection of nicotine (left) and of saline (right). The calcium signal, or lack of a calcium signal, elicited by a pressure injection of nicotine or saline, is shown in red.

RESULTS

EXPERIMENT 1: OCTOPAMINE MODULATES OLFACTORY RESPONSES WHEN PAIRED WITH ODOR

When 10mM OA was pressure injected above the antennal lobe in the odor conditioning paradigm, the post-conditioning odor response was strengthened compared to the pre-conditioning odor trial (Figures 8 and 10). This was true for not only the initially responsive glomeruli, but also for the initially silent glomeruli, which became responsive. The responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.141 +/- 0.011$ to $\Delta F_0/F = 0.471 +/- 0.195$ in the post-OA conditioning condition. The non-responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.031 +/- 0.004$ to $\Delta F_0/F = 0.407 +/- 0.112$ post-OA conditioning (Figure 10).

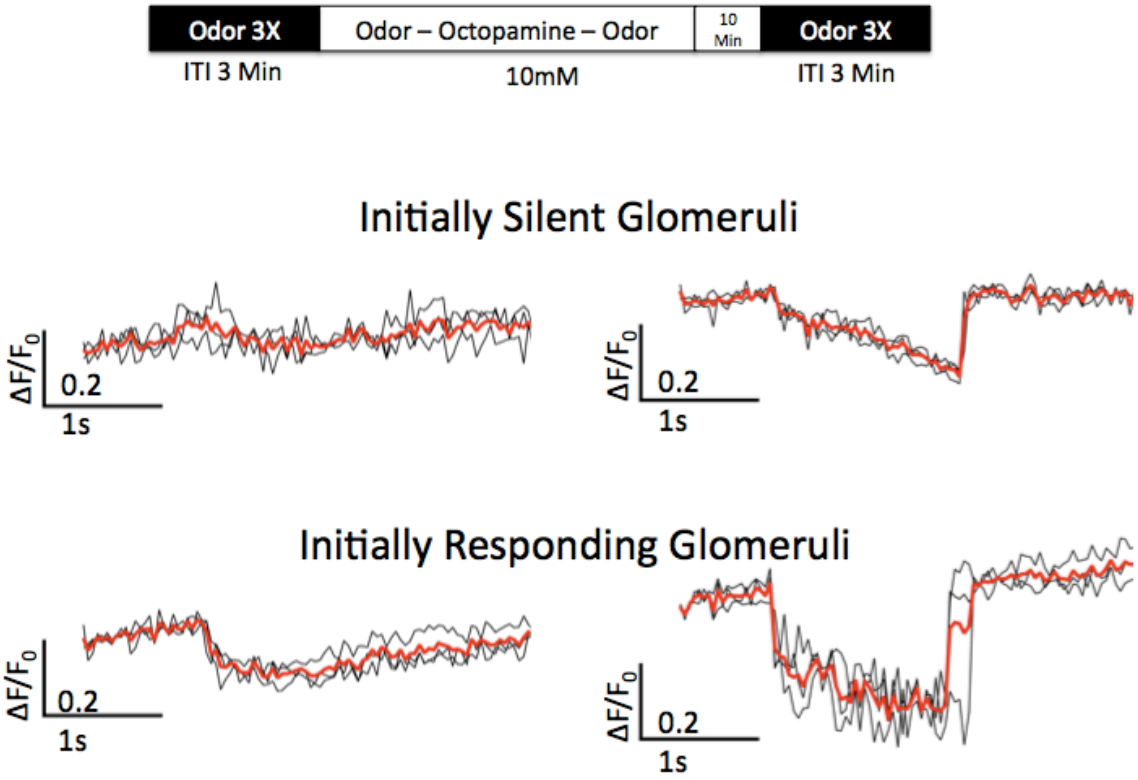

FIGURE 8: Raw traces of calcium signals from a single initially silent glomerulus (top traces) is shown as well as a single initially responsive glomerulus (bottom traces). The traces on the left are the pre-OA conditioning scans. The traces on the right are the post-OA conditioning scans. Each of the three calcium signal scans is shown as the black traces. The average of the three odor trials for each paradigm in each glomerulus is shown as a red traces

EXPERIMENT 2: OCTOPAMINE ALONE DOES NOT EVOKE A SIGNIFICANT CALCIUM REPSONSE

Neither 1mM (n=3) nor 10mM (n=10) OA injected above the AL neuropil, without the CS, evoke significant calcium signal changes in PN dendrites ($\Delta F/F_0<0.05$; Figure 9). All recordings were within background noise levels.

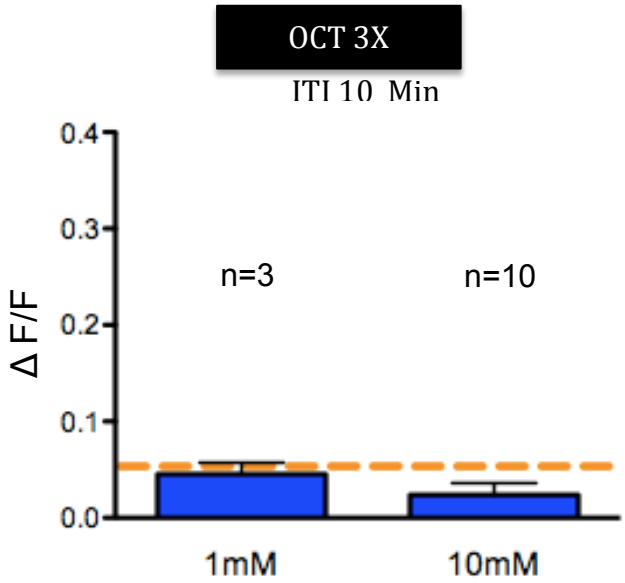

FIGURE 9: OA application failed to elicit a significant calcium signal 1mM (n=3) and 10mM (n=10) when it was injected above the AL neuropil. The significant calcium signal is defined as a change of $\Delta F/F_0>0.05$ as indicated by the orange dotted line; everything below the line is within background noise levels. The pharmacological application timing is shown above the chart. Shown is the mean +/- SEM.

EXPERIMENT 3: RP-Br-8-cAMPS BLOCKS OCTOPAMINE INDUCED PLASTICITY IN THE ANTENNAL LOBE

The cAMP blocker, RP-8-Br-cAMPS, blocks the plasticity that is induced by pairing an odor with the pharmacological application of OA. The responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.141 +/- 0.011$ to $\Delta F_0/F = 0.471 +/- 0.195$ in the post-OA conditioning condition. The non-responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.031 +/- 0.004$ to $\Delta F_0/F = 0.407 +/- 0.112$ in the post-OA conditioning condition. The responding glomeruli in the RP-8-Br-cAMPS pre-OA conditioning did not significantly change from $\Delta F_0/F = 0.260 +/- 0.034$ to $\Delta F_0/F = 0.215 +/- 0.034$ in the post-OA conditioning condition. It is notable that these glomeruli still showed a response above baseline, but this response did not increase as it did under saline. This means that RP-8-Br-cAMPS did not diminish the normal odor response; it only blocked the expected increase. The non-responding glomeruli in the RP-8-Br-cAMPS pre-OA conditioning did not significantly change from $\Delta F_0/F = 0.028 +/- 0.011$ to $\Delta F_0/F = 0.024 +/- 0.006$ in the post-OA conditioning condition (Figure 10).

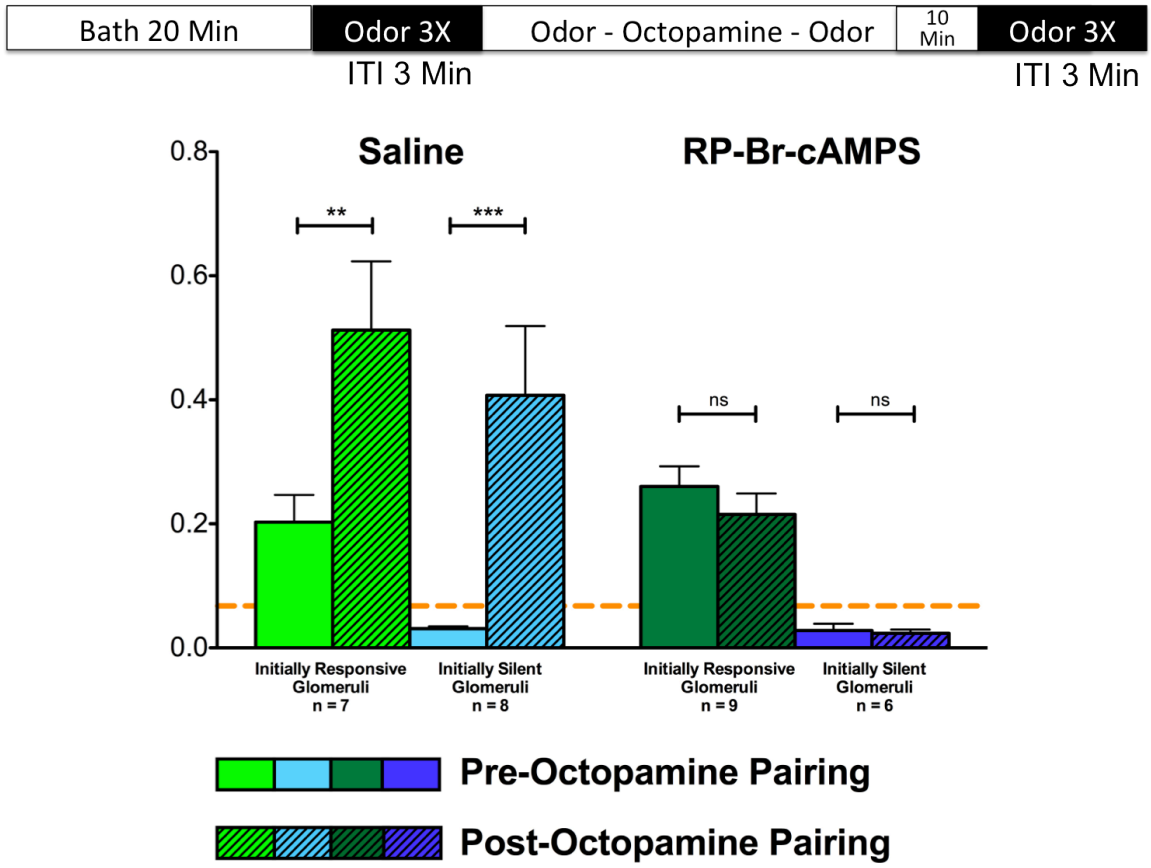

FIGURE 10: cAMP blockade blocks the OA induced plasticity when paired with odor relative to saline treatment. The responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.141$ +/- 0.011 to $\Delta F_0/F = 0.471$ +/- 0.195 in the post-OA conditioning condition. The non-responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.031$ +/- 0.004 to $\Delta F_0/F = 0.407$ +/- 0.112 in the post-OA conditioning condition. The responding glomeruli in the RP-8-Br-cAMPS pre-OA conditioning did not significantly change from $\Delta F_0/F = 0.260$ +/- 0.034 to $\Delta F_0/F = 0.215$ +/- 0.034 in the post-OA conditioning condition. The non-responding glomeruli in the RP-8-Br-cAMPS pre-OA conditioning did not significantly change from $\Delta F_0/F = 0.028$ +/- 0.011 to $\Delta F_0/F = 0.024$ +/- 0.006 in the post-OA conditioning condition. Shown is the mean +/- SEM.

EXPERIMENT 4: SPECIFICITY OF ODOR RESPONSES AFTER OCTOPAMINE PAIRING

The odor presentation did not have a significant effect on the results of the odor-evoked calcium responses. The glomeruli that had PN dendrites show increased excitability following OA pairing with an odor, did not show significant calcium responses to the dissimilar odor. (Figures 11 and 12).

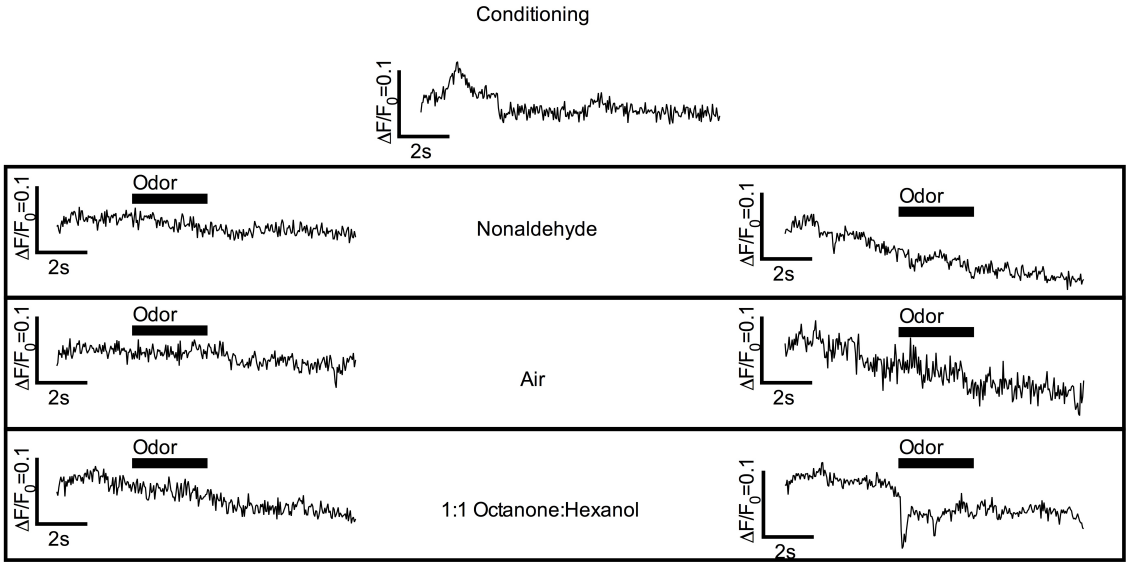

FIGURE 11: The odor-evoked responses of a single trial from a single glomerulus in a single bee are shown. The bars above each trace show the odor stimulus presentation. The conditioning trace in which the odor is paired with a pressure injection of OA is shown above all the traces in the middle. The pre-OA-odor pairing odor-evoked calcium response traces are shown on the left. The post-OA-odor pairing odor-evoked calcium response traces are shown on the right. The odor used during the conditioning trial which was paired with the OA was a mixture of 1:1 2-octanone:1-hexanol. There were no significant calcium responses to either the control (air) or to nonaldehyde.

