Mechanisms that underlie experience-dependent assembly of neural circuits

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular, Cellular, and Developmental Biology)
in the University of Michigan
2016

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DEDICATION

To all my animals, past and present.

ACKNOWLEDGEMENTS

funds towards the exhibit.

Of course, I must thank my parents for their unwavering support and encouragement over the years. Dr. Ian Macara, thank you for giving me the ambition and self-confidence to successfully pursue my goals. Dr. Deb Lannigan, you are my hero and role model. By example you have shown me that through hard work and determination you can achieve your dreams (no matter how unique or quirky they might be). Thank you both for instilling in me a love for nature by allowing me to roam free with my pack of dogs around the woods and fields near our house in Vermont, as well as inspiring my sense of adventure through our many travels around the world. Our trekking adventures taught me to be resilient and persevere even through the toughest of circumstances and I'll never forget those (often life-threatening) experiences. Throughout my life you both have encouraged me to follow my passions, to always be enthusiastic about new opportunities, and to be wildly independent. Finally, thank you both for spending many hours in science museums (particularly in the geology sections) with me, putting me to work stuffing pipette boxes in your labs for no pay (this must have violated some child labour laws!), and reading entomology books to me on long plane flights as a child - those early experiences have profoundly influenced my life.

To my friends, thank you for the many laughs and beers we have shared over our time at graduate school. Thank you Dr. Corinne Weisheit for being the very definition of a best friend, I honestly could not have done this without you.

I especially need to thank all my animals, past and present. To love and receive love is all that you have asked of me. Helix, you have taken a piece of my heart but you have given me some of yours in return. Leela, you are my stable companion and I admire your tenacity. Anders, your love for life is infectious. Mel, I know you secretly love me and I secretly love you too. Clock and Doubletime, you taught me that invertebrates really do have emotions and personalities. Doodles, you were quite the little adventurer. To all my other animals, I may have rescued you but you have rescued me many times over. I'd also like to give a special thanks to those at the Creature Conservancy for giving me the opportunity to volunteer at their sanctuary. That experience allowed me to persevere through a very difficult time in my life and having the chance to interact with your animals was very therapeutic.

Finally, I must thank my incredibly supportive, hilarious, and patient partner Nathan Raynard. Thank you for giving me a beer to drink and a shoulder to lean on whenever I needed it. I know that whatever insane and risky goal I set myself, I will succeed because you will always be there supporting me. I am so excited to begin our next adventure together. *Let's always be stupid. Forever!*

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ABSTRACT

I aim to understand the interaction between the environment and the developing brain through investigating how sensory experience shapes behavior. Sensory experience modifies neural connections through activity-dependent plasticity, enabling animals to cope with environmental variability. Classic work, particularly those in vertebrate visual systems, has provided important insights into the mechanisms that underlie experience-dependent plasticity of the developing circuit. However, there are significant gaps explaining the mechanism by which sensory experience shapes circuit function during development. This dissertation examines how sensory experience during development changes the sensorimotor circuit to shape behavior in *Drosophila melanogaster*. *Drosophila* provides a relatively simple and genetically amenable model for analyzing both neural development and mechanisms underlying behaviors, and is thus a powerful model for discovering basic principles underlying experience-dependent plasticity during development. To address how sensory stimuli alters the sensorimotor circuit, I have established a calcium live-imaging technique to physiologically measure neural dynamics within larval brains and have developed a behavioral assay to probe motor output. Through the use of these experimental techniques, I have determined that sensory experience during a sensitive period in development modifies behavior through an intrinsic program of circuit development. These findings suggest that the

development of the larval sensorimotor circuit is shaped by sensory input and neural

activity.

CHAPTER 1

Introduction to the assembly of neural circuits

Introduction

Sensory experience shapes both vertebrate and invertebrate neural circuit development. This plastic nature of the brain allows animals the ability to cope with environmental variability. Detection of noxious stimuli is a conserved survival mechanism across the animal kingdom. The ability of the brain to store the memory of past harmful experience is a critical adaptation for continued survival in a hostile environment. Such environmental stimuli can lead to synaptic modifications that alter behavior, as exemplified in *Aplysia*. However, there are significant gaps in the explanation of how changes in synaptic transmission during development affect behavior in the mature animal. This dissertation examines how noxious sensory experience during development shapes nociceptive synaptic transmission to modify behavior in *Drosophila melanogaster*. In order to investigate how this process occurs, we first need to be able to assess the communications between neurons.

Measuring neural activity

A major goal in neuroscience is to understand how the nervous system senses external stimuli, processes the sensory inputs, and instructs the movements of the motor system. Investigating functional neural communication is integral to understanding the circuit dynamics of the nervous system. Many tools have been developed to study neural networks; major advances include electrophysiology, optogenetics, and calcium sensors.

Neural activity

A neuron is an electrically excitable cell that communicates information through neural activity. Neural activity is created by the flow of ions across the plasma membrane. Outside of the cell there are higher concentrations of sodium, calcium and chloride ions than on the inside. In contrast, potassium ions are higher in concentration on the inside of the cell than on the outside. This creates an electrochemical gradient with the cell being more negative on the inside and more positive on the outside. At rest, the electrochemical gradient across the membrane determines the membrane potential. The cell membrane is made permeable by the expression of different types of ion channels that allow the movement of specific ions across the membrane in a regulated fashion (Barnett and Larkman, 2007; Neher and Sakmann, 1976).

When a neuron is activated, a signal is generated in the form of an action potential. About 70 years ago, Huxley and Hodgkin first performed experiments on the giant squid axon uncovering the ionic mechanism of action potentials (Hodgkin and

Huxley, 1939). The large size of the axon made this system amenable to electrophysiological recordings. The giant squid axon controls muscle contraction to rapidly expel water through the siphon propelling the animal forward (Young and Keynes, 2005). Hodgkin, Huxley and Katz discovered that this contraction is initiated by the opening of voltage-gated ion channels on the axon membrane, allowing the influx of sodium and calcium (Hodgkin and Huxley, 1939; Katz, 1996). This changes the ion permeability and eventually reverses the electric polarity of the cell membrane, leading to an action potential (Baker et al., 1971; Rojas and Taylor, 1975). Those channels then close and potassium channels open, returning the membrane to resting state. Meanwhile, sodium is actively pumped out while potassium is being transported back into the cell, maintaining resting potential. This wave of activity or action potential, moves down the axon (Barnett and Larkman, 2007).

Once the action potential reaches the axon terminal where the neuron forms specialized connections, termed synapses, with the next neuron in the circuit, voltage-gated calcium channels open and calcium enters the synapse. Early electrophysiological recordings on the giant squid synapse revealed that this influx of calcium triggers a cascade of signaling events that result in the release of vesicles containing neurotransmitters and neuropeptides (Baker et al., 1971). Once released, the neurotransmitters and neuropeptides bind to their respective receptors on the downstream or postsynaptic neuron. This activates the postsynaptic neuron and, if the signal is intense enough, will trigger another action potential (Barnett and Larkman,

2007). In this way, the activation of neurons elicits an electrical response that can be recorded.

Recording neural activity

Electrophysiology

Electrophysiological recordings of the collective responses in discrete regions of the nervous system (e.g. field potential recordings), nerves (suction recordings of nerves), and individual neurons (intracellular and whole-cell recordings) have led to many important insights into how sensorimotor circuits operate (Paulk et al., 2013; Zhang and Stewart, 2010). Electrophysiology has been used in large invertebrate nervous systems to investigate neural connectivity, where one electrode is used to activate a neuron of interest while another electrode is used to measure electrophysiological responses in the postsynaptic neuron (Kandel et al., 1967; Willows and Hoyle, 1969). Limitations for electrophysiology include the feasibility of such multi-electrode experiments in *Drosophila* due to the small size of neurons in the CNS. Additionally, electrophysiology does not offer a comprehensive view of how the entire nervous system or large ensembles of neurons operate in behavior.

Calcium indicators

Recent advances in imaging-based recordings have changed the scenario by allowing us to record whole-brain activity or responses in groups of neurons simultaneously. The most common of these imaging-based techniques measure the calcium dynamics within neurons. Calcium transients occur during neuronal activity

(Baker et al., 1971; Burgoyne, 2007). Monitoring calcium with genetically encoded

calcium indicators (GECIs) can be used as a proxy for measuring neural activity (Baird

et al., 1999). GECIs are used to non-invasively record the activity across populations of

neurons as well as activity patterns across entire central nervous systems. The most

optimized GECI is the single green-fluorophore sensor GCaMP (Nakai et al., 2001).

GFP fluorescence increases upon Ca^{2+} binding to the calmodulin and calmodulin-

binding peptide M13 domains of GCaMP (Baird et al., 1999). An increase in GCaMP

fluorescence serves as a proxy readout for an increase in neural excitation. In the

absence of calcium, GCaMP has a low basal fluorescence. Over the years, GCaMPs

have been modified to improve fluorescence signal, dynamic range, kinetics and

sensitivity to neural activity (Akerboom et al., 2012; Nakai et al., 2001; Tian et al., 2009)

as well as expanded the spectral options that can be used in combination with

optogenetics (Marvin et al., 2012; Dana et al., 2016; Inoue et al., 2015).