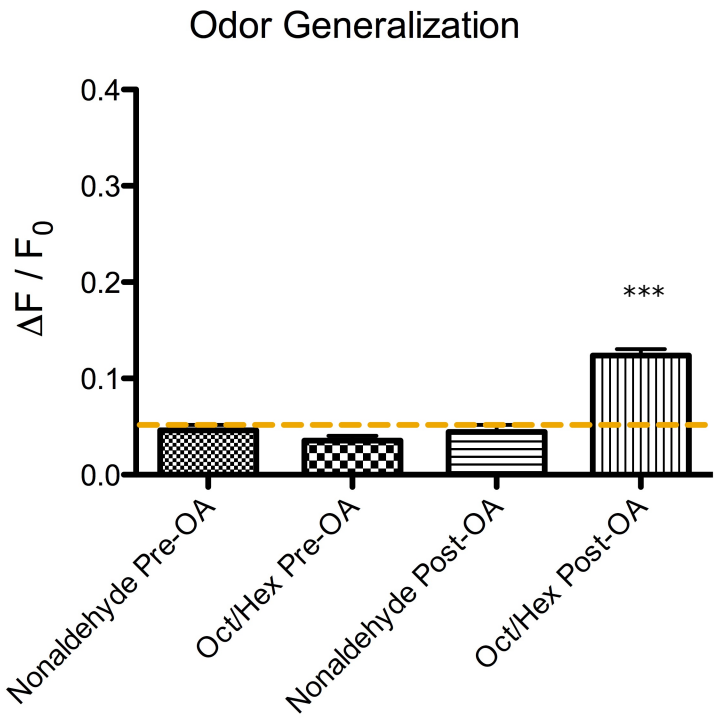

FIGURE 12: Summary of the specificity of the conditioned odor response. The pre-octopamine nonaldehyde odor response was within noise levels ($\Delta F_0/F$ = 0.046 +/- 0.006) and did not significantly differ from the post-octopamine nonaldehyde odor response ($\Delta F_0/F$ = 0.044 +/- 0.007). The pre-octopamine octanone/hexanol odor response was also within noise levels ($\Delta F_0/F$ = 0.035 +/- 0.005) but after octopamine pairing to the odor mixture, the post-octopamine octanone/hexanol odor response significantly differed ($\Delta F_0/F$ = 0.124 +/- 0.007). Shown is the mean +/- SEM. $p<0.001$, n=12.

EXPERIMENT 5: nACh RECEPTOR BLOCKADE BLOCKS ODOR RESPONSES

The non-responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.031 +/- 0.004$ to $\Delta F_0/F = 0.407 +/- 0.112$ in the post-OA conditioning condition. When the bath of the nACh receptor antagonist, α-BTX (1mM), which blocks α7 nACh receptors was applied, the non-responding glomeruli in the α-BTX pre-OA conditioning showed no significant calcium signal above baseline and was unchanged from $\Delta F_0/F = 0.017 +/- 0.009$ to $\Delta F_0/F = 0.038 +/- 0.011$ in the post-OA conditioning responses. When the nACh receptor antagonist, MEC (1mM), which blocks α2> α7 nACh receptors, was bath applied, the non-responding glomeruli in the MEC pre-OA conditioning also showed no significant calcium signal and was also unchanged from $\Delta F_0/F = 0.022 +/- 0.013$ to $\Delta F_0/F = 0.022 +/- 0.012$ in the post-OA conditioning responses. (Figure 13)

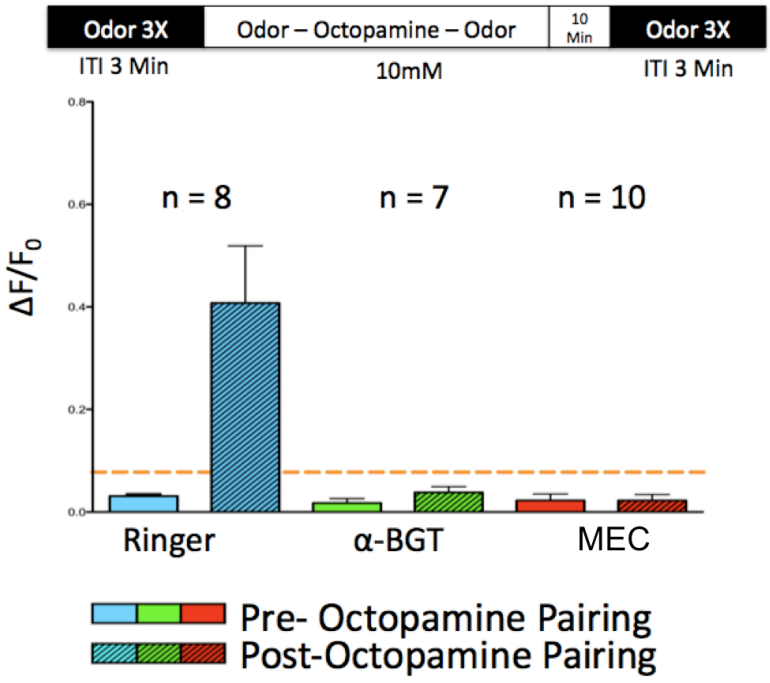

FIGURE 13: nACh receptor antagonists block odor-evoked responses. The non-responding glomeruli in saline pre-OA conditioning increased from $\Delta F_0/F = 0.031$ +/- 0.004 to $\Delta F_0/F = 0.407$ +/- 0.112 in the post-OA conditioning condition. The non-responding glomeruli in the α-BTX pre-OA conditioning had no significant calcium signals and was unchanging from $\Delta F_0/F = 0.017$ +/- 0.009 to $\Delta F_0/F = 0.038$ +/- 0.011 in the post-OA conditioning responses. The non-responding glomeruli in the MEC pre-OA conditioning had no significant calcium signals and was unchanging from $\Delta F_0/F = 0.022$ +/- 0.013 to $\Delta F_0/F = 0.022$ +/- 0.012 in the post-OA conditioning responses. Shown is the mean +/- SEM.

EXPERIMENT 6: OCTOPAMINE PAIRING WITH ODOR FAILS TO SIGNIFICANTLY CHANGE NICOTINE RESPONSES

When 10mM OA is pressure injected above the antennal lobe in the odor conditioning paradigm (Figure 14), the post-pharmacological conditioning odor response was consistently and significantly strengthened in amplitude (Pre-OA $\Delta F_0/F = 0.157 +/- 0.034$ to Post-OA $\Delta F_0/F = 0.398 +/- 0.083$, Figure 15) as well as the response area (Pre-OA $\Delta F_0/F^2 = 0.153 +/- 0.064$ to Post-OA $\Delta F_0/F^2 = 0.245 +/- 0.134$, Figure 16). No consistent changes in nicotine-evoked calcium signals were detected in amplitude of response (Pre-OA $\Delta F_0/F = 0.087 +/- 0.023$ to Post-OA $\Delta F_0/F = 0.065 +/- 0.026$, Figure 17) or total response area (Pre-OA $\Delta F_0/F^2 = 1.708 +/- 1.094$ to Post-OA $\Delta F_0/F^2 = 1.359 +/- 1.177$, Figure 18). In order to more finely analyze the responses to both odor and nicotine, we used both the signal amplitude as well as the area.

Although there were no significant differences in the nicotine-evoked responses pre- and post-conditioning, there were variable responses. Figure 19 shows the average (of three trials) amplitude of both odor-evoked responses and nicotine-evoked responses by glomerulus and bee.

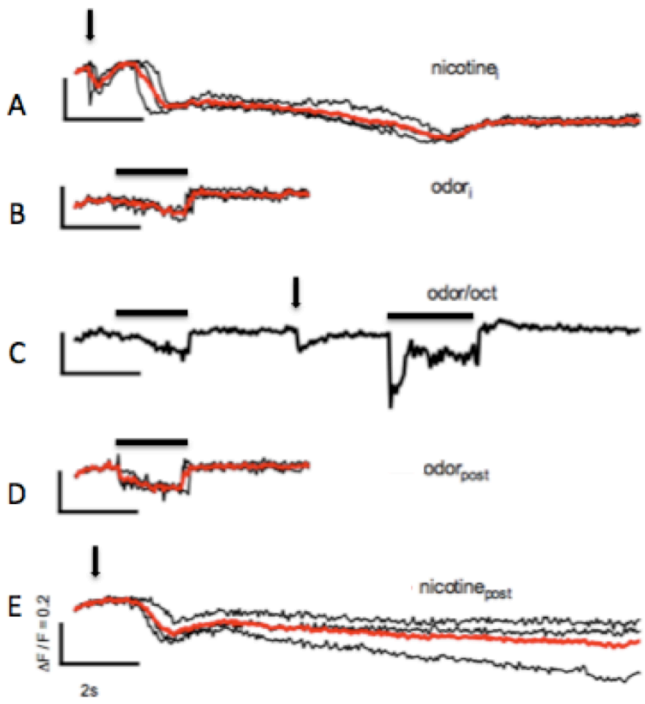

FIGURE 14: Nicotine responses pre- vs. post- odor-OA pairing. A) Nicotine (1mM) was injected at the point indicated by the arrow. The black traces are the raw traces of 3 trials within the same glomerulus. The average of the three traces is shown in red. B) The pre-conditioning odor responses are shown. The bar above the traces indicates the odor stimulus delivery. The three trials of the odor responses are shown in black. The average of the three traces is shown in red. C) The pharmacological conditioning paradigm is shown and the bars above the trace indicate the odor stimulation while the arrow indicates the pressure injection of OA (10mM). D) The post-conditioning odor responses are shown in the same format as in 'B'. The post-conditioning response is strengthened as compared to the pre-pharmacological conditioning response shown in B. E) Nicotine (1mM) injection repeated as in 'A'. The total change in fluorescence does not significantly differ from the nicotine calcium responses shown in A but the temporal response may change.

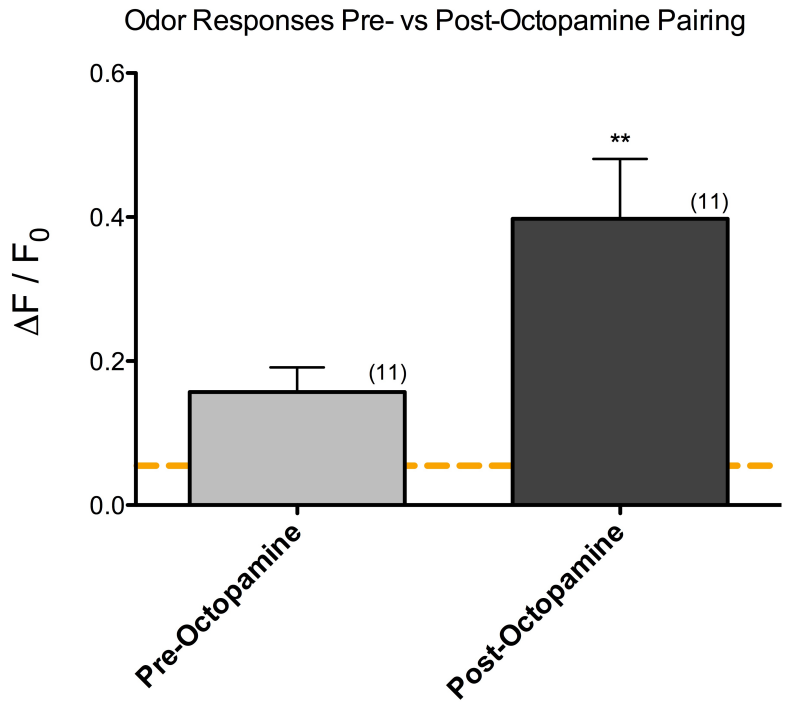

FIGURE 15: $\Delta F_0/F$ of odor-evoked calcium responses from pre-OA-odor pairing condition compared to post-OA-odor pairing condition. All glomeruli were responsive pre-OA conditioning. The pre-OA odor-evoked responses ($\Delta F_0/F$ = 0.157 +/- 0.034) were significantly lower than the post-OA odor-evoked responses ($\Delta F_0/F$ = 0.398 +/- 0.082). The orange line indicates threshold for significant calcium signals. ** indicates $p < 0.01$. Shown is the mean +/- SEM.

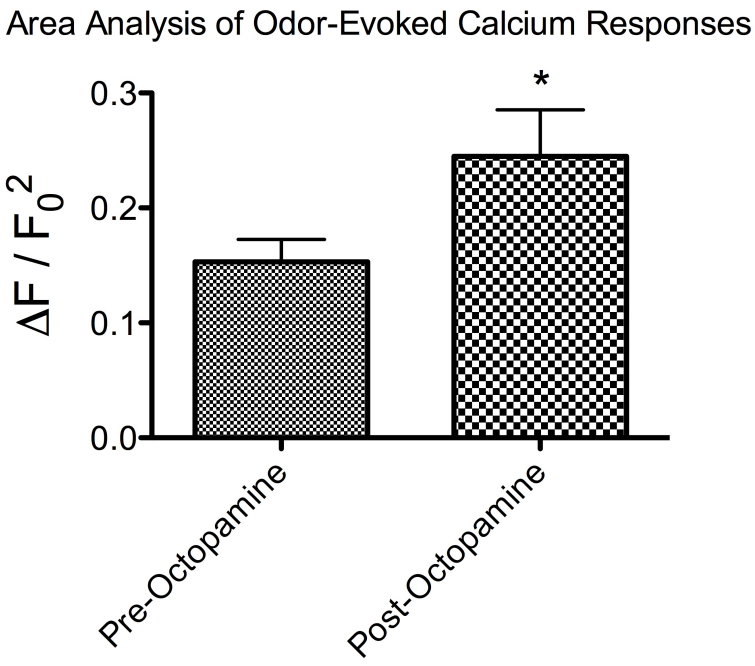

FIGURE 16: Area of odor response pre-OA-odor pairing compared to post-OA-odor pairing. All glomeruli were responsive pre-OA conditioning. The pre-OA odor-evoked response ($\Delta F_0/F^2 = 0.153$ +/- 0.064) was significantly lower than the post-OA odor-evoked response ($\Delta F_0/F^2 = 0.245$ +/- 0.134). * indicates $p < 0.05$. Shown is the mean +/- SEM.

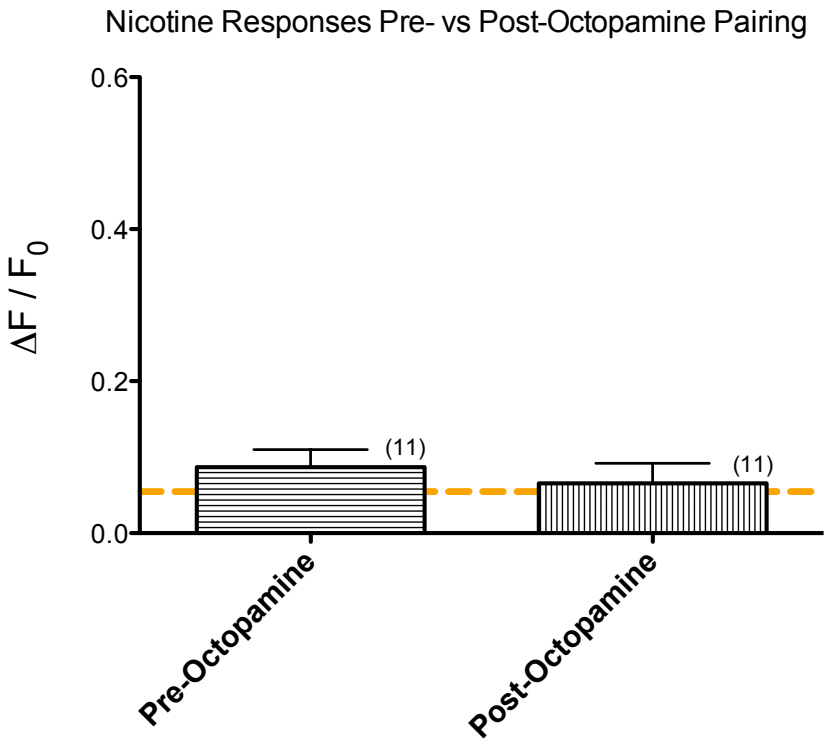

FIGURE 17: Peak $\Delta F_0/F$ of nicotine-evoked calcium responses from pre-OA-odor pairing condition compared to post-OA-odor pairing condition. The pre-OA nicotine-evoked responses ($\Delta F_0/F^2 = 0.153$ +/- 0.064) did not significantly differ from the post-OA nicotine-evoked responses ($\Delta F_0/F^2 = 0.245$ +/- 0.134). The orange line indicates threshold for significant calcium signals ($p>0.05$). Shown is the mean +/- SEM.

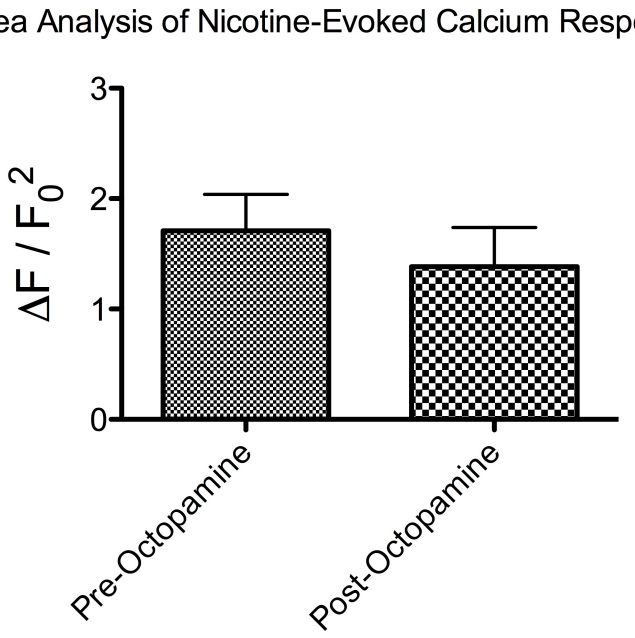

FIGURE 18: Area of nicotine-evoked response pre-OA-odor pairing ($\Delta F_0/F^2$ = 1.708 +/- 1.094) compared to post-OA-odor pairing ($\Delta F_0/F^2$ = 1.359 +/- 1.177) analyzed for only the nicotine studies. The post-OA pairing nicotine-evoked response did not change significantly from pre-OA pairing nicotine-evoked response. $p>0.05$. Shown is the mean +/- SEM.

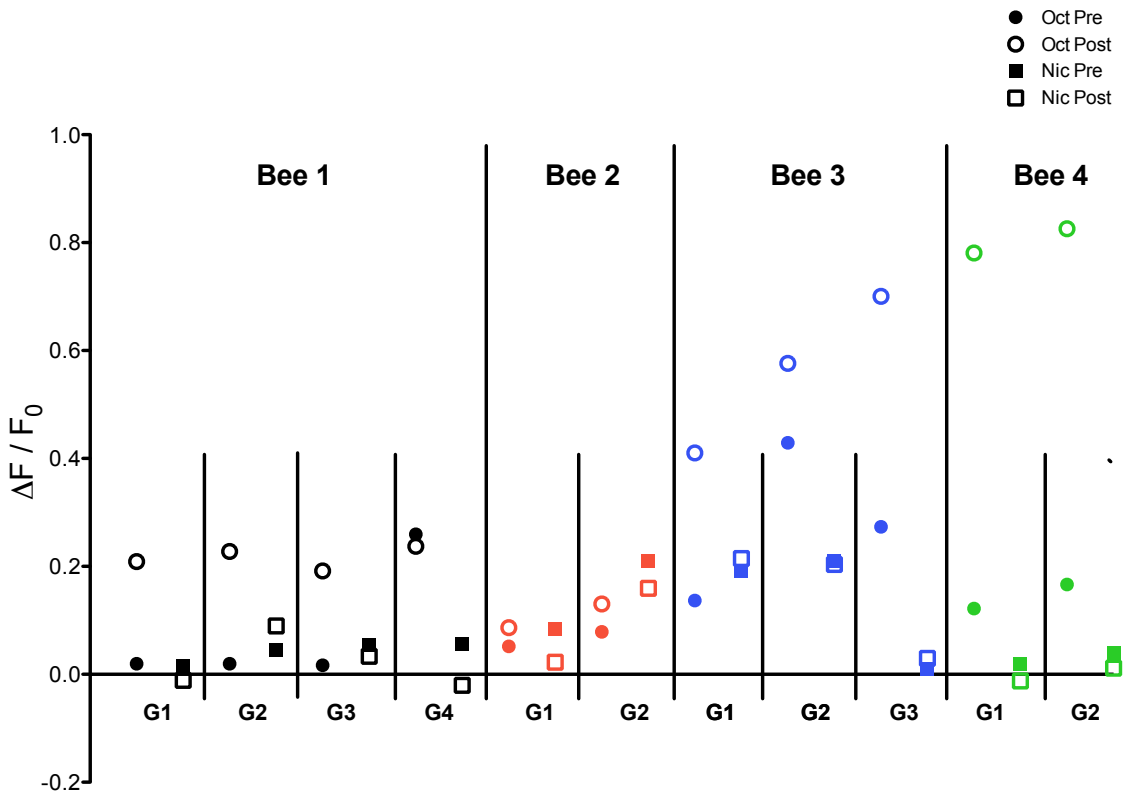

FIGURE 19: Scatterplot showing variable responses of glomeruli to odor input or nicotine application pre- and post-odor-OA pairing. There were four bees analyzed in these experiments. The raw data amplitudes of both odor-evoked responses (circles) and nicotine-evoked responses (squares) are shown for each glomerulus. The solid symbols on the scatterplots represent the averaged amplitude of the three odor or nicotine trials in the pre-OA-odor conditioning paradigm. The hollow symbols represent the averaged amplitude of the three odor or nicotine trials post-OA-odor pairing conditioning paradigm.

DISCUSSION

We have shown that a pharmacological application of the biogenic amine, OA, when coupled with an odor, can induce increased excitability of the PN dendrites to the odor stimulus. This increase was consistent in both initially responding and non-responding glomeruli. In fact, the responses in the initially silent glomeruli became as strong as the initially responsive glomeruli. It should be noted that each of the three odor response trials were very similar in their spatiotemporal response profile and robustness of response for the period in which they were recorded (pre – vs. post-OA-odor conditioning).

OA itself did not increase excitability in the PN dendrites. In order to test for this, we pressure injected a low and high dose of OA and neither evoked a significant calcium response. This result implies that the AmOA1 receptor is not involved in excitability of PN dendrites. If that were the case, we should have recorded increases in calcium with OA alone, because this receptor drives the release of calcium from intracellular stores. This result corresponds to recent immunocytochemical analyses of the distribution of AmOA receptors in the AL. It occurs on different cell types, and mostly on GABA-ergic components of the AL network (LINs and a few PNs that project to a different area of the brain and would not be labeled by fura-2-dextran in our method). If we develop a methodology for filling GABA-ergic LINs with a calcium indicate, we would be able to test this hypothesis in future studies.

The lack of a direct OA-mediated increase in calcium could mean that the mechanism driving plasticity we report in the PNs requires confluence of calcium input via cholinergic receptors (odor input) with an increase in cAMP stimulated by activation

of AmOAβ receptors. We tested this hypothesis by performing experiments under blockade of cAMP by RP-Br-8-cAMPS. Under cAMP blockade, the increased excitability we observed under a saline, when OA was paired with odor, was completely abolished in both initial response types of glomeruli.

Behaviorally conditioning honey bees to odor produces robust responses to that odor that can last a bee's lifetime. That response also generalizes to odors that have similar chemical makeups, either in the form of molecular structure (B. H. Smith & Menzel) or mixtures (Fernandez et al., 2009). However, the response does not strongly generalize to odors that are chemically very different. If the AL plasticity we report in PN dendrites is linked to behavioral conditioning, it should not generalize to novel odors to which the behavioral response would not generalize. To test this hypothesis, we coupled the mixture of 1:1 2-octanone:1-hexanol with the pharmacological application of OA and tested with a dissimilar odor, nonaldehyde. We indeed found that the glomeruli that showed increased excitability to the paired odor did not respond to the dissimilar odor.

The primary transmitter from sensory axons to PN dendrites in ACh, which interacts with postsynaptic nACh receptors (Barbara et al., 2008; J. Dupuis et al., 2012; J. P. Dupuis et al., 2011). Because nACh receptors allow both calcium and sodium to enter PNs (Gotti & Clementi, 2004), it is conceivable that the plasticity induced by association of odor with OA could result from changes in the nACh receptor or its distribution in PN dendrites (Ping & Tsunoda, 2012). We therefore first confirmed that a blockade of nACh would block the odor-driven response. The first nACh receptor antagonist was α-bungarotoxin which blocked α7 nACh receptors. The second nACh receptor antagonist was mecamylamine which blocked α2>α7 nACh receptors. Both antagonists

successfully blocked all odor-evoked responses. This could mean that both the α7 and α2 nACh receptors are responsible for the odor-evoked responses or it could mean that the dose of mecamylamine blocked the α7 nACh receptors and that the α7 nACh receptors are responsible for the odor-evoked responses.

Having confirmed that the odor response was due to activation of nACh receptors, the next experiment was designed to test for changes in calcium when PNs are driven by nicotine, which is an agonist of nACh receptors. The last experiment tested the effects of OA-odor pairing on the cholinergic system by comparing the pre-conditioning nicotine-evoked and odor-evoked responses to the post-conditioning odor-evoked and nicotine-evoked responses. As before, the post-conditioning odor-evoked responses were strengthened as compared to the pre-conditioning odor-evoked responses. This was true for both the total amplitude as well as the total area of the odor-evoked responses. The nicotine-evoked responses however were not consistently strengthened or weakened in regard to either amplitude or total area of response. The responses were extremely variable from glomerulus to glomerulus and bee to bee. The raw data amplitudes of both odor-evoked responses and nicotine-evoked responses show the variability in the scatterplot (Figure 19). This variability led to a lack of significance in the resulting data on amplitudes of nicotine-evoked calcium responses as well as the total area of nicotine-evoked calcium signals to be not significant.

However, the spatiotemporal response profile was slightly variable from pre- to post-conditioning responses. This was more difficult to quantify, and there were no significant differences, but this could be subject to more detailed analysis in the future as future experiments could reveal more about these subtle changes in cholinergic response

profiles. Experiments performed on mammals have shown that there are variable cholinergic response profiles to both agonists and antagonists dependent on the nACh receptor subunit composition and affinity (Briggs et al., 2006; Collins, Salminen, Marks, Whiteaker, & Grady, 2009; Gotti et al., 2008; Marks et al., 1999; McClure-Begley et al., 2009; Moroni, Zwart, Sher, Cassels, & Bermudez, 2006) as well as experiments on honey bees (J. P. Dupuis et al., 2011). Different subunit compositions of nACh receptors leads to different Ca^{2+} permeability of the channel (Fucile, 2004). Also, plasticity has been shown to be regulated by nACh receptors (McKay, Placzek, & Dani, 2007).