Activators of neural activity

Optogenetics

To stimulate specific groups of neurons, multi-electrode approaches become

challenging. Optogenetics, on the other hand, allows for the stimulation of specific types

of neural groups using light and is widely used to study behavioral output (Honjo et al.,

2012). Optogenetics uses light to activate neurons that are genetically engineered to be

sensitive to light. A modified photoreceptor from green algae, Channelrhodopsin-2

(ChR2), is used to sensitize neurons to light. ChR2 is a blue light-activated cation

channel (Nagel et al., 2003). *Drosophila* larvae have a clear cuticle that allows light to

penetrate through to the nervous system, making optogenetics a powerful tool in this model organism. However, the use of optogenetics becomes limited when used in combination with genetically fluorescent sensors such as GECIs. This limitation is partly due to the overlap in light wavelengths, resulting in unwanted stimulation prior to recording or bleaching of sensors during live-imaging.

Ectopic expression of cation channels

Neurons can also be activated through the genetically targeted expression of cation channels such as TrpA1, TRPM8 and P2X2. The two thermal-gated cation channels, TrpA1 and TRPM8, can be ectopically expressed to activate neurons. The heat-gated cation channel, *Drosophila* TrpA1 (dTrpA1), is activated and depolarizes neurons expressing it at temperatures above 25^oC (Hamada et al., 2008). Whereas the cold-sensitive rat TRPM8 channel is activated in response to reductions in temperature in the range of about 25 to 10^oC (Peabody et al., 2006). The ionotropic purinoreceptor, P2X2, is a nonspecific cation channel that is activated by ATP binding. *Drosophila melanogaster* lacks the purinoreceptor gene and is therefore normally unresponsive to ATP application. A responder UAS-transgene encoding the trimeric rat P2X2 channel has been created (Zemelman et al., 2003) and can be used in combination with a neuronal specific GAL4 driver in order to ectopically express the P2X2 channel in a subpopulation of neurons. Upon ATP application the P2X2 channel becomes activated causing a robust increase in neuronal excitation (Huang et al., 2011; Lima and Miesenböck, 2005).

This wide range of tools available enables researchers to interrogate the nervous system like never before. Hence, with the relative simplicity of the nervous system and genetic malleability in combination with the vast array of available tools, *Drosophila* is the ideal model to interrogate the sensorimotor network.

Sensorimotor systems

We experience the world through our sensory systems. Populations of sensory neurons respond to different types of environmental stimuli. The sensory neurons transmit this environmental information to the central nervous system where it is processed. The central nervous system then relays the message back to the periphery via motor neurons, which in turn excite specific muscle cells to produce an appropriate behavioral output. The sensory and motor neurons, together with the interneurons between them, form the somatosensory circuit. Whereas human brains have about 100 billion neurons, *Drosophila* larvae have only about 100,000 neurons (Chiang et al., 2011). Therefore, *Drosophila* provides a relatively simple and genetically amenable model for analyzing sensorimotor circuits.

Drosophila larval sensory inputs

The *Drosophila* larval peripheral nervous system contains sensory neurons termed dendritic arborization (da) neurons, which elaborate their dendrites on the body wall and project their axons to the ventral nerve cord (VNC) (Brewster and Bodmer, 1995). There are four classes of da neurons (I-IV) that can be distinguished by their branching patterns, with each subclass containing different sensory properties. Class III

(C3da) and Class IV (C4da) neurons, which are mechanosensors and nociceptors, respectively (Hwang et al., 2007; Yan et al., 2013), are unique in their relatively complex dendritic branching patterns that cover a wide territory without overlapping (Grueber et al., 2002). There are three C4da neurons in each hemi-segment (half of one segment). The C4da neurons project their axons into the ventral VNC neuropil at regular intervals and branch into anterior, posterior, and contralateral projections that together form a ladder-like structure (Kim et al., 2013). These peripheral sensory neurons relay environmental information to the VNC where it is then processed to produce an appropriate motor output.

Drosophila larval sensorimotor outputs

Sensory information is detection through the type II peripheral neurons in the *Drosophila* larval peripheral nervous system. The function of class I and II neurons have yet to be fully determined, although some evidence suggests that class I is involved in proprioception (Hughes and Thomas, 2007). The class III da neurons (C3da) are mechanosensory and respond to light touch (Yan et al., 2013). The class IV da neurons (C4da) are nociceptors and are tuned to detect hazardous chemical, noxious mechanical and harmful thermal stimuli (Hwang et al., 2007). The C4da neurons can be activated directly through the use of genetic activators of neural activity or through natural stimulation to elicit a robust and measurable motor output. Therefore, the C4da neurons are a useful tool for uncovering the mechanisms underlying experience-dependent circuit assembly.

Nociception

Whether or not the subjective experience of pain is universal among animals is debatable; on the other hand, nociception, the ability to detect harmful stimuli, is an objective protective neuronal mechanism and an evolutionary necessity for survival. Examples of nociception include both vertebrates and invertebrates.

Nociceptive behavior

Nociception is defined by characteristic behavioral responses in which a noxious stimulus evokes a reflex response that moves the animal away from the source of harm (Bromm and Treede, 1980). Nociception and nociceptive responses can be observed in both vertebrates and invertebrates. The nematode *C. elegans* will stop and reorient away from the direction of the noxious stimulus (Wittenburg and Baumeister, 1999). *Aplysia* have a robust defensive behavior that involves the retraction of the delicate siphon and gill when startled or presented with a noxious cue (Kandel, 2001). Upon noxious stimulation, zebrafish larvae exhibit an escape reflex common to both fish and amphibians known as a C-start. The C-start consists of two sequential stages starting with the head moving away from the stimulus causing an acute decrease in body angle to form a C shape, followed by the animal moving away from the stimulus (Kimmel et al., 1974). Confronted with noxious chemicals on hind-paws, rodents will lick, bite, flinch and guard their inflicted area (Caterina et al., 2000; Meseguer et al., 2008). Thus, nociception is an evolutionary-conserved protective mechanism that is crucial for survival in a harmful environment.

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Previous studies have described that *Drosophila* display a rolling escape response, defined as a complete 360-degree rotation around the anterior/posterior axis, which is distinct from peristaltic locomotion. Rolling increases larval speed to a higher velocity (3-5 mm/s) than typical peristalsis (1 mm/s) (Hwang et al., 2007), potentially providing rapid escape. One hypothesis is that rolling behavior is an escape mechanism used to maneuver away from ovipositor attack by natural predators of *Drosophila*, such as the small parasitoid wasp of the superfamilies, *Chalcidoidea* and *Ichneumonoidea*. However, rolling also leads to fast lateral shift of the body, which may allow the larva to quickly escape from noxious heat or chemicals. Targeted optogenetic or thermogenetic activation of all the larval nociceptors produces rolling behavior (Hwang et al., 2007; Zhong et al., 2012). Thermogenetic activation of random subsets of nociceptors indicated that only 5-10 nociceptors are needed to trigger escape behavior (Zhong et al., 2012). As shown in Chapter 3, our further characterization has described nociceptive behavior as including multiple steps and levels of intensity.

Nociception has directionality. For example, the C-start observed in larval zebrafish has directionality bias depending on the location of the noxious stimuli. The head moves away from the stimulus to form a C-like curvature of the body which then allows the animal to propel itself away from the noxious cue (Burgess and Granato, 2007) . Third instar *Drosophila* larvae display a variety of nociceptive behaviors such as thrash, roll, whip seizure and paralysis when the whole body is subjected to noxious heat (Chattopadhyay et al., 2012). However, when touched by a heat probe, larvae will roll towards the direction of the stimulus (Neely et al., 2011). Rolling directionality is also

biased to harsh mechanical stimuli (Hwang et al., 2007). Larvae have a strong tendency to propel themselves toward the noxious stimulus. This paradoxical directionality is hypothesized to be a defense mechanism against parasitoid wasp attack allowing larvae to successfully evade the wasp ovipositor (Robertson et al., 2013), however, this does not explain this behavior that occurs after heat probe stimulation (Hwang et al., 2007) . Presumably, rolling toward the source of heat is not a useful survival mechanism. This paradoxical response may be explained by the convergence of both mechano- and nociceptive sensory inputs from the heat probe (Ohyama et al., 2015).

Another important feature of nociception is sensitization after noxious stimulation. Sensitization can be divided into two types: allodynia when an innocuous stimulus elicits nociceptive response after repeated noxious stimulation (such as when you gently touch a sunburn), and hyperalgesia when there is an exaggerated responsiveness to noxious stimuli. Mammalian studies show allodynia and hyperalgesia after injury or inflammation (Gold and Gebhart, 2010), but sensitization is not limited to vertebrates. Studies in invertebrates, such as in leeches (Pastor et al., 1996), moths (Walters et al., 2001) and *Aplysia* (Kandel, 2001), have also demonstrated sensitization of nociceptive behavior. For example, in *Aplysia*, the gill withdrawal reflex is induced by a novel stimulus, such as touch, and becomes sensitized after noxious stimulation. One recent study demonstrated that attacks by natural predators of *Aplysia* induce the sensitization of the gill withdrawal reflex suggesting that this type of sensitization to pain is an ecologically-relevant response (Kandel, 2001). Work in other mollusks have shown that sensitization to pain reduces risk of predation (Crook et al., 2013, 2014). *Drosophila* also exhibit both

allodynia and hyperalgesia after exposure to UV light. After third instar larvae are

exposed to acute UV light, the normally innocuous temperature of 38°C increases

nociceptive response in the larvae (Babcock et al., 2009).

Nociceptors

Nociceptors are specialized sensory neurons that are specifically tuned to

respond to noxious environmental cues, such as intense radiation, chemical or

mechanical stimuli. Nociceptors are usually multimodal, meaning that they can respond

to noxious cues of multiple sensory modalities. A nociceptor expresses different

receptor molecules that are respond to noxious cues from distinct modality. While

chemical sensors detect tissue-damaging chemicals, mechanical sensors detect excess

pressure, incisions, or deformation and thermal sensors detect noxious heat or cold,

these receptors often overlap. For example, the transient receptor potential A1 (TrpA1)

is a polymodal sensor for environmental irritants and is able to detect two of these

modalities (chemical and thermal). The *Drosophila* TrpA1 is an orthologue to the

mammalian molecular receptor (Kang et al., 2010). Two isoforms of dTrpA1 (dTrpA1-C

and dTrpA1-D) are specifically expressed in the nociceptors, and the dTrpA1-C isoform

is responsive to noxious heat (Zhong et al., 2012). *Drosophila* TrpA1 is also responsive

to reactive electrophiles such as allyl-isothiocynate, n-methylmaleimidem and

cinnamaldehyde . Reactive electrophiles are tissue-damaging compounds produced by

both plants and animals as deterrents and are aversive to both vertebrates and

invertebrates (Kang et al., 2010).

Some receptor molecules are modality-specific. The DEG/ENaC superfamily, based on the names of the first two identified subfamilies degenerins (DEG) and epithelial sodium channel (ENaC), is an ion channel class that is involved in a number of processes including nociception (Mano and Driscoll, 1999). In *Drosophila*, the *pickpocket* gene encodes a DEG/ENaC subunit and is required for mechanical but not thermal nociception in larvae. Pickpocket is expressed in nociceptors (Zhong et al., 2010). Pickpocket shows homology with multiple mammalian channels involved in mechanical pain perception such as the amiloride-sensitive cation channel 3 (Chen et al., 2002). Another mechanosensitive ion channel is the Piezo family, found in mammals and *Drosophila*. The *Drosophila* Piezo (Dmpiezo) mediates noxious mechanical stimuli but not to other noxious stimuli or to gentle touch. Knockdown of Dmpiezo in the nociceptors suppresses the nociceptive response to harsh mechanical stimuli even with the expression of pickpocket in those neurons (Kim et al., 2012).

Nociceptive circuit

The *Drosophila* larval nociceptive circuit consists of a large number of neurons, with the nociceptors themselves contacting multiple target neurons (Ohyama et al., 2015; Vogelstein et al., 2014). A large scale electron microscopy reconstruction of a section of the *Drosophila* larval VNC revealed mechanosensory and nociceptive circuit convergence. This multisensory circuit reconstruction uncovered other nociceptor downstream target neurons. One set of downstream targets were four lineage-related Basin-neurons (Basin-1-4). The Basin-neurons are segmentally repeated with projections in the ventral sensory domain of the VNC in proximity to the C4da axon

terminals. Basin-1 and -3 mostly receive inputs from the mechanosensory neurons but very few inputs (less than 1% of total inputs) from the nociceptor axon terminals. Basin-2 and -4 are multi-modal and receive inputs from both mechanosensory and nociceptor neurons. Functionally, Basin-4 responded to both nociceptor and mechanosensory input and responded synergistically to simultaneous sensory input. There are two main pathways of this multisensory circuit that controls rolling behavior. The first pathway remains in the nerve cord, were Basin-2 neurons synapse onto the abdominal A05q neurons, which then synapse onto a single pair of thoracic neurons called Goro. The Goro neurons are thought to be command-like neurons for rolling behavior, since direct activation of the Goro neurons triggers rolling. The second pathway enters the brain through multiple interneurons downstream of Basin-4 with the final stop back in the nerve cord on the Goro neurons. Both pathways are referred to as the Basin-Goro pathway, the convergent circuit the mechanosensory and nociceptive sensory inputs (Ohyama et al., 2015).

Another set of potential downstream targets of the nociceptors were found using a large optogenetic screen. This screen found several candidate neurons within the VNC that displayed an escape behaviotype probability distribution similar to that of the C4da nociceptors. These escape lines include; GMR50H05, GMR75C05, and GMR82E12. The corresponding neurons within each line include; AVM001b, AVM002b located in the brain, and A08n located in the VNC. The A08n projections overlap tightly with the nociceptor axon terminals, possibly forming synaptic connections and therefore are potential downstream targets of the nociceptors (Vogelstein et al., 2014). However,

the A08n neurons have yet to be fully characterized as functional and synaptic partners to the nociceptors.

Development of nociceptive circuits

      Drosophila larva has a relatively simple nervous system, is amenable to genetic manipulations, and has extensive tools available to interrogate neural circuits. Investigating the sensorimotor circuit development in *Drosophila* therefore may provide insight into the mechanisms underlying nociception circuit assembly and the consequences of early life nociception and, possibly, pain.

      Nociceptors are specified early in development. In mammals, nociceptor sublineages are determined even before neural crest cells become committed to neural or glial fates (Fitzgerald, 2005; Zirlinger et al., 2002). The nociceptor-fated neural crest cells split off from the neural tube and nociceptors are then generated in the dorsal part of the neural tube, the dorsal root ganglia (DRG). The development of the peripheral innervation of the skin by these primary sensory axons originating from the DRG occurs before birth (Jackman and Fitzgerald, 2000). Defining characteristics of nociceptors present in the DRG sensory neurons occur at an early embryonic stage. For example, the expression of a nociceptive chemical and thermal sensor and TRP channel family member, vanilloid receptor 1 (TrpV1), begins at postnatal day 2 and occurs at similar levels as in the adult (Guo et al., 2001). Recordings of DRG sensory neurons revealed physiological characteristics specific to those of nociceptors. The nociceptors form axon terminals in the central nervous system at postnatal day 5 (Pignatelli et al., 1989).

Peripheral nociceptive innervation density and nociceptor sensitization can be modified

due to environmental conditions during early postnatal life. For example, neonatal skin

wounds can result in long-term sensory hyperinnervation (Reynolds et al., 1991). These

data indicate that nociceptors are functional from the first few days of postnatal life and

reveal the potential impacts on peripheral sensory development after exposure to early

life pain. Preterm infants admitted to the Neonatal Intensive Care Unit spend for about a

month where they undergo multiple invasive procedures per day, the majority of which

produce pain. Long-term consequences to early life pain include alterations in

somatosensory processing and impaired response to stress. Such physiological

changes in stress response are associated with disorders of anxiety, depression, and

obsessive compulsion (Fitzgerald, 2005). Comparison to similar phenomena in non-

mammalian species may provide insight into the mechanisms underlying how noxious

sensory experience shapes circuit function and subsequent behavior.

Similarly, in *Drosophila*, the nociceptors neurons form during development. The

nociceptor axons reach into the VNC during early embryonic development and the axon

terminals maintain the ladder-like structure in the VNC throughout larval development

(Grueber et al., 2007). Expression of painless mRNA appears at stage 13 of embryonic

development. The dendritic arborizations of the C4da neurons begin to elaborate at

embryonic stage 13 and painless localizes to the dendritic projections (Gao and Chess,

1999; Tracey et al., 2003). The DEG/ENac protein required for mechanical nociception,

pickpocket, is expressed at stage 15 (Kaneko and Ye, 2015). *Drosophila* exhibits a

variety of nociceptive behaviors dependent on the stage of development. Avoidance

behavior occurs as early as first instar larva, indicating that the nociceptors are functional from the onset of early development (Sulkowski et al., 2011). The nociceptor topographic projections are established in an activity-dependent manner during the 2nd and early 3rd instar larval stages (Yang et al., 2014). Rolling behavior emerges at the later developmental stages during late second-instar and remains throughout third-instar (Sulkowski et al., 2011). It is interesting to note that rolling behavior emerges during the same developmental period that is preferred by female wasps to lay their eggs (Hwang et al., 2007). Finally, in adult *Drosophila*, heat probes induce a jump or avoidance in response to the noxious thermal stimulus (Neely et al., 2011; Xu et al., 2006). Together, these data suggest that larvae are able to detect, transmit and respond to nociceptive information as early as the 1st instar stage.

Neuromodulators

Neuromodulators mediate a number of behaviors and physiological processes including sensorimotor integration (Taghert and Nitabach, 2012) and circuit development (Daubert and Condron, 2010). Neuromodulators diffuse broadly and have widespread effects, mediating groups of neurons whereas classic neurotransmitters have short-ranged effects, diffusing between synapses from a neuron to an effector cell. While neurotransmitters produce short-term response in their effector cells, neuromodulators mediate long-term change in their target cells (Marder, 2012; Nadim and Bucher, 2014). Neuromodulators include serotonin (Ciranna, 2006), dopamine (Puig et al., 2014), acetylcholine (Picciotto et al., 2012), histamine (Montesino et al., 1995), and norepinephrine (O'Donnell et al., 2012).