We cannot attribute the changes we observe to PNs alone, because they are still connected to other components of the network, which may respond to OA (Sinakevitch et al., 2013). We now need to complete these experiments on isolated PNs *in vivo* to determine if the plasticity is intrinsic to the PNs, which we suspect it is. We also need to complete these experiments on isolated LINs if we can develop a method to do so which can prove to be very challenging. Once complete, we can evaluate how summation of calcium from different sources modifies PN responses to odor by targeting downstream calcium-sensitive processes.

CHAPTER 3

PROPERTIES OF PROJECTION NEURONS STUDIED *IN VIVO* BY WAY OF ISOLATION IN AN INTACT NETWORK

Spatiotemporal processing in the mammalian olfactory bulb (OB) and insect antennal lobe (AL) is modified by both nonassociative and associative plasticity. It is important to study how these different forms of plasticity are implemented in the OB and AL networks through modulation of any of the several different cell types embedded in the network. Projection neuron (PN) dendrites in the honey bee AL show transient increases in calcium in response to odor stimulation, and these increases are altered by different types of association of an odor with food reinforcement. However, is it unclear what cells in the network are the targets of modulation that produces the changes reported in the PNs. Here we show how pharmacological manipulation can be used to isolate PNs *in vivo* in the honey bee AL such that plasticity can be studied in cells that have developed in an intact network. This manipulation promises to reveal cellular properties of plasticity that likely apply to both the OB and AL because of common mechanisms of plasticity.

INTRODUCTION

Neural encoding of odors in the mammalian olfactory bulb (OB), and in its functional analog, the invertebrate antennal lobe (AL), is subject to plasticity driven by association of odors with release of biogenic amines associated with important events (Fernandez et al., 2009; Wilson et al., 2004). Rapid plasticity in coding is important, because information about odors related to important events will change very quickly and

often within an animal's lifetime. For example, floral perfumes associated with nectar change often within and between days and weeks, potentially several times in the foraging lifetime of a honey bee. These rapid changes in information value require that honey bees quickly learn which odors indicate, or no longer indicate, food. This behavioral plasticity to odors maps to plasticity in the antennal lobes of the brain, which serves to separate and potentially make more distinct spatiotemporal activity patterns for odors that take on important meanings (Fernandez et al., 2009). There are two advantages to using the honey bee as a model for olfactory plasticity. The honey bee brain is significantly smaller, more accessible and simpler in structure relative to a mammalian brain (Galizia & Rossler, 2010). More glomeruli can be imaged at a faster temporal and spatial resolution than in a mammalian OB. Furthermore, honey bees can perform associative conditioning tasks under controlled laboratory conditions under which neural activity elicited by odors and associations with food can be closely monitored *in vivo* (Fernandez et al., 2009; Locatelli et al., 2013).

Studying neuronal activity *in vivo* is an important tool for understanding neuronal processing and coding properties, and then relating those properties to behavior. There have been several studies of plasticity in the honey bee AL (Faber et al., 1999; Fernandez et al., 2009; Locatelli et al., 2013). We employ two-photon excitation microscopy (2PE) using fluorescent calcium indicators to investigate the potential sources of plasticity as revealed by calcium imaging in AL projection neurons (PNs), which are the functional analogs of mitral cells in the OB. PN dendrites show calcium increases in response to odor stimulation (Sachse & Galizia, 2003). Calcium enters AL interneurons via nicotinic

acetylcholine (nACh) receptors, which are driven by acetylcholine release from sensory neuron terminals, as well as through voltage-gated calcium channels. nACh receptors are the most abundant acetylcholine receptors in the honey bee brain (J. Dupuis et al., 2012). nACh receptors have been studied in cultured honey bee kenyon cells (Goldberg, Grunewald, Rosenboom, & Menzel, 1999). However, *in vitro* rearing in cell culture can alter properties of neurons relative to cells reared in an intact network *in vivo* (Maletic-Savatic, Lenn, & Trimmer, 1995). To date no one has developed a means for studying cellular properties in vivo, which would allow investigations of cells that have developed in an intact network.

In order to study intrinsic properties of PNs separate from network properties, we need to isolate PNs in the AL network after they have developed *in vivo*. Cadmium competes with calcium for entry via calcium channels (Hinkle, Kinsella, & Osterhoudt, 1987). Cadmium chloride ($CdCl_2$) has been used in an *in vitro* electrophysiology to presynaptically block vesicle release in order to study nicotine-evoked post-synaptic neuronal activity (Santos-Torres, Slimak, Auer, & Ibanez-Tallon, 2011). We investigate the post-synaptic activity in the honey bee PNs using the same approach in an *in vivo* system, which has further applications for studying other intrinsic properties of specific cell types *in vivo*.

MATERIALS AND METHODS

SPECIMEN PREPARATION AND DYE LOADING

Worker honey bees (*Apis mellifera*) were prepared for imaging following a modified protocol of (Galizia et al., 1997). Honey bees were collected at the entrance to the hive the day preceding the imaging session and immediately placed on ice to briefly anesthetize them. Individuals were then mounted in plexiglass frames with wax and fed 1M sucrose-water solution to satiation. The bees were kept in a humid chamber until 12 hours before the imaging session, at which time a rectangular window in the cuticle was made from compound eye to compound eye and from antennae to medial ocellus. The glands and trachea were carefully pushed aside until a distinct morphological landmark in the brain, the alpha lobe (Galizia & Rossler, 2010) could be seen. A bolus of fura-2-dextran dye on the tip of a quartz glass microelectrode was used to create a lesion along the medial and lateral antennocerebral tracts (Galizia & Rossler, 2010), which carry the axons of PNs from the AL to other brain regions, at a point close to where they enter the mushroom bodies. The removed cuticle from the window was placed back on the head and covered with wax to close the window. The bee was fed again and placed back in the humid chamber for 12 hours to allow the dye to travel along the tracts and backfill the PN dendrites.

PHARMACOLOGICAL AGENTS

All pharmacological agents were dissolved in bee saline solution (130mM NaCl, 7mM $CaCl_2$, 6mM KCl, 2mM $MgCl_2$, 160mM Sucrose, 25mM Glucose, 10mM Hepes, 0.02M Ascorbic Acid). Cadmium Chloride ($CdCl_2$; *Sigma-Aldrich*), a voltage-gated

calcium channel antagonist, was bath applied in the head capsule at a low dose (200μM) or a high dose (500μM). Tetrodotoxin (TTX; *Sigma-Aldrich*) was bath applied (1μM) in the head capsule for 30 minutes prior to imaging to block Na^+ channels and therefore synaptic transmission. (-)-Nicotine hydrogen tartrate salt (*Sigma-Aldrich*) was pressure injected (1mM, 0.04pL) directly next to the honey bee AL neuropil. Treatment with blank saline solution was used for control groups.

BLOCKADE OF ODOR-EVOKED RESPONSE

PN dendrites in the honey bee ALs were imaged *in vivo* under saline to test for responsiveness to a 1:1 mixture of 2-octanone (0.02M):1-hexanol (0.02M). Three odor presentations were given with an inter-trial interval (ITI) of 3 minutes. Following the initial odor trials, TTX or a low or high dose of $CdCl_2$ was bath applied for 30 minutes. The same initially responsive glomeruli were then imaged and additional three odor stimuli were presented with an ITI of 3 minutes.

TEMPORAL EFFECTS OF $CdCl_2$

PN dendrites in the honey bee ALs were imaged under saline to test for responsiveness to a 1:1 mixture of 2-octanone (0.02 M):1-hexanol (0.02 M). Three odor presentations were made at an ITI of 3 minutes. Following the initial odor trials, $CdCl_2$ (500 μM) was bath applied for 10,20,25, and 30 minutes. The same initially responsive glomeruli were imaged at those time-points to determine the temporal effects of the $CdCl_2$.

INTRINSIC CHOLINERGIC PROPERTIES OF PROJECTION NEURONS

PN dendrites in honey bee ALs were imaged under saline during and after pressure injection of nicotine. Three pressure injections were applied with an ITI of 10 minutes. Following the initial nicotine applications, $CdCl_2$ (500μM) was bath applied for 30 minutes. The same initially responsive glomeruli were imaged upon another three pressure injections of nicotine with an ITI of 10 minutes to determine the intrinsic cholinergic properties of the PN dendrites.

DATA COLLECTION AND PROCESSING

A Prairie View Ultima IV was used for imaging and was equipped with 6mm galvanometer scanners. A Ti:Sapphire laser (830 nm, 80 MHz, <100 fs pulse width; Mai Tai HP, Spectra Physics, Mountain View, California) pumped by an integrated 14W Millenia® pump laser (Spectra Physics) was used for 2PE through A 20X water-immersion objective (Olympus XLUMPLFL20XW/IR-SP, 0.95 NA, 1.9 mm working distance). PrairieView software was utilized to control scan and filter settings, laser wavelength and power, PMT gain, and pre-session wavelength spectra optimization. Emission light was collected using multi-alkali photomultiplier tubes (Hamamatsu, Bridgewater, New Jersey). The ImageJ MBF "ImageJ for Microscopy" collection was used for fluorescence over time image analysis.

STATISTICAL ANALYSIS

The calcium signals were calculated as $\Delta F/F_0$, where the initial fluorescence is F_0, and is calculated by averaging the first 0.5s of the time series preceding the odor stimulus

onset. ΔF is the change of fluorescence, which is defined as the maximum response amplitude during the stimulus presentation compared to F_0. The calculated $\Delta F/F_0$ is proportional to intracellular calcium concentrations. Two-tailed t-tests were used to test for significant differences between control (saline) and treatment (cadmium) groups upon nicotine application.

RESULTS

BLOCKADE OF ODOR-EVOKED RESPONSES

Under saline, OERs were significantly above baseline (mean $\Delta F/F_0 = 0.191$, n=13). TTX blocked all OERs and brought calcium signals close to background levels ($\Delta F/F_0 = 0.067$, n=2). The low dose (200μM) of $CdCl_2$ only partially blocked the OERs ($\Delta F/F_0 = 0.131$, n=3). The high dose (500μM) reduced OERs so that the calcium signals were equal to signals under TTX and undetectable from background ($\Delta F/F_0 = 0.048$, n=5). (Figure 20A, B)

TIME COURSE OF CADMIUM CHLORIDE EFFECTS

The high dose (500μM) of $CdCl_2$ started to attenuate the OERs at 10, 20, and 25 minutes post-$CdCl_2$ application. The OER was abolished by 30 minutes post-$CdCl_2$ application. (Figure 20C)

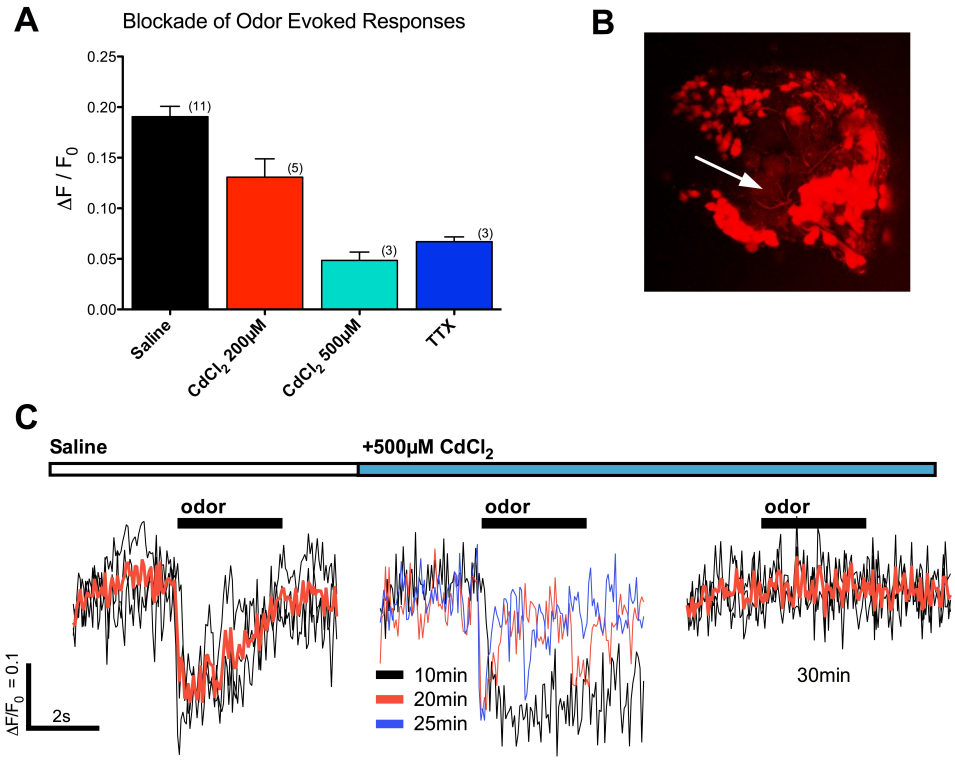

FIGURE 20: Blockade of OERs. 20A**)** The inter-trial intervals of the odor presentations were 3 minutes. In a ringer (saline) bath, the OERs were significant calcium signals ($\Delta F/F_0 = 0.191$, n=13). TTX blocked all OERs and brought calcium signals down to background levels ($\Delta F/F_0 = 0.067$, n=2). The low dose (200μM) of $CdCl_2$ only partially blocked the OERs ($\Delta F/F_0 = 0.131$, n=3) while the high dose (500μM) blocked the OERs so the calcium signals were undetectable from background levels ($\Delta F/F_0 = 0.048$, n=5). Shown is the mean +/- SEM. 20B) Temporal effects of $CdCl_2$.**:** The glomerulus analyzed for fluorescence over time ($\Delta F/F_0$) is indicated by the arrow. 20C) In a ringer bath, the OERs were significant calcium signals ($\Delta F/F_0 = 0.202$). The high dose (500μM) of $CdCl_2$ started to attenuate the OERs at 10, 20, and 25 minutes post-$CdCl_2$ application although the response was not abolished until 30 minutes post-$CdCl_2$ application.