Neuromodulators in sensorimotor integration

Innate behavior is a robust and stereotyped response that is genetically programmed and is dictated by dedicated neural circuits. Although innate behavior is considered to be hardwired, it can be mediated through neuromodulation (Su and Wang, 2014). About half a century ago, the characterization of two basic central pattern generators in the crustacean stomatogastric ganglion led to the idea that invertebrate neural networks are hardwired. However, it was found that modulation of this simple network of 30 neurons creates a multitude of motor outputs more than just the two basic rhythmic motor patterns (Marder and Bucher, 2007). This ability to generate a diverse array of behaviors from a simple network is due to neuromodulation.

The serotonergic system is involved in the behavioral plasticity of the mature nervous system in various species including mammals, *Aplysia,* and *C. elegans* (ref). Serotonin is an evolutionarily ancient signaling molecule (Turlejski, 1996). It is derived from the essential amino acid tryptophan. The biochemical reaction converts tryptophan to 5-hydroxytryptophan (5-HT) by the enzyme tryptophan hydroxylase (TRH), 5-hydroxytryptophan (5-HTP) is subsequently converted to 5-HT by aromatic acid decarboxylase. Serotonin exerts its effects on 7 distinct receptor families in mammals and only 3 known receptor families in *Drosophila*. Serotonin can be either excitatory or inhibitory depending on the differential expression of receptors on the postsynaptic neuron. Of the 3 families, there are five known *Drosophila* serotonin receptor subfamilies: $5HT_{1A}$ and $5HT_{1B}$ are negatively coupled to adenylyl cyclase, $5HT_{2A}$, $5HT_{2B}$

and $5HT_7$ are positively coupled to adenylyl cyclase (Johnson et al., 2009; Majeed et al., 2016; Saudou et al., 1992).

Studies in invertebrates have shown that neuromodulators, such as serotonin, mediate the gating of a particular behavioral state, as exemplified by the recent work of Flavell and colleagues. In this work, serotonin was found to mediate the feeding state in *C. elegans*. Serotonin, in opposition to another neuropeptide, initiates and extends dwelling states in which the worm stays in one location (Flavell et al., 2013). Behavioral states are gated through serotonin in *Drosophila* as well. In early development, larvae are photophobic but gradually become photo-neutral during 3rd instar. The activity of serotonergic neurons regulates this change in locomotor behavior state in larvae through $5HT_{1A}$ receptors (Rodriguez Moncalvo and Campos, 2009).

Neuromodulators also play a key role in regulating the gain of behavior response. Serotonin modulates the transmission of nociceptive information in vertebrates, for example, through descending inhibition (Bardin, 2011). However, few studies have investigated the role of serotonin in invertebrate nociception. Orb weaving spiders are known to undergo leg autotomy (removal of leg) once injected by serotonin, a component found in venom (Eisner and Camazine, 1983). Similarly, injections of serotonin increases stress response in crayfish (Fossat et al., 2015). The *Aplysia* gill withdrawal reflex becomes sensitized in response to noxious stimuli such as electric shock which is mediated through presynaptic facilitation by serotonin (Kandel, 2001). In *C. elegans*, serotonin facilitates aversive response to the repellent octanol through the

SER-5 receptor, which is closely related to mammalian 5-HT6 receptors. Elevated 5-HT

in adult *Drosophila* leads to heightened aggression, perhaps an aspect of an aversive

response (Curran and Chalasani, 2012). Additionally, there are 84 identified

serotonergic neurons in the *Drosophila* larva that are distributed in clusters throughout

the brain and VNC. The serotonergic neurons in clusters labeled A1-7, and A8 in the

VNC are segmentally repeated with projections that lay in close proximity to the

nociceptor axon terminals (Vallés and White, 1988). Together, these studies highlight

the importance of serotonin signaling and the need to further investigate the role of

serotonin in mediating aversive behavior.

Neuromodulators in development

Although the serotonergic system is involved in modulating behavior of the

mature nervous system, it is additionally crucial for the proper development of neural

networks. Serotonin is synthesized in early development leading to the speculation of its

role in mediating network assembly. Serotonin receptor expression in mammals

appears in unique temporal and spatial patterns during early development further

implicating the role of the serotonergic system as a key developmental molecule

(Muller et al., 2016). There is also considerable evidence that links the disruption of

serotonin signaling to various neurodevelopmental and psychiatric disorders.

Serotonin regulates basic neural morphology and modification of synapse

formation in the developing animal (Lesch and Waider, 2012). In the rodent, the barrel

cortex is part of the somatosensory region that is responsible for integrating whisker

sensory input. Reducing 5-HT by genetic manipulation delays the timing of barrel cortex development (Vitalis et al., 2013). Serotonin also regulates the spacing of neuronal processes. Reduction of serotonin levels in *Drosophila* leads to an increase in serotoninergic neuron branching whereas administration of 5-HTP to raise 5-HT levels results in reduced branches (Budnik et al., 1990; Diefenbach et al., 1995). Serotonin controls the formation and retraction of growth cones through voltage-dependent calcium channels (Kater and Mills, 1991) and phenocopies the inhibition of growth cones through electrical activity (Cohan and Kater, 1986), suggesting the possibility that 5-HT may regulate some aspects of neural morphology through an activity-dependent mechanism. Serotonin also likely plays a key role in the developmental process of long term potentiation (LTP) in the formation of the sensorimotor circuit. Neural connections are formed and strengthened during the coordinated spontaneous activity waves that are critical for proper circuit development, for example, the retinal waves that occur prior eye opening and development of visual responses (Firth et al., 2005). Serotonin levels show a transient increase during early development in mammals. This increase in serotonin correlates with a decrease in LTP during critical period of the rat visual cortex development (Edagawa et al., 2001). These studies indicate that 5-HT may be involved in the modification of synapse formation through mediating neural activity, at least in the visual system.

Few studies have investigated how serotonin modifies behavioral output. During the first part of larval development the animal forages for food avoiding light. Third instar wandering larvae leaves the food, switching its photobehavior from photophobic to light

neutral (Sawin-McCormack et al., 1995). A pair of neurons that are regulated by

serotonergic neurons were found to be required to maintain the photophobia in

developing larvae (Rodriguez Moncalvo and Campos, 2009). In mammals, altering the

levels of 5-HT through disrupting the serotonin transport system leads to disruption in

sensory processing. For example, genetic modification of serotonin uptake using

serotonin transporter mouse models disrupts whisker sensory processing. Exposure to

a serotonin reuptake inhibitor in early life disrupts the stress response and increases

anxiety-like behavior in adult mice. Interestingly, the long-term consequences to early

life pain in humans include alterations in somatosensory processing and impaired

response to stress (Lesch and Waider, 2012). The serotonergic system is closely linked

to pain and disruption of serotonin signaling during development leads to impaired

sensory processing therefore, serotonin may be involved in the development of the

nociceptive sensory system.

Plasticity

Brain function is ultimately dependent on the proper formation of the neural

network to establish precise communication. The brain must also be able to adapt. This

active and dynamic process of neural plasticity occurs throughout life and allows

animals to cope with environmental variations.

Mechanisms underlying experience-dependent synaptic plasticity

Whereas neural plasticity refers to the ability of the circuit to change, a classical

neural plasticity is synaptic plasticity, which is the modification of the pre- and post-

synapse. Studies in the sensorimotor system of the gastropod *Aplysia californica* have

led to insights into the mechanisms of how experience-dependent plasticity modifies

synapses to affect behavior. *Aplysia* produce a stereotypical and robust defensive

response to noxious input known as the gill and siphon withdrawal reflex. Applying a

weak tactile stimulus to the siphon withdraws both the delicate siphon and gill into the

mantle cavity for protection under the mantle shelf (Kandel, 2001).

The gill withdrawal reflex is widely used to examine the synaptic mechanism of

associative learning, sensitization and habituation. The classical conditioning paradigm

used to observe associative learning pairs a weak tactile stimulus to the siphon as the

conditioned stimulus (CS) with an electric shock to the tail as the unconditioned stimulus

(US). Specific temporal pairing of CS and US heightened the gill withdrawal reflex upon

CS stimulation alone. During tail shock, the tail sensory neuron activates serotonergic

interneurons that presynaptically modify siphon-gill synaptic transmission. Sensitization

resembles that of classical conditioning in that exposure to a noxious stimulus enhances

the response of another pathway. Sensitization occurs through the strengthening of the

synapses between the sensory neuron in the siphon and motor neuron in the gill

through the presynaptic facilitation on the sensory neuron. This type of plasticity

requires serotonin which acts through the cAMP-PKA pathway, stimulating the

production of cAMP as well as activating calcium channels in the synapse to facilitate

sensory neuron-motor neuron synaptic transmission (Kandel, 2001). Habituation is the

decline of response intensity to repeated innocuous stimulus. Habituation is

characterized by the presynaptic depression of the sensorimotor pathway as repeated

stimulus results in reduced neurotransmission between sensory neuron and motor neuron (Carew et al., 1981; Castellucci et al., 1970).

Known mechanisms that underlie developmental plasticity

Developmental plasticity occurs when neural connections remodel and are susceptible to plasticity. A theme in the development of neural circuits is an initial phase of synaptic overgrowth followed by refinement of synaptic connections. The refinement may include structural or physiological remodeling of the connections. Genetic programs control the differentiation, migration, and axon growth to form the structural neural connections which then can be further modified through activity-dependent plasticity. In the developing retina, for example, genetic programs establish a coarse retinotopic map (Luo and Flanagan, 2007). On the other hand, endogenously driven periodic activity bursts that propagate across the developing retina are required for the activity-dependent refinement of the retinotopic map during early development (Firth et al., 2005). In addition to instructions by the genome, which dictates both wiring that is independent of neural activity and that depends on spontaneous activity in the circuit, sensory experience during development also refines the neural circuitry.

Hebbian mechanisms, such as long-term potentiation (LTP) and long-term depression (LTD), are important in establishing developmental plasticity. The Hebbian forms of synaptic plasticity require the correlated firing of pre- and post-synapses results in modification of only those synapses (Morris, 1999) . For example, monocular deprivation during a critical period in the development of the mammalian visual cortex

leads to enhanced responsiveness in the open-eye to stimulation which is known as ocular dominance (Gordon and Scott, 2009; Wiesel and Hubel, 1963). However, Hebbian plasticity alone would provoke a positive feedback process that would increase activity uncontrollably within the nervous system, breaking down the specificity of the plasticity (Pozo and Goda, 2010; Turrigiano, 2008). The importance of maintaining circuit stability becomes evident when considering an uncontrolled system of feedforward potentiation, where each following neuron has enhanced activity compared to the previous. In this system, the synaptic strengths are driven towards their maximum, eventually leading to saturation, and because of this positive feedback the synapse specificity of these synaptic plasticity mechanisms break down so that information about the original stimulus is lost (Turrigiano and Nelson, 2004). Therefore, there is a need for a non-Hebbian mechanism that sets excitation and inhibition at appropriate levels. This problem is avoided by homeostatic plasticity.

Homeostatic plasticity is the process by which neurons restore baseline function in the presence of chronic stimulation to compensate for the destabilizing effects of the Hebbian synaptic changes that occur during activity-dependent plasticity (Blankenship et al., 2009; Davis, 2013). Homeostatic plasticity is important not only for reducing synaptic strength under conditions of elevated excitability but also for enhancing synaptic strength to prevent synapse elimination (Turrigiano and Nelson, 2004). The mechanisms underlying homeostasis include intrinsic control of neuronal excitability, synaptic-scaling, and regulation of neurotransmitter release. Chronic activity suppression in cultured neurons led to compensatory changes in synaptic strength that

returned activity levels to control values, suggesting that neurons have an internal activity set point (Turrigiano et al., 1998). Such compensatory changes in synaptic strength are mediated by synaptic scaling. Synaptic scaling has been well described in excitatory glutamatergic synapses in the mammalian CNS. Evidence from these studies suggests that neurons regulate their activity level through calcium-dependent sensors to mediate glutamate receptor trafficking at the synapse (Turrigiano, 2008). The larval neuromuscular junction (NMJ) has been a powerful model in investigating the homeostatic regulation of presynaptic function. Postsynaptic modifications, including manipulation of glutamate receptors and muscle excitability, results in the compensatory change of presynaptic neurotransmitter release (Davis, 2013). Homeostatic plasticity thusly counteracts the changes brought about by Hebbian potentiation, stabilizing neural circuit activity.

Recent studies indicate that the development of the mammalian CNS involves the interplay of Hebbian and homeostatic mechanisms (Vitureira and Goda, 2013). For example, short monocular deprivation of one eye reduces the deprived-eye responses to stimulation whereas the open-eye has enhanced response to stimulation, consistent with that of Hebbian ocular dominance. However, longer monocular deprivation results in the strengthening of not only the open-eye response to input but also in the neurons receiving input of the deprived-eye consistent with homeostatic synaptic strengthening (Mrsic-Flogel et al., 2007). In another study, genetically suppressing activity in a single neuron within the mammalian CNS reduced the synapses formed on that neuron during development but after synapses had already formed, reducing neural activity resulted in

a homeostatic increase in synaptic input (Burrone et al., 2002). These differential effects suggest that neural plasticity is regulated by both Hebbian and homeostatic mechanisms, although separated over developmental time.

Forms of developmental plasticity in Drosophila

In *Drosophila*, activity influences synapse formation, morphology and neural homeostasis. The larval NMJ undergo structural and functional changes in response to modulations in neural and muscle activity (Budnik et al., 1990; Davis, 2013). For example, reduction of postsynaptic activity results in presynaptic arbor growth and increased neurotransmitter release (Korkut et al., 2013; Mosca et al., 2005; Paradis et al., 2001). Structure also regulates function, such that genetically decreasing the number of synapses leads to a compensatory increase in postsynaptic response (Turrigiano and Nelson, 2004). Second messenger signaling plays a role in the activity-dependent changes in synaptic growth. Enhanced neural activity leads to calcium influx and subsequently affects cAMP signaling to regulate synaptic growth (Budnik et al., 1990; Zhong et al., 1992). While the NMJ is an excellent model for synaptic development and plasticity, its use in determining the impact of plasticity on behavior is limited.

Understanding how neural plasticity affects behavior requires investigating the mechanisms underlying circuit development in the central nervous system. Studies manipulating neural activity have demonstrated that activity affects the morphology and wiring of central neurons. For example, central neurons undergo postsynaptic structural

modifications to compensate for changes in presynaptic activity (Tripodi et al., 2008).

Additionally, work in our lab has demonstrated that genetic activation of the nociceptors

modifies the axon terminal topography within the VNC (Yang et al., 2014). The functions

of such morphological adjustments remain to be determined.

Sensory inputs may impact the structure or physiology of developing neural

circuits to affect behavior. For example, prolonged exposure to CO_2 during a critical

developmental window enlarged the structure of a CO_2-specific glomerulus that was

induced by the chronic neural activity in the CO_2 olfactory sensory neurons. The chronic

activity of the olfactory sensory neurons resulted in enhanced activity of inhibitory

GABAergic interneurons. Postsynaptic projection neurons displayed a reduced

response to CO2 stimulation due to inhibition from the GABAergic interneurons resulting

in a subdued behavioral response to CO_2 exposure (Sachse et al., 2007). In another

study, the ventral lateral neurons, postsynaptic targets of the larval photoreceptors,

have been shown to be modified by visual input. Prolonged light exposure results in

shortened dendrites and reduced calcium responses in the postsynaptic neurons

whereas reduced light results in the opposite structural and functional modifications

(Yuan et al., 2011). More recently, genetic activation of dopaminergic interneurons

increased calcium within specific postsynaptic targets in the *Drosophila* mushroom body

(Boto et al., 2014), although the homeostatic mechanism to determine the input-

specificity of this activity-dependent change has not been examined.

Drosophila nociceptive circuit as a model for studying developmental plasticity

Despite these major progresses in our understanding of the mechanisms underlying developmental plasticity of neural circuits, how experience-dependent changes in synaptic transmission during development affect behavior in the mature animal are poorly understood. This knowledge gap motivated us to develop a system that links synaptic physiology to behavior in *Drosophila* to interrogate the mechanisms underlying experience-dependent assembly of neural circuits.

Research objectives

This dissertation seeks to understand the mechanisms underlying sensory-experience-dependent plasticity in the development of the *Drosophila* larval nociceptive circuit. To address how noxious environmental stimuli alter the function of the nociceptive circuit, I use a combination of live-imaging and behavioral assays. In Chapter II, I describe my role in the optimization and establishment of a calcium live-imaging technique to physiologically measure neural activity within adult and larval *Drosophila* brains. This technique bypasses the need for multi-electrode recordings and allows us to observe communication between specific groups of neurons to whole-brain activity. In Chapter III, I investigate how sensory experience shapes nociceptive sensorimotor circuit development. Specifically, I discuss how noxious sensory experience during development changes synaptic transmission in the nociceptive circuit to shape larval behavior. The studies described in Chapter III utilize the technique developed in Chapter II to investigate the functional assembly of the nociceptive circuit. Finally, in

Chapter IV I summarize the main contributions of my work to the field of developmental neurobiology and discuss some of the unanswered questions in the field.

studies on neuronal morphology. E.F. and Z.D. set up the optogenetic system for studying larval nociceptive behavior. A.M.M, T.K., Y.H., and Z.D. performed behavior analyses. S.H. and J.Z. developed the method and software for quantifying body angles in larval nociceptive curling. A.G. and R.Y. provided initial data suggesting that serotonergic neurons are downstream of the nociceptors. B.Y. supervised the project. A.M.M., T.K. and B.Y. wrote the paper.

Some material from each of these papers has also been incorporated into this introductory Chapter.

Animal welfare considerations

Due to the lack of knowledge surrounding the emotional experience of pain in invertebrates, the animals used in the preparation of this dissertation were respected as necessary sacrifices towards the progress of understanding the complicated process of nociception and will hopefully provide insight to alleviate animal suffering in the future.

CHAPTER 2

Analysis of functional neuronal connectivity in the *Drosophila* brain

Abstract

Drosophila melanogaster is a valuable model system for the neural basis of complex

behavior, but an inability to routinely interrogate physiologic connections within central

neural networks of the fly brain remains a fundamental barrier to progress in the field.