BLOCKED PROJECTION NEURONS STILL RESPOND TO NICOTINE

Under saline, the nicotine-evoked calcium responses were significantly above baseline ($\Delta F/F_0 = 0.177$, n=11). The high dose (500μM) of $CdCl_2$ (at a time when it blocked OERs) had no effect on the nicotine-evoked calcium signals in the AL PN dendrites ($\Delta F/F_0 = 0.169$, n=5). (Figure 21)

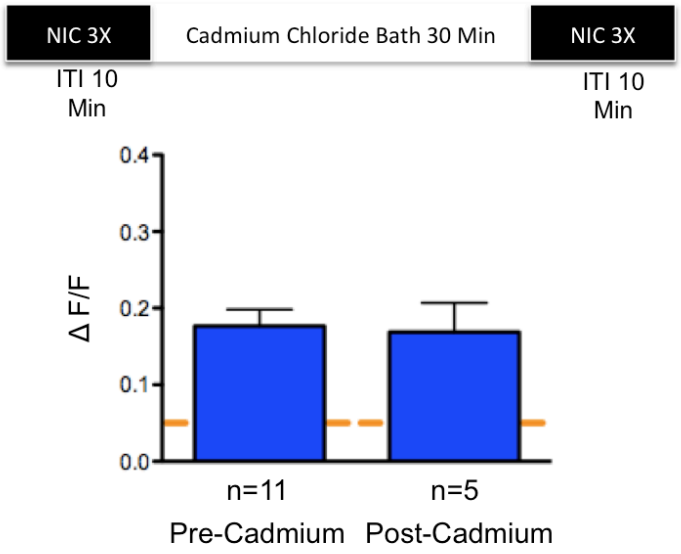

FIGURE 21: Nicotine was pressure injected 3 times with intertribal interval before and after the $CdCl_2$ bath. In a ringer bath, the nicotine-evoked calcium responses were significant ($\Delta F/F_0 = 0.177$, n=11). The high dose (500µM) of $CdCl_2$ did not affect the post-synaptic nicotine-evoked calcium signals in the AL PN dendrites ($\Delta F/F_0 = 0.169$, n=5). p>0..05. Shown is the mean +/- SEM.

DISCUSSION

Calcium can enter PN dendrites through voltage-gated calcium channels as well as via nACh receptors, which are driven by ACh release from sensory neuron terminals (J. Dupuis et al., 2012). Olfactory receptor neurons contain voltage gated calcium channels, which contribute to synaptic transmission (Laurent, Masson, & Jakob, 2002) and OERs. We have found that blockade of voltage gated calcium channels with $CdCl_2$ (500μM) can successfully block OERs after 30 minutes in bath applications in the honey bee head capsule (Figure 20). nACh receptors are the most abundant ACh receptors in the honey bee brain (J. Dupuis et al., 2012) and are the primary cholinergic receptor in the AL, as the muscarinic binding sites are primarily located elsewhere in the brain (Cano Lozano, Armengaud, & Gauthier, 1995). So the nicotine-evoked signals we measure under cadmium treatment are from calcium entry via nACh receptors (Figure 21), although the type of nACh receptor is unknown. Therefore, we can use cadmium treatment to study the post-synaptic properties of PNs.

The main implication of our technique is that cadmium can now be used to study responses of intrinsic cells in neural networks of the honey bee AL after they have developed *in vivo*. This is useful for understanding how specific cell types are modulated by biogenic amines (Fernandez et al., 2009; Wilson et al., 2004). Without this blockade, it would be impossible to establish whether the changes in PN responses after nonassociative (Locatelli et al., 2013) or associative (Fernandez et al., 2009) conditioning are due to modulation of the PNs themselves or to modulation of other cells in the network, e.g., inhibitory local interneurons (Sinakevitch et al., 2013). Cadmium therefore

provides a unique way to study the intrinsic properties of a specific cell type in relation to associative and nonassociative learning in an *in vivo* system. This treatment would be very powerful when coupled to parallel studies of cellular properties in disassociated cell cultures (Grunewald, 2003).

CHAPTER 4

INTERNEURON STUDIES

INTERNEURON BACKGROUND IN THE HONEY BEE

Interneurons are important in the insect AL for sensory gating and modulating sensory responses to odors (Abel, Rybak, & Menzel, 2001; Hildebrand & Shepherd, 1997). The vast majority of interneurons in the AL are LINs, and they make connections among glomeruli within the AL. A recent study has shown that AmOA1 receptors are expressed on GABA-ergic LINs in the honey bee AL (Sinakevitch et al., 2013). This makes studying the pharmacological manipulations of odor responses with octopamine very important in regards to calcium imaging of LINs. A few studies have tried to collect data from LINs with some success (Galizia & Kimmerle, 2004; Sun, Fonta, & Masson, 1993). However, it is extremely difficult and time consuming to make intracellular electrophysiological recordings from LINs, and most recordings are so brief that they do not allow for pharmacological assays. A more efficient approach, which allows long-term recordings, to studying response characteristics of LINs *in vivo* needs to be developed to better understand the roles of LINs in the AL network. Toward that goal, I undertook several studies to characterize LINs and develop a means to record from them in a way that would allow for the same pharmacological manipulations I used on PNs in Chapter 2.

ANTENNAL LOBE GLOMERULI NEURANATOMY

Arnold et al. (1985) studied the neurantomy of the honey bee AL and describes it in detail according to the following nomenclature. There are four populations of glomeruli, and they arise from different tracts of axons fibers, or combinations of tracts, that emerge from the antennal nerve. The first population includes 71 glomeruli in the dorsal region and arises from the T1 antennal nerve tract. The second population is known as the intermediary region and arises from the T2 tract; it includes 6 glomeruli, which are laterally located. The third population is known as the ventral region and arises from the T3 tract. It includes 81-90 glomeruli and is innervated by three tract bundles T3-1, T3-2, and T3-3. The last population includes 7 glomeruli in the posterior region and arises from the T4 tract. Overall, there are on average 166 glomeruli in ALs of worker honey bees.

LOCAL INTERNEURONS

Aside from the projection neurons, there are two different populations of AL GABA-ergic interneurons. The first population contains approximately 12 output interneurons (Fonta, Sun, & Masson, 1993). These output interneurons project to the ipsilateral protocerebrum as well as the contralateral AL. The second group of GABA-ergic interneurons are LINs, which have processes localized to the AL. Estimates of the number of LINs in the AL have ranged from 109-750 (Fonta et al., 1993; Schafer & Bicker, 1986). All LINs are periglomular and each population has unique morphological features. LINs comprise at least two, and possibly more, subpopulations. Homogeneous

LINs are characterized by regular and uniform arborizations in many glomeruli; they account for 13% of all LINs (Fonta et al., 1993). The average size of the cell bodies is 15.5 μm in diameter ranging from 10.9μm to 25μm in diameter. Heterogeneous LINs are characterized by dense arborizations in a single glomerulus and then sparse projections to many other glomeruli (Fonta et al., 1993). The Hetero LIN cell bodies are smaller than homogenerous LIN, as the average size is 11.5μm, ranging from 6.3μm to 19.7μm in diameter. The hetergoeneous LINs innervate the ventral, dorsal, and intermediate populations of glomeruli.

GABA-ERGIC IMMUNOHISTOCHEMISTRY

INTRODUCTION

This study focused on organization of the inhibitory network in the glomerular neuropil of the honey bee AL, the functional analog to the mammalian OB. Within a glomerulus, there are groups of cells comprised of LINS and PNs. We employ whole-mount immunohistochemistry using anti-GABA antibodies to stain the GABA-ergic processes in glomeruli. Other GABA-ergic immunohistochemistry studies of the honey bee AL have been completed in brain sections (Bicker, 1999; Kreissl, Strasser, & Galizia, 2010; Schafer & Bicker, 1986; Sinakevitch et al., 2011). There have been differing counts of the number of GABA-ergic cells in the AL. Bicker (1999) describes the location of the GABA-ergic cell bodies throughout the brain but does not give a cell count of the GABA-ergic cells within the AL. Kreissl et. al (2010) described colocalization of allostatin with GABA in interneurons within the AL but not all GABA-ergic cells colocalized with allostatin, and this study also did not provide cell counts. Schafer & Bicker (1986) describe GABA-immunoreactivity throughout the entire brain and provide a cell count of 750 GABA-ergic somata within the AL. Sinakevitch et al. (2011) completed GABA immunohistochemistry on honey bee brain slices and found that the GABA-ergic cell bodies colocalized with AmOA1 receptors. Whole-mount immunostaining has not been used yet with anti-GABA staining in the AL to perform cell counts. Therefore, our aim was to estimate the number and location of these cells in the AL using a more accurate whole-mount technique.

MATERIALS AND METHODS

Immunohistochemistry was performed on forager honey bees, which were caught at the base of the hive on the first day of tissue processing. The honey bees were anesthetized by briefly cooling to 4˚C. Brains were removed from the head capsule and fixed in 1% glutaraldehyde, 2.5% Paraformaldehyde/0.1M sodium cacodylate trihydrate buffer [pH 7.0; cat#12300, Electron Microscopy Sciences: Hatfield, PA, USA.] with 1% sodium metabisulfite [SMB; Sigma: St. Louis, MO]. The tissue was then washed in Tris HCl SMB (1 x 10 minutes). The tissue was then processed with 0.5% sodium borohydride [$NaBH_4$; Sigma] in 0.05 M Tris-HCl [pH 7.4; Sigma] buffer with 0.45% SMB for 15 minutes. The tissue was subsequently washed in Tris HCl SMB (4 x 10 minutes) then PBS [0.01 M phosphate buffered saline, pH 7.4; cat # P4417, Sigma-Aldrich] (3 X 1 minute). The tissue was treated with 0.03% collagenase in PBS for 15 minutes. The samples were washed in Tris HCl SMB (2 X 10 minutes) and Tris HCl SMB Triton X-100 0.5% [cat# VW3929-2, VWR International: West Chester, PA, USA] (6 X 1 hour then left overnight). The next the day, the tissue was blocked with normal goat serum 0.1% in Tris HCl SMB TX 0.5% for 12 hours at 4*C. The tissue was then incubated for 5 days at room temperature on a shaker platform with a rabbit anti-GABA primary antibody, which uses GABA-BSA as the immunogen [1:100, GEMAC, Talence, France]. Brains were then washed in Tris HCl Tx 0.5% (5 x 2 hours) and subsequently the secondary antibody, goat anti-rabbit IgG Alexa Fluor® 555 [cat# A-21430, Invitrogen: Eugene, Oregon, USA] in Tris HCl SMB TX 0.5%, was applied and the tissue was incubated at room temperature for 5 days on a shaker platform. The tissue was then washed with Tris HCl SMB TX 0.5% (5 X 2 hours and left overnight). The next day, the

tissue was washed in Tris HCl (6 X 30 minutes). The brains were then fixed in 4% paraformaldehyde for 1 hour and washed in PBS (3 X 10 minutes) prior to whole mounting. To clear the samples post-fixation, the tissue specimens were treated in 50% glycerol/ 50% PBS for 1 hour followed by a 80% glycerol/ 20% PBS treatment for 1 hour. The brains were then whole mounted using a polyvinyl alcohol embedding media, Elvanol, protocol modified from (Rodriguez & Deinhardt, 1960). Antibody specificity and staining controls in the bee brain were described in detail previously (Sinakevitch et al., 2011).

IMAGING OF SPECIMENS

Digital images were captured with a Leica TCS SP2 confocal laser scanning microscope (Leica, Bensheim, Germany) using a Leica HCX PLAPO CS 40X oil-immersion objective (NA:1.25) Stacks of 1µm optical sections were processed using Leica software. Sizes of final images and maximum intensity projections were compiled using the ImageJ MBF "ImageJ for Microscopy" collection.

RESULTS

Once I was able to successfully develop a protocol to complete whole-mount GABA-ergic immunohistochemistry in the honey bee brain, the project was completed by Irina Sinakevitch, Ph.D. in my lab. There were 402 ± 30 cells distributed as follows: medial group (n = 30 ± 5, Figure 22) and medial-ventral group (n = 18 ± 5, Figure 24)

and lateral group (n =350 ± 25, Figure 23) (Protas, Sinakevitch, & Locatelli, 2010; Sinakevitch et al., 2013).

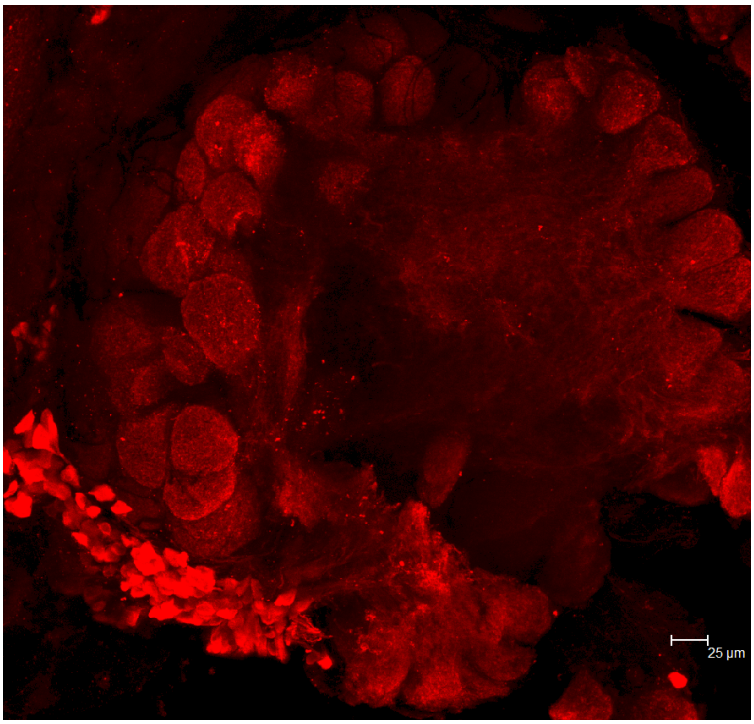

FIGURE 22: The medial group of LINs as stained with anti-GABA in the honey bee AL whole-mount preparation. Shown is a 20X magnification. There were found to be 30 +/- 5 neurons in this group.