To address this problem, we have introduced a simple method of measuring functional

connectivity based on the independent expression of the mammalian P2X2

purinoreceptor and genetically encoded Ca^{2+} within separate genetically defined

subsets of neurons in the adult brain. We show that such independent expression is

capable of specifically rendering defined sets of neurons excitable by pulses of bath-

applied ATP in a manner compatible with high-resolution Ca^{2+} imaging in putative

follower neurons. This technically facile approach can now be used in wild-type and

mutant genetic backgrounds to address functional connectivity within neuronal networks

governing a wide range of complex behaviors in the fly. Furthermore, the effectiveness

of this approach in the fly brain suggests that similar methods using appropriate

heterologous receptors might be adopted for other widely used model systems.

Introduction

Despite its relative simplicity, the nervous system of *Drosophila melanogaster* is capable of producing a remarkable repertoire of complex behaviors (Weiner, 1999). Work on *Drosophila* has identified discrete networks of neurons that govern circadian timekeeping (Nitabach and Taghert, 2008), courtship (Villella and Hall, 2008), memory (McGuire et al., 2005), sleep (Crocker and Sehgal, 2010), feeding (Melcher et al., 2007), and decision-making (Dickson, 2008; Peabody et al., 2009). The study of these and other neural networks in the fly continues to enrich and inform our understanding of the neural control of animal behavior. For many of these central brain networks the pattern and physiologic basis of their constituent connections have been proposed; however, due to the electrophysiological inaccessibility of much of the fly CNS, many aspects of these network models remain unchallenged experimentally. The development of technically feasible methods to test for the presence and physiologic nature of connections between defined neuronal classes of the fly CNS will therefore be critical for progress in the field.

The ability to address the nature of connections between pairs of identified neurons has been one of the great strengths of large invertebrate model systems (Kandel 1976). The stereotyped and large neurons of these organisms are accessible to multiple recording and stimulating electrodes, making it possible to stimulate activity in a neuron of interest while measuring electrophysiological responses in putative follower neurons (Kandel et al., 1967; Willows and Hoyle, 1969; Fig. 2.1). Unfortunately, such

multielectrode experiments are not feasible for most central neural networks of the

Drosophila brain. The electrophysiological inaccessibility of many central fly neurons

has been surmounted somewhat by the use of genetically encoded sensors for

neuronal excitation and second-messenger signaling (Lissandron et al., 2007; Ruta et

al., 2010; Shafer et al., 2008; Tian et al., 2009; Tomchik and Davis, 2009; Wang et al.,

2003) and the physiologic responses of single deeply situated neurons can now be

routinely observed in the fly brain using live imaging techniques. Combining these

techniques with an ability to acutely activate subsets of neurons would allow for existing

models of neural connectivity to be tested and the downstream targets of neurons of

interest to be identified physiologically.

Several genetically encoded triggers of neural excitation have been successfully

used in *Drosophila* in conjunction with various chemical or physical triggering methods

(Venken et al., 2011). The first instance of such triggering in the fly used the

photochemical excitation of neurons expressing transgenic P2X2 receptor, a

mammalian ATP receptor that is not encoded by the *Drosophila* genome (Lima and

Miesenböck, 2005; Littleton and Ganetzky, 2000). The mammalian thermosensitive

TRPV1 channel has been used to excite fly sensory neurons using its ligand capsaicin

(Marella et al., 2006) and ectopic expression of the *Drosophila* thermosensitive TRPA1

channel has also been used to activate multiple neuron types with pulses of high

temperature (Parisky et al., 2008). Furthermore, the mammalian cold-sensitive TRPM8

channel has been used with both low-temperature pulses and menthol vapor as

exogenous excitation triggers in the fly (Peabody et al., 2009). Finally, several groups

have used the bacterial opsin Channelrhodopsin-2 (ChR2) to trigger neuronal excitation in Drosophila with blue light (Pulver et al., 2009; Schroll et al., 2006; Zimmermann et al., 2009). The fact that ChR2 is maximally activated by blue wavelengths makes it problematic for use in live imaging experiments, since GFP-based sensors must be excited with the same wavelengths that activate opsin conductance (Guo et al., 2009). The recent development of red-shifted optogenetic controls (Yizhar et al., 2011) and Ca^{2+} sensors (Zhao et al., 2011) may ultimately circumvent this problem, but these newly developed tools have not yet been successfully introduced to *Drosophila*. The use of temperature pulses to trigger the opening of TRPA1 or TRPM8 channels during live imaging experiments is also problematic, because acute shifts in temperature can cause significant movement of imaging targets within the explanted brain during high resolution imaging, which makes the analysis of single-neuron somata difficult (Q. Zhang and O. Shafer, unpublished observations). For these reasons we have opted for ligand-gated triggering of transgenic receptors as a means for acute neuronal excitation. The feasibility of combining ATP excitation of P2X2-expressing fly neurons to attain biologically relevant neural excitation during behavioral and physiologic experiments has already been established for both larval and adult nervous systems (Hu et al., 2010; Lima and Miesenböck, 2005). We have therefore chosen ATP/P2X2 excitation for use in our live-imaging experiments.

In *Drosophila* the Gal4/UAS system is a powerful and versatile method of transgene expression that has been the tool of choice for directing sensor expression in specific neuronal classes within the fly brain (Brand and Perrimon, 1993; Venken et al.,

2011). The recent development of alternative binary expression systems, the LexA and

Q systems (Lai and Lee, 2006; Potter et al., 2010), now makes it possible to

independently direct P2X2 and sensor expression within different neuronal classes.

Here, we have used the simultaneous use of the Gal4 and LexA systems for the dual

binary expression of P2X2 and genetically encoded sensors of Ca^{2+}, thereby allowing

for the acute excitation of defined neuronal populations during the simultaneous live

imaging of Ca^{2+} dynamics (Fig. 2.1).

Here, we establish the feasibility of the simultaneous use of the GAL4 and LexA

systems to render defined groups of neurons excitable by pulses of bath-applied ATP

while simultaneously and independently expressing the Ca^{2+} sensor GCaMP3.0 in

putative follower neurons. The LexAop-P2X2 and LexAop-GCaMP3.0 lines we have

used for these studies, along with large and growing number of existing GAL4, UAS,

and LexA lines, constitute a useful and technically facile toolkit for the interrogation of

central neuronal networks in the *Drosophila* brain.

Results

Controlled excitation of P2X2-expressing deep brain neurons with perfused ATP is
compatible with high-resolution live-imaging.

Previous work has established that neurons expressing transgenic P2X2

receptor in *Drosophila* can be excited at biologically relevant levels through the global

uncaging of ATP in freely moving flies (Lima and Miesenböck, 2005) or through the

puffing of ATP on explanted brains during electrophysiological recordings of superficial

brain neurons (Hu et al., 2010). We wondered if the simple perfusion of ATP across the

explanted brain could provide a reliable and technically facile means of exciting deeply

situated adult neurons in a manner compatible with high-resolution live imaging. We

therefore used a Pdf-Gal4 driver to co-express UAS-GCaMP3.0 (Tian et al., 2009) and

UAS-P2X2 (Lima and Miesenböck, 2005) in the small ventral lateral neurons (s-LN$_v$s).

These cells are critical circadian pacemaker neurons whose small somata and deep

position within the central brain make them difficult neurons to investigate

electrophysiologically (Cao and Nitabach, 2008). Compared with vehicle controls (Fig.

2.2 A), 30-s perfusions of 1 or 2.5 mM ATP resulted in significant GCaMP3.0

fluorescence increases, thereby revealing acute excitation of the s-LN$_v$s (Fig. 2, B, C, E,

F). In contrast, 30-s perfusions of 2.5 mM GTP did not result in significant increases in

on GCaMP3.0 fluorescence, instead causing very small decreases in fluorescence

during perfusion (Fig. 2.2, D and E). The latencies of the s-LN$_v$ responses to 1 mM ATP

were less consistent compared with the responses to 2.5 mM, although a few s-LN$_v$s did

display relatively late responses to the higher dose (Fig. 2.2, B and C). Many of the

GCaMP3.0 fluorescence increases displayed by the s-LN$_v$s following 1 mM ATP

perfusion were markedly bimodal, unlike the majority of responses to 2.5 mM (Fig. 2.2,

B and C). This was reminiscent of s-LN$_v$ GCaMP3.0 responses to nicotinic

acetylcholine. Carbachol (CCh) excitation of s-LN$_v$ nicotinic acetylcholine receptors

(nAChRs), which like P2X2 are expected to gate both Na$^+$ and Ca^{2+} upon ligand

binding, results in bimodal GCaMP3.0 responses at low CCh concentrations but in

single fluorescence peaks at high concentrations (Lelito and Shafer, 2012). It is possible

that, in the case of bimodal responses, the first peak reflects the direct gating of Ca^{2+} through P2X2, whereas the second peak represents Ca^{2+} entry through voltage-gated Ca^{2+} channels or the release of intracellular Ca^{2+}.

The *Drosophila* genome does not encode a P2X2 receptor homolog and previous studies suggest that there are no acute behavioral or physiologic effects of ATP in the absence of transgenic P2X2 (Lima and Miesenböck, 2005; Littleton and Ganetzky, 2000). Nevertheless, it is still possible that bath-applied ATP might have previously uncharacterized effects on the physiology of fly neurons, possibly through effects on the conserved ATP sensitive K^+ channel (Kim and Rulifson, 2004), or might have effects on properties of the genetically encoded sensors themselves (Willemse et al., 2007). We therefore treated brains expressing UAS-GCaMP3.0 in the absence of transgenic P2X2 expression with 30-s perfusions of 2.5 mM ATP to determine if ATP had measurable effects on GCaMP3.0, which is directly proportional to Ca^{2+}. The 30-s perfusions of 2.5 mM ATP resulted in very small but consistent transient decreases in GCaMP3.0 fluorescence relative to vehicle controls (Fig. 2.2, G and H). We therefore conclude that bath-applied ATP has only small and easily accounted for effects on GCaMP3.0 fluorescence.

Taken together, these these results indicate that deeply situated P2X2 expressing neurons can be excited by the controlled perfusion of ATP across the explanted brain in a manner compatible with high-resolution GCaMP3.0 live-imaging within single neuronal somata. Furthermore, the absence of ATP excitation in neurons

lacking transgenic P2X2 expression confirms that, as expected from previous work (Lima and Miesenböck, 2005), ATP did not excite the s-LN$_v$s in the absence of specifically directed P2X2 expression and had only minor effects on genetically encoded sensors.

Bath-applied ATP reliably and repeatedly activates P2X2-expressing neurons of the adult brain.

Having acquired the genetic elements necessary for dual binary control of P2X2 and sensor expression, we sought to determine the most reliable means of exciting deep brain neurons expressing UAS-P2X2 and LexAop-P2X2 elements using bath-applied ATP. We first imaged the somata of three different classes of neuron co-expressing P2X2 and GCaMP3.0: the s-LN$_v$s and DN1p clock neurons [using Pdf(bmrj)-GAL4 and Clock(4.1M)-Gal4, respectively] and the olfactory projection neurons [PNs; using Cha(7.4)-Gal4] and compared the effects of 30-s perfusions of a range of ATP concentrations on GCaMP3.0 fluorescence (Fig. 2.3 A). For all three neuron types, 30-s perfusions of 0.1 mM ATP had no significant effects on GCaMP3.0 fluorescence. Higher concentrations resulted in dose-dependent increases in Ca^{2+} responses, with the s-LNvs and DN1ps displaying sigmoidal response curves and the PNs (the most superficial of the neurons tested) displaying a biphasic response curve with diminished response magnitudes at doses > 5 mM (Fig. 2.3 A). We also compared the effects of these ATP concentrations between s-LN$_v$s expressing GCaMP3.0 and P2X2 using either the GAL4 or LexA expression system. The LexA-expressing s-LN$_v$s displayed significant GCaMP3.0 responses over the same range of ATP concentrations as their

GAL4-expressing counterparts, but did so with lower response amplitudes, most likely

due to lower levels of transgene expression (Fig. 2.3 E). Nevertheless, the LexA-

expressing s-LN$_v$s displayed maximum fluorescence changes (ΔF /F0) approaching

100%, amplitudes on par with the GCaMP3.0 responses displayed by fly sensory

neurons subjected to acute sensory excitation (Tian et al., 2009). As shown in Fig. 4, C

and D, the excitatory responses of single P2X2-expressing neurons to a series of

increasing ATP doses were proportional to the concentration of perfused ATP. Thus,

the excitatory responses of single neurons can be controlled through the manipulation

of ATP dose, thereby making it possible to excite neurons at a range of intensities.

Our results suggest that 30-s perfusions of 1-5 mM ATP result in significant

neuronal excitation for all three neuron types we tested. To gauge the reliability of such

ATP/P2X2 excitation we analyzed how often each of these 30-s ATP treatments failed

to excite the P2X2 expressing s-LN$_v$s, DN1p s, and PNs. We defined a failure

conservatively as any ATP treated neuron displaying less than a 25% maximal increase

in GCaMP3.0 fluorescence. For all three neuron types, failure rates were < 50% for 1

mM ATP perfusions and approached zero at higher concentrations (Fig. 2.3 B). Our

choice of 30-s perfusions was based on previous experiments involving the bath

application of neurotransmitters and receptor agonists (Lelito and Shafer, 2012). We

wondered if shorter applications of ATP might still yield sufficient excitation of the s-

LN$_v$s, the most deeply situated of the neurons tested, using both the LexA and Gal4

expression systems. We therefore determined the failure rates for various durations of

2.5 mM ATP for s-LN$_v$s coexpressing GCaMP3.0 and P2X2 with either LexA or Gal4

drivers. For perfusion durations of 10 to 20 s, failure rates for both genotypes were all near 30%. Failure rates reached zero at perfusion durations of 25 s for LexA s-LN$_v$s and at 30 s for GAL4 s-LN$_v$s (Fig. 2.3 F).

The ability to excite the same set of P2X2-expressing neurons repeatedly would allow for multiple sets of putative follower neurons residing in different focal planes to be investigated in the same brain preparation. We therefore asked if P2X2-mediated excitation by bath-applied ATP could be used to repeatedly stimulate deep brain neurons. Indeed, repeated 30-s perfusions of 2.5 mM ATP resulted in reliable repeated excitation of s-LN$_v$s expressing either GAL4- or LexA-driven P2X2 (Fig. 2.3, G and H). Although the baseline fluorescence of these neurons displayed a slow and steady drop in intensity, there was no significant difference in the mean maximum GCaMP3.0 fluorescence increases displayed in response to the first and last (fifth) 30-s perfusion of ATP, when compared with the baseline fluorescence preceding each ATP pulse. For repeated excitation using the GAL4 system to co-express GCaMP3.0 and P2X2 expression (Fig. 4G), the first ATP perfusion caused a mean maximum GCaMP3.0 increase of 126.6 ± 32.9% and the fifth and final pulse caused a mean increase of 114.5 ± 21.9% (P = 0.8438 by Mann-Whitney U test). For repeated excitation using the LexA system (Fig. 2.3H) the first ATP perfusion caused a mean maximum GCaMP3.0 increase of 145.3 ± 19.1% and final pulse caused a mean increase of 94.1 ± 18.8% (P = 0.0524 by Mann-Whitney U test). Thus, P2X2-expressing neurons can be repeatedly activated in the same dissected brain without a significant rundown in excitation.

Based on these results, we conclude that 30-s perfusions of 1-5 mM ATP result in robust, reliable, and repeatable excitation of deep brain P2X2-expressing neurons, using either the GAL4 or LexA expression system to drive the expression of P2X2. However, we note that different neuronal types display differing profiles of excitation, indicating that specific excitation parameters should be determined empirically for every neuron class and genotype to be excited.

Discussion

Animal behavior is an emergent property of neural networks and is shaped by the pattern and nature of the connections between their constituent neurons. Connectivity is therefore an abiding problem in neuroscience, and understanding how it governs complex behavior is a fundamental goal of the field (Lichtman and Sanes, 2008). Here we have introduced a method for addressing the physiologic connections between discrete neuronal classes in Drosophila. We have shown that the dual binary expression of the vertebrate purinergic P2X2 receptor and genetically encoded sensors makes possible the specific excitation of neuronal classes of interest while simultaneously imaging Ca^{2+} dynamics.

Although the method we introduce here makes possible the detection of neural connections in regions of the brain where multi-electrode electrophysiological experiments are not possible, it is important to note its limitations relative to electrophysiological techniques. For example, the use of bath-applied ATP to excite P2X2-expressing neurons does not offer the fine temporal control associated with the

depolarization of neurons by brief current injections (Fig. 2.1). Likewise, genetically

encoded sensors of neural signaling have not yet attained the sensitivity and temporal

resolution of electrodes for detecting small changes in membrane voltage or modest

excitatory/inhibitory responses. Thus, connections producing subthreshold excitatory

input or only very weak excitation in follower neurons might be missed using the

approach we have described. Furthermore, some inhibitory connections may not be

detectable using existing genetically encoded sensors (Lelito and Shafer, 2012). Thus,

for any pair of neuronal classes, the absence of Ca^{2+} responses in a putative follower

neuron is not in itself compelling evidence for a complete lack of connection.

The ultimate cellular resolution afforded by this approach is currently limited by

the number of available highly specific LexA and Gal4 drivers for directed P2X2

expression. This is no less true for the widespread use of these same drivers for the

experimental manipulation of neuronal function and behavior, a limitation that has not

prevented the field from learning a great deal about the neuronal classes underlying a

wide range of behaviors (Simpson, 2009). Nevertheless, the current supply of specific

drivers allows for many hypothesized connections between neuronal classes to be

experimentally tested using the approach we have described, and the production of

highly specific genetic drivers continues apace (Bohm et al., 2010; Luan and White,

2007; Pfeiffer et al., 2008, 2010). Furthermore, in instances when sufficiently specific

drivers prove unattainable, increased specificity of ATP/P2X2 excitation can be realized

through localized puffing of ATP (Hu et al., 2010; Huang et al., 2010) or through the

focal liberation of caged ATP using focused laser light (Z. Yao and O.T. Shafer,

unpublished observations).