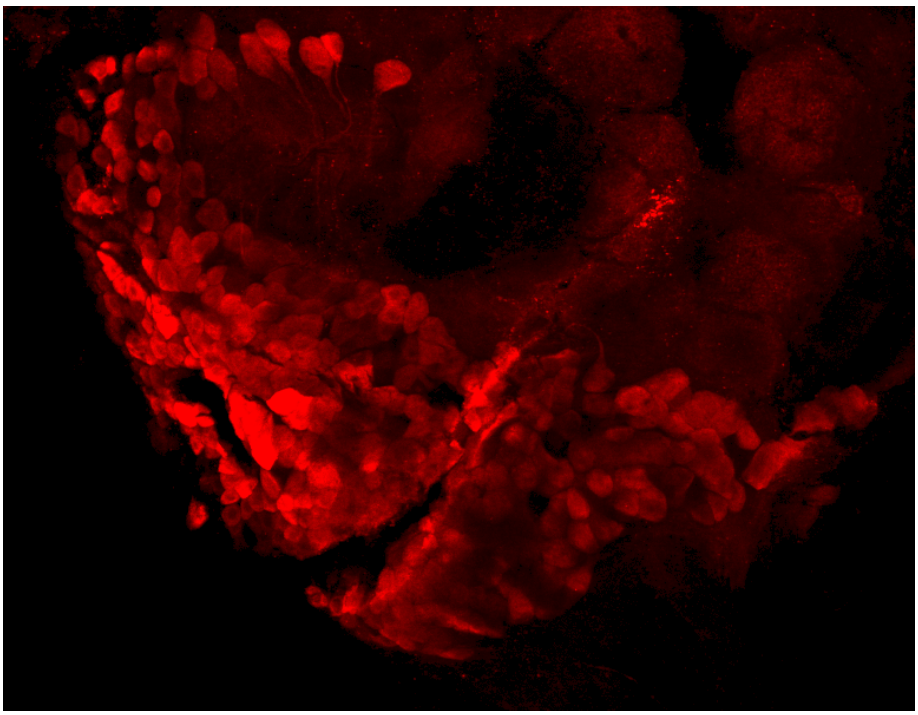

FIGURE 23: The lateral group of LINs as stained with GABA in the honey bee AL whole-mount preparation. Shown is a 20X magnification. There were found to be 350 +/- 25 neurons in this group.

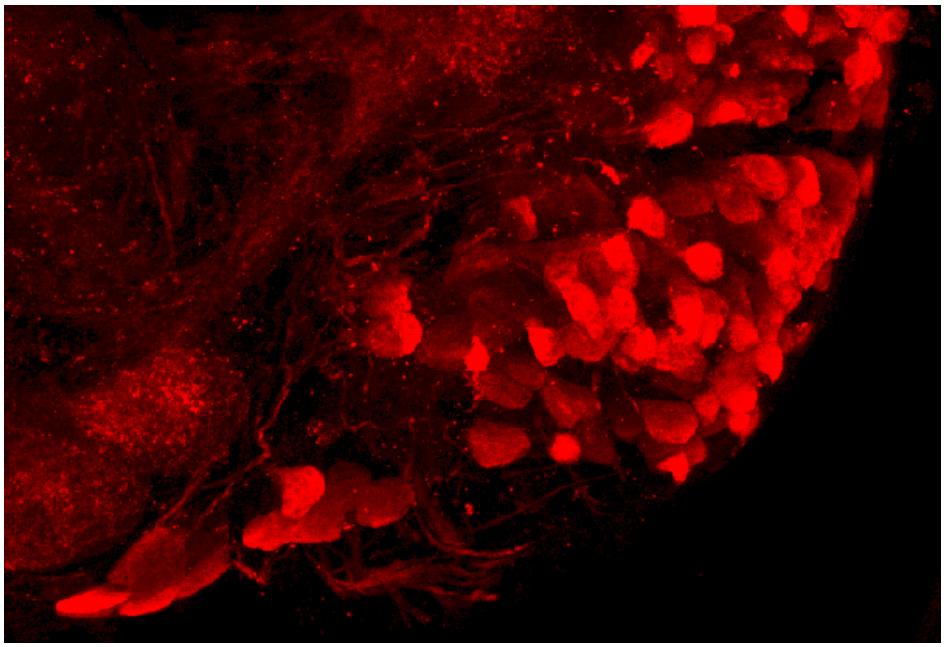

FIGURE 24: The medial-ventral group of LINs as stained with GABA in the honey bee AL whole-mount preparation. Shown is a 40X magnification. There were found to be 18 +/- 5 neurons in this group.

DISCUSSION

There were significantly fewer GABA-ergic cell bodies in the honey bee AL than the previously published 750 by the Schaffer & Bicker study (1986) but more than a previously published Fonta et al. (1993) study with an estimated 109 LINs. There are still three main clusters of GABA-ergic cell bodies in the AL including the medial, the medial-ventral, and the lateral groups. The whole-mount staining protocol was successfully developed such that it can now be used with other staining protocols in order to maintain the integrity of cellular localization of receptors, the cellular positions and number in the honey bee AL. This would make for easier development of three dimensional reconstructions of the honey bee AL. Knowing the relative number and location of these cell bodies is important for my next experiments in which I will employ blind sharp electrode electrophysiological recordings of these interneurons *in vivo* within the honey bee AL

INTRACELLULAR RECORDINGS

INTRODUCTION

Due to the development of fluorescent calcium indicators coupled to dextrans, we have the ability to backfill projection neuron dendrites in the AL by creating a lesion with a bolus of dye along the medial and lateral antennocerebral tracts in the honey bee brain (Sachse & Galizia, 2002). Use of these dyes with this method allows us to selectively target PNs in experiments since they are retrogradely labeled with the calcium indicator. Presently, because of this technique much more is know about PNs than LINs of the honey bee. Other invertebrates have LINs which do not spike, such as the locust (MacLeod & Laurent, 1996). A few studies have shown that LINs spike in honey bees. This spiking occurs in either an excitatory manor or an inhibitory manner (Flanagan & Mercer, 1989; Galizia & Kimmerle, 2004; Sun et al., 1993). LINs have not to date been specifically targeted *in vivo* in the honey bee for electrophysiological or calcium imaging studies. They have only been incidentally recorded from and filled electrophysiologically when the electrode by chance, and usually only briefly, penetrated a LIN. Because of their importance for functioning of the AL neural network, development of a technique to record from LINs would greatly facilitate an understanding of the AL and how the network processes odors.

In order to study LINs, sharp electrode recordings in the AL have been completed by other groups who use a 'blind' (i.e. chance) method of recordings. Galizia and Kimmerle (2004) completed this type of sharp electrode electrophysiology on honey bee AL neurons and ionophoretically injected a calcium indicator when they recorded spikes

within the AL. They then performed limited odor experiments while imaging the calcium dynamics and reconstructed the stained neurons using a confocal to identify the neuron as either a PN or LIN. Our objective was to replicate and further develop this procedure by including octopamine pharmacological manipulations coupled with the odor experiments as I have done for PNs (chapter 2).

MATERIALS AND METHODS

SPECIMEN PREPARATION AND DYE LOADING

Bees were prepared for imaging following a modified protocol of (Galizia & Kimmerle, 2004). Forager honey bees (Apis *mellifera*) were collected at the entrance to the hive the day of the imaging session and immediately placed on ice to briefly anesthetize them. The bees were then mounted in plexiglass frames with wax and fed 1M sucrose to satiation. The bees were kept in a humid chamber until preparation for electrophysiological recording. At this point, a rectangular window in the cuticle was made from compound eye to compound eye and from antennae to medial ocellus. The glands and trachea were removed and the glial sheath surrounding each AL was carefully removed. Saline was applied to the head capsule to prevent the brain tissue from drying out.

ELECTROPHYSIOLOGICAL RECORDING

Borosilicate glass electrodes with a filament (BF100-78-10, Sutter Instruments) were pulled with a horizontal puller (P-2000, Sutter Instruments). The electrodes were

backfilled with the calcium indicator, calcium green-1 dextran (3000MW, 4% in 5mM KOH in HEPES; Invitrogen). The back of the electrode was filled with 1M K-acetate. We slowly drove the sharp electrodes into the AL neuropil using a micromanipulator (MP-225, Sutter Instruments) at a 45° angle until there was a drop in the baseline, indicative of cellular penetration. At this point, there was either detectable background spiking or we would apply a small depolarizing current to induce spiking to ensure that our electrode was indeed in the cell. This procedure would ensure that we could record cellular activity before pharmacological experiments would take place. Current clamp experiments were performed in bridge mode using an Axoclamp 2B amplifier (Axon Instruments). Recordings were analyzed using pClamp (Axon Instruments).

DATA COLLECTION AND PROCESSING

An Olympus Fluoview FV-300 laser scanning confocal microscope (Olympus America, Inc., Center Valley, Pennsylvania, USA). Excitation of calcium green-1 dextran was performed using the 488nm laser-line of an argon laser. Time-series images were acquired using a 20x (0.5NA) Olympus UMPlanFL water-immersion lens in bee saline. The ImageJ MBF "ImageJ for Microscopy" collection was used for fluorescence over time image analysis.

RESULTS

We recorded from several preparations that contained a neuron, which had low background spiking activity and upon an odor application, would start spiking (Figure 26, Table 1). We also recorded from several neurons that had high background spiking

activity and, upon odor application, would stop spiking (Figure 27, Table 2). In these preparations, the odor to which the neuron would respond to varied from neuron to neuron. There were only two preparations in which OA could be applied and the neuron could be recorded from post-OA application (Figures 26 and 27). The problem lies in the fact that we were trying to mimic our other OA-odor pairing experiments and a small amount of OA (0.4pL) needed to be pressure injected (10psi, Picospritzer III, Parker Instruments) directly over the AL neuropil. This created a problem because upon pressure injection application, we would loose the cell we were recording from. This was most likely due to the pressure application creating too many vibrations for the sharp electrode to stay in the cell. We were able to record information from two cells using this method. Cell 1 responded with increased spike rate to geraniol while cell 2 responded by inhibitory spiking during the odor stimulus of heptanone. The most disheartening part of the project was that it took over 6 months to get this data and we were unsuccessful in loading the microruby dextran (Invitrogen) to label the cell in order to discriminate whether the cell was a PN or LIN. In fact, in all of the preparations we recorded data from, we were unsuccessful in loading the cellular tracer into the neurons. However, we knew we could load the microruby into the cells because we had preparations that we did not collect olfactory data from and just tried to stain once we knew we could record from the cell; we successfully labeled the cells in these instances (Figure 25).

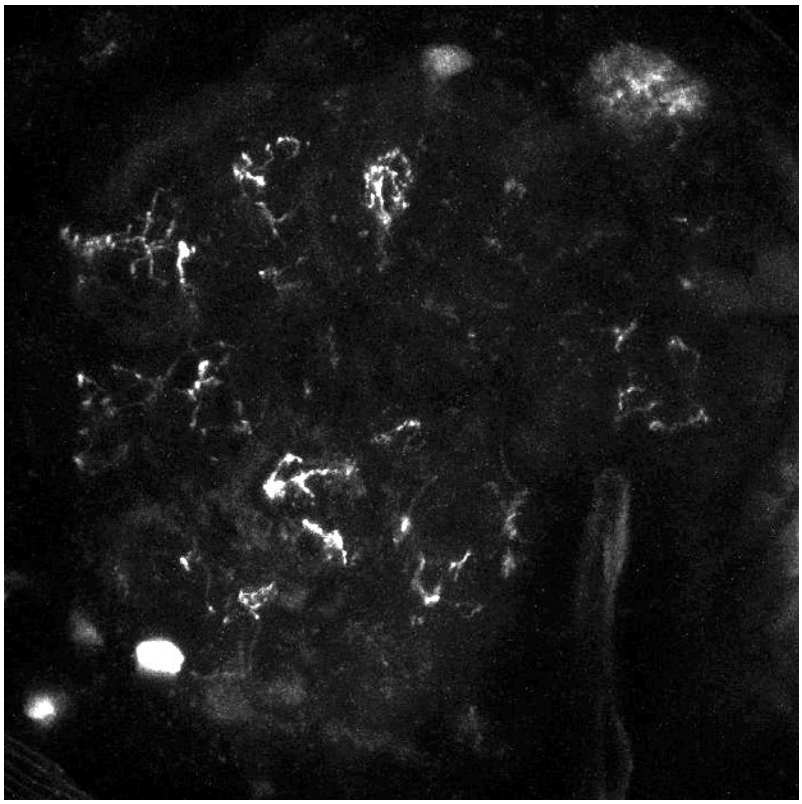

FIGURE 25: Stained LIN in the honey bee AL. The labeled cell is on the left with processes extending out to other glomeruli. No electrophysiological data was collected form this cell prior to staining with microruby dextran.

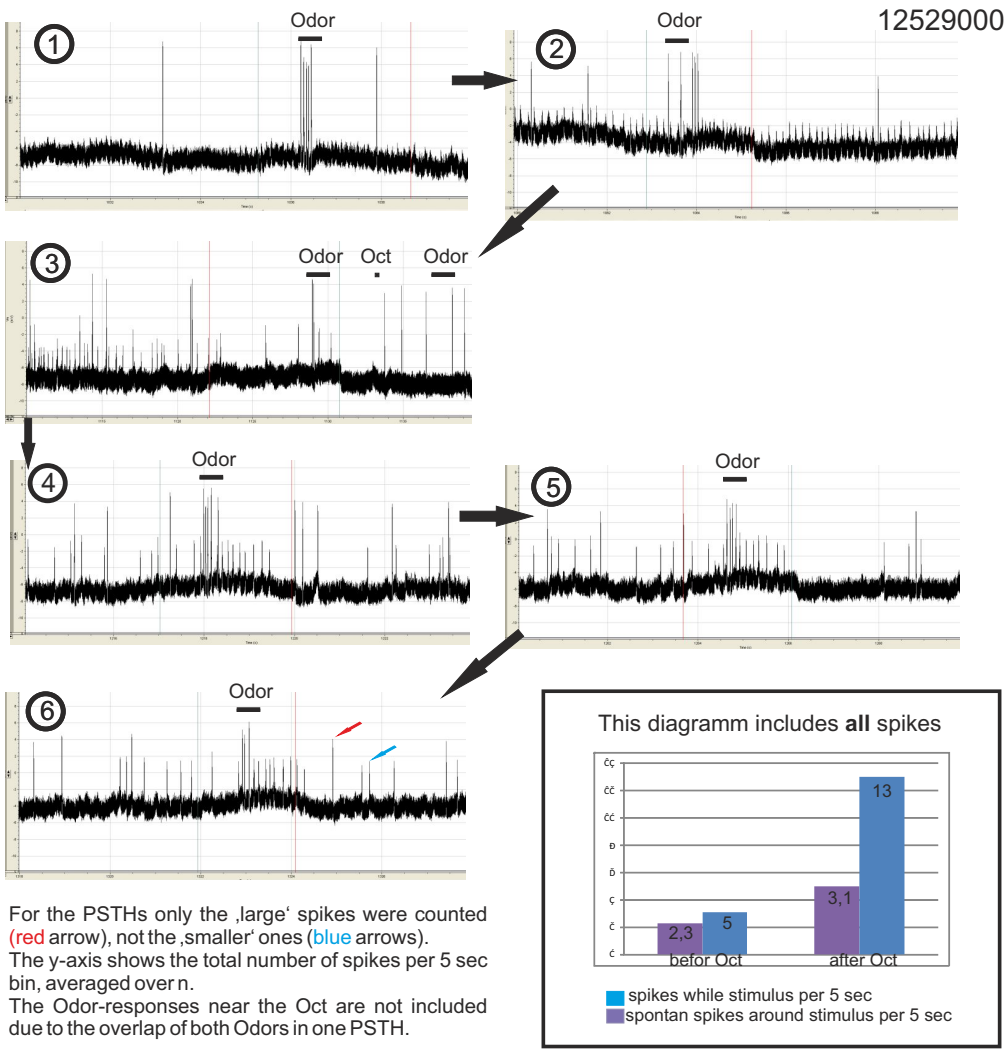

FIGURE 26: Cell 1 increased its firing rate in response to the odor stimulus, geraniol. The bars above. Pre-odor conditioning odor responses are shown in parts 1 and 2. The conditioning protocol is shown in part 3. Parts 4-6 show the post-odor conditioning odor responses. Part 6 shows the two different spike amplitudes seen following the conditioning protocol. Only the larger (red arrow) spike amplitudes were used in the PSTH analysis.

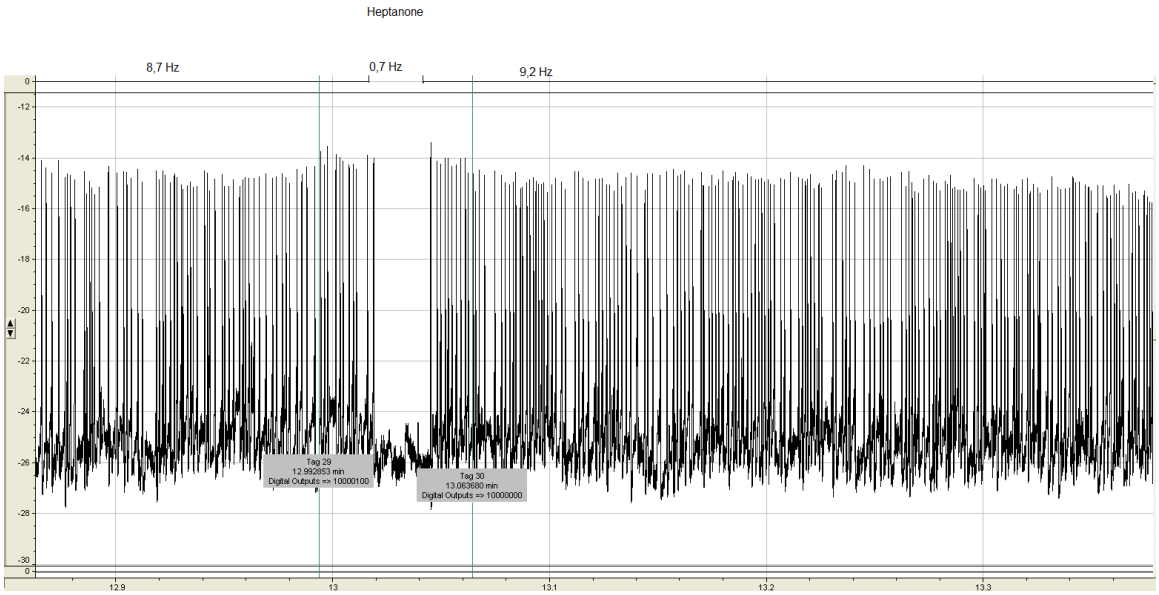

FIGURE 27: The intracellular recording of a single neuron (cell 2) which stops firing in response to the odor, heptanone. The Odor stimulus is indicated by a bar above the recording.

Pre-Conditioning		***Post-Conditioning***	
Background Spiking	*Spiking During Stimulus*	*Background Spiking*	*Spiking During Stimulus*
0	*14.8*	*10.7*	*13.5*
0	*11.8*	*3.5*	*15.5*
8.2	*15.6*	*0*	*14.7*
		0	*13*
Average Spike Rate			
2.733	***14.067***	***3.55***	***14.175***

TABLE 1: The chart shows the background spike rate and stimulus spike rate of a single neuron (cell 1) pre-OA odor pairing and post-odor-OA pairing. The baseline was variable from -30mV to -45mV. The mean spike amplitude was 35mV. The odors tested during the recording of this cell were geraniol, hexanol, heptanol, and octanone. The cell responded to geraniol. OA was coupled to geraniol for the pharmacological conditioning paradigm in this case.

Pre-Conditioning		***Post-Conditioning***	
Background Spiking	*Spiking During Stimulus*	*Background Spiking*	*Spiking During Stimulus*
15	*7*	*10*	*2.7*
18	*15*	*10*	*1.4*
19	*8*	*10*	*0.7*
12	*12*	*9*	*5.6*
16	*1*	*11*	*2.7*
		11	*1.6*
		10	*8*
		11	*4*
		11	*3.6*
Average Spike Rate			
16	***8.6***	***10.33***	***3.367***

TABLE 2: The neuron stopped/ slowed firing rate when the stimulus was given that it would respond to (Heptanone). The odors tests on this cell were geraniol, hexanol, heptanone, octanone, and mineral oil (control). The odor that the neuron responded to was heptanone and this odor was used in the conditioning trial. The baseline was between -23mV and -28mV with a mean of -26mV. The amplitude ranged from 8-12mV.

DISCUSSION

After 6 months of little results, we found this method to be extremely complicated and time consuming and we did not collect a substantial amount of data that would inform us about the LINs. We contemplated ionophoretically delivering the OA directly into the AL neuropil but this would be such a drastic change of protocol that it wouldn't be able to be compared to the years of data collected using the pressure injection method. Bath application of OA may differ from our low-volume of picospritzed application in that it could change properties of the entire system. In another study, it made all of the PNs more excitable (Rein, Mustard, Strauch, Smith, & Galizia, 2013).

ELECTROPORATION

INTRODUCTION

It is important to study how different forms of plasticity are implemented in the OB and AL networks through modulation of both PNs and LINs. As PNs are more accessible and easily isolated for imaging experiments, LINs have remained of interest. Projection neuron (PN) dendrites in the honey bee AL show transient increases in calcium in response to odor stimulation, and these increases are altered by different types of association of an odor with food reinforcement. We have recently shown that these changes can be pharmacologically induced by a pressure injection of OA. However, is it unclear what the LINs' role is in this network. Recently, a group has developed an electroporation method for imaging neurons in the silkmoth brain (Fujiwara, Kazawa, Haupt, & Kanzaki, 2009). We would like to adapt this method to easily and rapidly identify and image from LINs in the honey bee brain under the same conditioning paradigms which allowed for pharmacological conditioning of the PN dendritic processes of the AL.

MATERIALS AND METHODS

SPECIMEN PREPARATION AND DYE LOADING

Bees were prepared for imaging following a modified protocol of (Galizia & Kimmerle, 2004). Forager honey bees (*Apis mellifera*) were collected at the entrance to the hive the day of the imaging session and immediately placed on ice to briefly

anesthetize them. The bees were then mounted in plexiglass frames with wax and fed 1M sucrose to satiation. The bees were kept in a humid chamber until 12 hours before the imaging session, at which time a rectangular window in the cuticle was made from compound eye to compound eye and from antennae to medial ocellus. The glands and trachea were carefully pushed aside until a distinct morphological landmark in the brain, the alpha lobe (Galizia & Rossler, 2010) could be seen. A bolus of dye on the tip of a quartz glass microelectrode was used to create a lesion along the medial and lateral antennocerebral tracts (Galizia & Rossler, 2010), which carry the axons of PNs from the AL to other brain regions, at a point close to where they enter the mushroom bodies. The removed cuticle from the window was placed back on the head and covered with wax to close the window. The bee was fed again and placed back in the humid chamber for 8 hours to allow the dye to travel along the tracts and backfill the projection neuron dendrites until the electroporation dye loading took place. Right before the electroporation, the glands and trachea were removed and the glial sheath surrounding each AL was carefully removed. Saline was applied to the head capsule to prevent the brain tissue from drying out.

Electroporation was completed using extracellular borosilicate electrodes pulled on the P-2000 (Sutter Instruments). The dye was iontophoretically injected using a cathodal current with a 20μA amplitude. The pulse duration was 25ms long and we delivered 150 pulses at 500ms intervals according to an adapted, previously published method using the silkmoth (Fujiwara et al., 2009). Following the electroporation, the

bees were fed 1M sucrose again and placed in the humid chamber for additional 4 hours to allow the dye to completely label the cells until 2PE imaging of the specimen would take place.

DYE LOADING TECHNIQUES

In order to differentiate the LINs from PNs, we could not just electroporate the calcium indicator. Although, if we only labeled a few cells, this would give us a good idea if they were LINs or PNs, it could not be said definitively what cell type they are. First, the PNs of the honey bee AL were labeled by making a lesion with a bolus of Texas-Red dextran (10,000MW, Invitrogen), a non-calcium indicator dye, on the tip of a quartz microelectrode along the medial and lateral antennocerebral tracts. 12 hours was allowed for the dye to travel and backfill the PN dendrites before imaging. In the same preparation, the head capsule was re-opened 4 hours prior to the imaging time in order to electroporate the calcium indicator, Calcium green-1 dextran (Invitrogen, 3000MW, anionic). The head capsule was waxed closed again with Eicosane®. After 12 hours post-Texas Red dextran (Invitrogen, 10000MW) injection and 4 hours post-calcium green-1 dextran electroporation, the head capsule was removed again and imaging could occur.

DATA COLLECTION AND PROCESSING

A Prairie View Ultima IV was used for imaging and was equipped with 6mm galvanometer scanners. A Ti:Sapphire laser (830 nm, 80 MHz, <100 fs pulse width; Mai Tai HP, Spectra Physics, Mountain View, California) pumped by an integrated 14W Millenia® pump laser (Spectra Physics) was used for 2PE through A 20X water-

immersion objective (Olympus XLUMPLFL20XW/IR-SP, 0.95 NA, 1.9 mm working distance). PrairieView software was utilized to control scan and filter settings, laser wavelength and power, PMT gain, and pre-session wavelength spectra optimization. Emission light was collected using multi-alkali photomultiplier tubes (Hamamatsu, Bridgewater, New Jersey). The ImageJ MBF "ImageJ for Microscopy" collection was used for fluorescence over time image analysis.

RESULTS

We found that we could successfully electroporation a fluorescent dextran of the same MW as calcium-green-dextran-1 (3000MW, Invitrogen). Many glomeruli were stained in this process (Figure 28). In control experiments, little staining was visible following insertion of electroporation electrode with no electrical stimulus delivered (Figure 29).

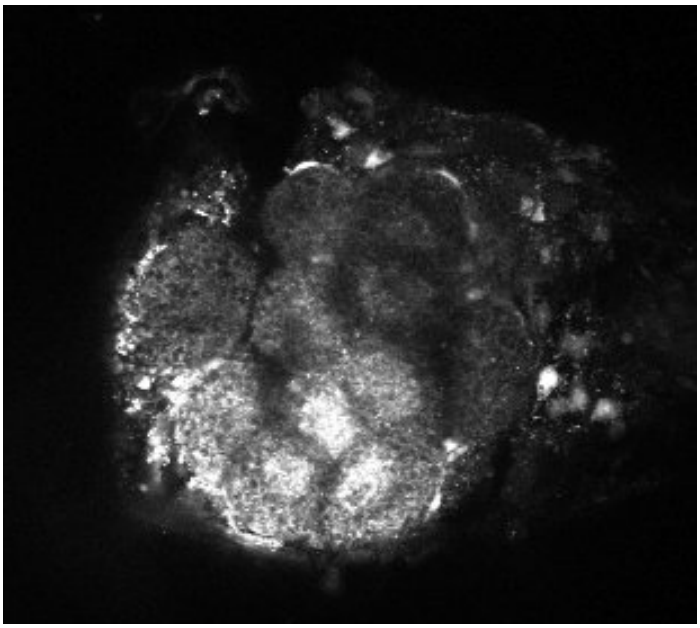

FIGURE 28: Right antennal lobe electroporated with microruby dextran. Many glomeruli were stained with little if any visible damage to the AL neuropil from the electroporation.

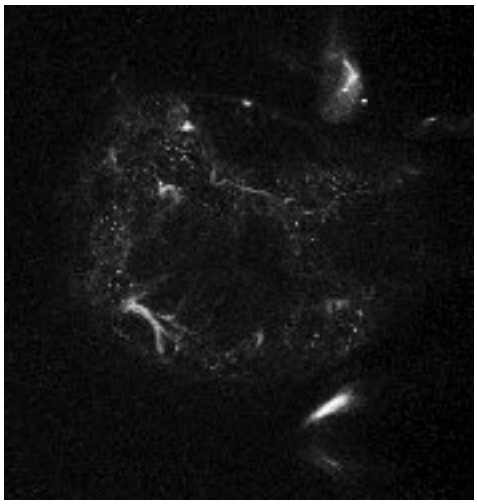

FIGURE 29: Left AL control injection. There is limited staining. The control consisted of a microruby filled electrode which was inserted gently into the AL neuropil and left for the same amount of time an injection would take if electroporation would take place. The electrode was then removed and compared to the injected side (the contralateral AL).

DISCUSSION

We found that we could successfully electroporation a fluorescent dextran (microruby, Invitrogen, 3000MW, anionic) of the same MW as calcium green-1 dextran (3000MW, Invitrogen, anionic). As we were able to stain many glomeruli in this process (Figure 28), the control experiments, where little staining was visible following insertion of electroporation electrode with no electrical stimulus delivered were successful (Figure 29) but could be improved upon. A negative holding current before electroporation would lessen the background staining and improve the problem with staining seen in the control experiments. The next experiments will consist of labeling the PN dendrites with a non-calcium indicator dextran of a different excitation/emission spectra than the electroporated calcium dye into the AL in order to differentiate the cell types. Once completed, we will repeat all odor-pairing experiments to determine the role of LINs in the plasticity we have observed in the neural network of the honey bee neuropil.

CHAPTER 5

DISCUSSION

Olfaction is relevant for many mammalian and invertebrate behaviors, including mate selection, food localization, and regulating emotional states (Wilson et al., 2004). Odors elicit powerful behavioral responses in both vertebrates and invertebrates, which have independently evolved functionally similar olfactory structures (Kaupp, 2010). Floral scents help bees to locate and forage on the nectar and pollen a flower offers in exchange for pollination (B.H. Smith et al., 2006). As in mammals, and in insects like the honey bee, information about food (e.g. what flowers are in bloom) will change very rapidly within an animal's lifetime. Since these food sources change from hour to hour, day to day, and week to week, a great deal of plasticity is exhibited in honey bee behavior, which maps to plasticity in the brain. OA is a key neuromodulator in areas important for learning and memory in the honey bee brain. Coincidence of calcium influx from ACh channels via activation from cholinergic ORNs, with activation of an OA receptor, may contribute to long-term modifications of the AL network.

We have shown that upon a pharmacological application of the biogenic amine, OA, when coupled with an odor, can induce increased excitability of the PN dendrites in all glomeruli, including in glomeruli that are initially not responsive to odor. In fact, responses of the initially silent glomeruli became approximately as strong as the initially responsive glomeruli. Each of the three odor response trials was very similar in its spatiotemporal response profile and robustness of response for the time period during which it was measured (pre– vs. post-OA-odor conditioning). Furthermore, the increased excitability failed to generalize to an odor that the behavioral response would also not

generalize to. Altogether, these results imply that the plasticity I have described could be an important basis for the behavioral plasticity to odors that has been well described in honey bees.

OA itself was not responsible for the increased excitability in the PN dendrites. In order to test for this, we pressure injected a low and high dose of OA and neither evoked a significant calcium response (Figure 9). This could mean that the mechanism driving plasticity in the PNs requires confluence of calcium input via cholinergic receptors (odor input) with an increase in cAMP stimulated by activation of AmOAβ receptors.

We next tested the hypothesis that the increased excitability observed in PN dendrites was at least in part cAMP dependent, which would be expected if the AmOAβ class of OA receptors were involved. Upon application of a cAMP antagonist, RP-Br-8-cAMPS, we found the effect of increased excitability we observed under a saline bath when the pharmacological application of OA was coupled with an odor response was completely abolished. Both the responsive glomeruli did not become more excitable and the non-responsive glomeruli stayed below baseline levels of detection for a calcium signal.

The primary sensory to PN dendrite transmission is due to nACh receptors. We first tested a blockade of nACh receptors with different affinities for different receptor subtypes. The first nACh receptor antagonist was α-BTX which blocks α7 nACh receptors. The second nACh receptor antagonist was MEC which blocks α2>α7 nACh receptors. Both antagonists successfully blocked all OERs. This could mean that both the α7 and α2 nACh receptors are responsible for the odor-evoked responses or it could

mean that the dose of MEC blocked the α7 nACh receptors and that the α7 nACh receptors are responsible for the OERs.

The next experiment was completed to test the effects of OA-odor pairing on cholinergic based synaptic transmission. As before, in this experiment the post-conditioning OERs were strengthened as compared to the pre-conditioning OERs. This was true for both the total amplitude as well as the total area of the OERs. The nicotine-evoked responses however were not strengthened. The responses were extremely variable from glomerulus to glomerulus and bee to bee. The raw data amplitudes of both odor-evoked responses and nicotine-evoked responses show the variability in the scatterplot. This variability effected the resulting data of the total amplitudes of nicotine-evoked calcium responses as well as the total area of nicotine-evoked calcium signals to be not significant.

Although neither the maximum amplitude, nor total area of the nicotine-evoked responses significantly differ from pre-conditioning to post-conditioning trials, the spatiotemporal responses showed slight variations. This was more difficult to quantify, but could be subject to more detailed analysis in the future to evaluate whether changes to network following OA-conditioning induce subtle changes in the nACh receptors and their subunit compositions. Experiments performed on mammals have shown that there are variable cholinergic response profiles to both agonists and antagonists dependent on the nACh receptor subunit composition and affinity (Briggs et al., 2006; Collins et al., 2009; Gotti et al., 2008; Marks et al., 1999; McClure-Begley et al., 2009; Moroni et al., 2006) as well as experiments on honey bees (J. P. Dupuis et al., 2011). Different subunit compositions of nACh receptors leads to different Ca^{2+} permeability of the channel

(Fucile, 2004). Also, plasticity has been shown to be regulated by nACh receptors (McKay et al., 2007)

We now need to complete these same experiments on isolated PNs *in vivo* to see what their intrinsic properties are compared to their network properties. We also need to complete these experiments on isolated LINs if we can develop a method to do so which can prove to be very challenging. Once complete, we can evaluate how summation of calcium from different sources modifies PN responses to odor by targeting downstream calcium-sensitive processes.

The experiments done under cadmium will us to examine these isolated components of the network. Calcium can enter PN dendrites through voltage-gated calcium channels as well as via nACh receptors, which are driven by ACh release from sensory neuron terminals (J. Dupuis et al., 2012). Olfactory receptor neurons contain voltage gated calcium channels, which contribute to synaptic transmission (Laurent et al., 2002) and OERs. We have found that $CdCl_2$ (500μM) can successfully block OERs after 30 mins in bath applications into in the honey bee head capsule. PNs are still responsive to nicotine after this block. Therefore, we can use cadmium treatment to study the post-synaptic properties of PNs. For example, we confirm here that the PNs are responsive to nicotine, which is an agonist of nACh receptors. It is been well established that the PNs in the honey bee AL receive cholinergic input from the olfactory receptor neurons and that the nACh receptors are the main post-synaptic receptor (J. Dupuis et al., 2012).

The main implication of our technique is that cadmium can now be used to study responses of cells in neural networks of the honey bee AL after they have developed *in vivo*. This is essential for understanding how modulation, for example, by biogenic

amines (Fernandez et al., 2009; Wilson et al., 2004), affects cellular responses. Without this blockade, it would be impossible to establish whether the changes in PN responses after nonassociative (Locatelli et al., 2013) or associative (Fernandez et al., 2009) conditioning are due to modulation of the PNs themselves or to modulation of other cells in the network, e.g., LINs (Sinakevitch et al., 2013). Cadmium therefore provides a unique way to study the intrinsic properties of a specific cell type in relation to associative and nonassociative learning in an *in vivo* system. This treatment would be very powerful when coupled to parallel studies of cellular properties in disassociated cell cultures (Grunewald, 2003).

We also aimed to isolate LINs in order complete pharmacological conditioning experiments and evaluate how summation of calcium from different sources modifies LIN and PN responses to odor by targeting downstream calcium-sensitive processes. We began by identifying the number and location of LINs in a whole mount preparation to clear up inconsistencies in the literature. We found that there were significantly less GABA-ergic cell bodies in the honey bee AL than the previously published 750 by the Schaffer & Bicker study (1986) but more than a previously published Fonta et al. (1993) study with an estimated 109 LINs (Protas et al., 2010). There are still three main clusters of GABA-ergic cell bodies in the AL including the medial, the medial-ventral, and the lateral groups. Knowing the relative number and location of these cell bodies was important for the blind sharp electrode electrophysiological recordings of these LINs *in vivo* within the honey bee AL.

During our intracellular recording experiments, we recorded from several preparations that contained a neuron that had low background spiking activity, and which

would start spiking upon an odor application. We also recorded from several neurons that had high background spiking activity and, upon odor application, would stop spiking. In these preparations, the odor to which the neuron would respond varied from neuron to neuron. There were only two preparations in which OA could be applied and the neuron could be recorded from post-OA application. The problem lied in the fact that we were trying to mimic our other OA-odor pairing experiments, which required that a small amount of OA needed to be pressure injected directly over the AL neuropil. This created a problem because upon pressure injection, we would loose the cell we were recording from. This was most likely due to the pressure application creating too many vibrations for the sharp electrode to stay in the cell. We were able to record information from two cells using this method. Cell 1 responded with increased spike rate to geraniol while cell 2 responded by inhibitory spiking during the odor stimulus of heptanone.

We were able to obtain recordings from 2 cells. Although we were unsuccessful in loading the microruby dextran to label the cell in order to discriminate whether the cell was a PN or LIN, we knew we could load the microruby into the cells because we had preparations that we successfully labeled. Ultimately, this method proved to be extremely complicated and time consuming. We contemplated ionophoretically delivering the OA directly into the AL neuropil, but this would be such a drastic change of protocol that it wouldn't be able to be compared to data collected using the pressure injection method. Bath application of OA may differ from our low-volume of picospritzed application in that it could change properties of the entire system. In another study, it made all of the PNs more excitable (Rein et al., 2013).

Recently, a group has developed an electroporation method for imaging neurons in the silkmoth brain (Fujiwara et al., 2009). We wanted to adapt this method to easily and rapidly identify and image from LINs in the honey bee brain under the same conditioning paradigms which allowed for pharmacological conditioning of the PN dendritic processes of the AL. We found that we could successfully electroporate a fluorescent dextran of the same MW as calcium green-1 dextran (3000MW, Invitrogen, anionic) into the AL of the honey bee brain. Many glomeruli were stained in this process. In control experiments, little staining was visible following insertion of electroporation electrode with no electrical stimulus delivered. Future experiments involving the coupling of this electroporation of the calcium green-1 dextran in the AL with the backfilling of PN dendrites with a fluorescent dye of a different emission spectra will allow for rapid identification of LINs *in vivo.* Coupling this dye loading technique with the same pharmacological conditioning paradigms will reveal much about the intrinsic properties of different cell types within the AL as well as network properties. Our studies have increased understanding of plasticity in several neural components of the honey bee AL and future studies will reveal more of these mechanisms.

CONCLUSION

These studies have revealed that we can pharmacologically condition olfactory responses in the honey bee AL as revealed by calcium imaging studies, when an odor is paired with OA to replace the US. This result confirms the involvement of OA as an important part of the US pathway in the honey bee brain, and it now shows this involvement at the cellular level. However, OA alone did not evoke significant calcium

signals. We have confirmed that these odor responses are evoked by cholinergic input from olfactory receptor neurons and also are at least in part cAMP dependent. This shows that the cholinergic input from olfactory receptor neurons and the co-activation of beta-adrenergic octopamine receptors (AmOAβ) are important for inducing this plasticity. Although cAMP has been implicated in AL plasticity in earlier studies (Müller, 1997, 2000) the results reported here localize the cAMP mechanism to PNs.

This work has important parallels to the study of plasticity in mammals, in which the co-activation of β-adrenergic and cholinergic receptors induce LTP (Watabe, Zaki, & O'Dell, 2000). Although we have failed to show significance or consistent responses in the nicotine-evoked responses from pre- to post-pharmacological conditioning states, future, more-detailed experiments may yet reveal significant changes to ACh receptors. The measurements we performed may not have been sensitive enough to detect changes to cholinergic transmission. Plasticity may occur through these means as different subunit compositions of nACh receptors leads to different Ca^{2+} permeability of the channels (Fucile, 2004).

This work also opens the possibility for future research to include electroporation of LINs in order to study their role in this exhibited plasticity. One possibility includes changes in excitability that allows more fine-tuned control of PN inputs, which would be similar to changes in dendritic excitability in motoneurons modulated by biogenic amines (Heckman, Lee, & Brownstone, 2003). The LINs in the honey bee may be hyperexcitable by octopamine, as the AmOA1 receptors have been found to be colocalized mostly on the LINs (Sinakevitch et al., 2011). Revealing the mechanisms by which the LINs modulate the plasticity in the AL is a next important step in this research. The honey bee remains

an excellent model to study plasticity due to its complex behavioral repertoire including learning about odors and due to its size and the tractability of imaging in the CNS.

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