The methods described here allow for connections between discrete neuronal classes to be detected and characterized, however they do not currently allow for a differentiation between monosynaptic (direct) and polysynaptic (indirect) connections. This limitation does not preclude the usefulness of the technique, which can nevertheless reveal the presence and physiologic nature of connections between defined neuronal classes, whether monosynaptic or polysynaptic. Furthermore, it may be possible in the future to adapt established pharmacologic methods for determining if a given downstream response to ATP/P2X2 excitation is monosynaptic or polysynaptic. For example, the use of bathing saline containing high concentrations of divalent cations (Kandel et al., 1967) or tetrodotoxin (Mizunami, 1990) to block the synaptic release from or the firing of interposed neurons could be compatible with this technique if P2X2, a nonselective cation channel, can drive sufficiently high Ca^{2+} in the presynaptic terminals of P2X2-expressing neurons in the presence of these manipulations. We are currently investigating these possibilities in multiple neuronal types.

Although other methods to detect physiologic connectivity have recently been used in the fly brain (Hu et al., 2010; Ruta et al., 2010), we feel that the approach outlined here has the virtue of a relative technical simplicity, requiring only standard confocal or epifluorescent microscopy and a means of delivering controlled perfusions of ATP solutions. Thus, the LexAop-driven P2X2 and GCaMP3.0 elements we describe here, in combination with the large number of available Gal4, UAS, and LexA elements,

constitute a flexible and technically facile toolkit for the interrogation of central neuronal networks in the fly. These tools can now be used to address functional connectivity within neuronal networks governing a wide range of behaviors in *Drosophila.* Furthermore, *Drosophila* photoreceptors and ligand-gated receptors have been successfully introduced into mammalian neurons (Morita et al., 2006; Zemelman et al., 2002), suggesting that an approach similar to the one described here using appropriate heterologous receptors could be used to investigate the physiologic connections between neuronal ensembles within other model systems.

Methods

Fly stocks and rearing. Flies were reared on cornmeal-yeast-sucrose media at 25°C under a 12:12 light:dark cycle or under the diurnal conditions of the lab. All Gal4 and UAS lines used in this study have been previously described: Pdf(M)-Gal4;; and ;Pdf(bmrj)-Gal4; (Renn et al., 1999), ;UAS-GCaMP3.0; (Tian et al., 2009), ;;UAS-P2X2 (Lima and Miesenböck, 2005), ;;Clock(4.1M)-Gal4 (Zhang et al., 2010, 2010), ;Clock (856[8.2/2])-Gal4; (Gummadova et al., 2009), ;c929-Gal4; (Hewes et al., 2000),;Cha(7.4)-Gal4/CyO; (Salvaterra and Kitamoto, 2001). The ;Pdf-LexA; line has also been described previously (Shang et al., 2008). The creation of the LexAop P2X2 and Lex-Aop-GCaMP3.0 lines is described in the following text. Stable lines carrying combinations of these elements were created using standard *Drosophila* genetic techniques.

Creation of LexAop P2X2 and sensor lines. We used the LexA response element

containing pLOT vector (Lai and Lee, 2006) for the creation of LexAop-GCaM3.0 and LexAop-P2X2 plasmids. GCaMP3.0 (Tian et al., 2009) was obtained in a pEGFP-N1 vector from Addgene (Cambridge, MA; plasmid #22692) and digested with EagI. The resulting GCaMP3.0-containing fragment was gel purified, digested with BglII, and subsequently PCR purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). In parallel, pLOT vector was digested with EagI and BglII, and treated with CIP alkaline phosphatase (New England Biolabs, Ipswich, MA) following manufacturer's instructions. The GCaMP3.0 fragment was ligated with the linearized pLOT vector with a Quick Ligation Kit from New England Biolabs. The P2X2 trimer (Lima and Miesenböck, 2005) was obtained as the Gateway entry clone pENTRA1_P2X2 from G. Miesenböck (Oxford University). We created a pLOT Gateway vector by cutting pLOT with KPN1, generating blunt ends using T4 DNA Polymerase (Invitrogen), and inserting the chloramphenicol/ccdB-resistant Gateway cassette A using T4 DNA Ligase following manufacturer's instructions (Invitrogen). We transformed OmniMAX 2T1R cells (Invitrogen) with the resulting pLOT-Gateway vector, selected ampicillin- and chloramphenicol-resistant clones for vector propagation, and purified the pLOT Gateway vector using a Qiagen Mini Prep kit (Qiagen). The transfer of the P2X2 trimer from pENTRA1_P2X2 to the pLOTGateway vector was accomplished via LR recombination reaction according to manufacturer's instructions (Invitrogen) using LR II clonase (Invitrogen).

All LexAop plasmids were extracted and purified using a Qiagen Mini Prep kit. Purified plasmids were sent to Genetic Services, Inc. (Cambridge, MA), where they

were injected into w1118 embryos. We isolated and mapped several independent

transgenic lines for each LexAop element using standard fly genetic techniques. The

specific lines used here were: w;LexAop-GCaMP3.0(4B);, w;LexAop-P2X2(7);, and

w;;LexAop-P2X2(1).

Dissections, solutions, and test compound delivery. Flies were anesthetized on CO_2

and brains were dissected into room temperature hemolymph-like saline (HL3)

consisting of (in mM): 70 NaCl, 5 KCl, 1.5 $CaCl_2$, 20 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose,

115 sucrose, 5HEPES; pH 7.1 (Stewart et al., 1994). For larval brain dissections, third

instar (non-wandering) larvae were removed from the food and brains were dissected

directly into HL3, keeping the eye disks and ventral nerve cord intact. Mouth hooks

continued to move after dissections and were therefore removed to prevent brain

movement during imaging experiments. All brains were allowed to adhere to the bottom

of 35-mm FALCON culture dishes (Becton Dickenson Labware, Franklin Lakes, NJ)

under a drop of HL3 contained within a petri dish insert (Bioscience Tools, San Diego,

CA) for directing perfusion flow. Brains were imaged 5 to 10 min after dissection to allow

for optimum baseline stabilization and settling of the brain to the dish. Perfusion flow

was established over the brain with a gravity-fed PS-8H perfusion system (Bioscience

Tools). Test compounds were delivered to mounted brains by switching perfusion flow

from the main HL3 line to another channel containing diluted compound for desired

durations followed by a return to HL3 flow. All test compounds were dissolved in HL3.

To control for the effects of switching channels, we perfused HL3 for 30 s from a second

vehicle channel as a vehicle control. Adenosine 5[prime]-triphosphate disodium salt

hydrate (ATP) and guanosine 5[prime]-triphosphate disodium salt hydrate (GTP) were purchased from Sigma-Aldrich (St. Louis, MO).

Live imaging and analysis. Live imaging was performed using an Olympus FV1000 laser-scanning microscope (Olympus, Center Valley, PA) under a X20 (0.50 N/A W, UMPlan FL N) or X60 (1.10 N/A W, FUMFL N) objective (Olympus, Center Valley, PA). Regions of interest (ROIs) were selected over single neuronal somata. For GCaMP3.0 imaging experiments, frames were scanned with a 488-nm laser at 1-10 Hz for 5 min and GCaMP emission was directed to a photomultiplier tube by means of a DM405/488 dichroic mirror. Scanning frequencies for GCaMP3.0 imaging were kept constant within experiments, but varied between experiments. Experiments involving multiple neuronal classes demanded larger scanning areas and therefore lower scan rates. For each neuron within an optical section, ROIs were drawn over somata using Fluoview software (Olympus). Raw intensity values for GCaMP3.0 emission was recorded as mean pixel intensities (value range: 0-4,095) for each ROI at each time point and exported from Fluoview. Data transformations (see details in the following text) were conducted using custom software developed in Matlab (The MathWorks, Natick MA). For GCaMP3.0 experiments, raw intensity traces were filtered with a 10-point moving average to remove high-frequency noise and then normalized to percentage fluorescence changes (ΔF/F0) using the following equation:

$$((F_n - F_0) / F_0) * 100\%$$

where F_n is a raw intensity value recorded at each point in time and F_0 is the baseline fluorescence value, calculated from the average of the raw intensity values in the first 10 s of recording from each trace. Maximum GCaMP3.0 fluorescence change values (max $\Delta F/F_0$) were determined as the maximum percentage change observed for each trace over the entire duration of each imaging experiment. Maximum values for each treatment and genotype were averaged to calculate the mean maximum change from baseline.

To statistically compare maximum changes in GCaMP3.0 fluorescence between the vehicle and test compounds, we used a Kruskal-Wallis one-way ANOVA with a Dunn's multiple comparison test. Pairwise comparisons of maximum changes in GCaMP3.0 fluorescence in response to test compound or vehicle perfusion were made using the Mann-Whitney U test. All plots and statistical tests were generated and performed using Prism 5 (GraphPad, San Diego CA). Figures were constructed in Adobe Illustrator and Photoshop (Adobe Systems, San Jose, CA). To obtain intensity mapped images representing select time points before, during, and after ATP/P2X2 stimulation, single frames were captured from intensity-mapped still images using Fluoview. These images were imported to Photoshop (Adobe Systems, San Diego CA), and trimmed to size.
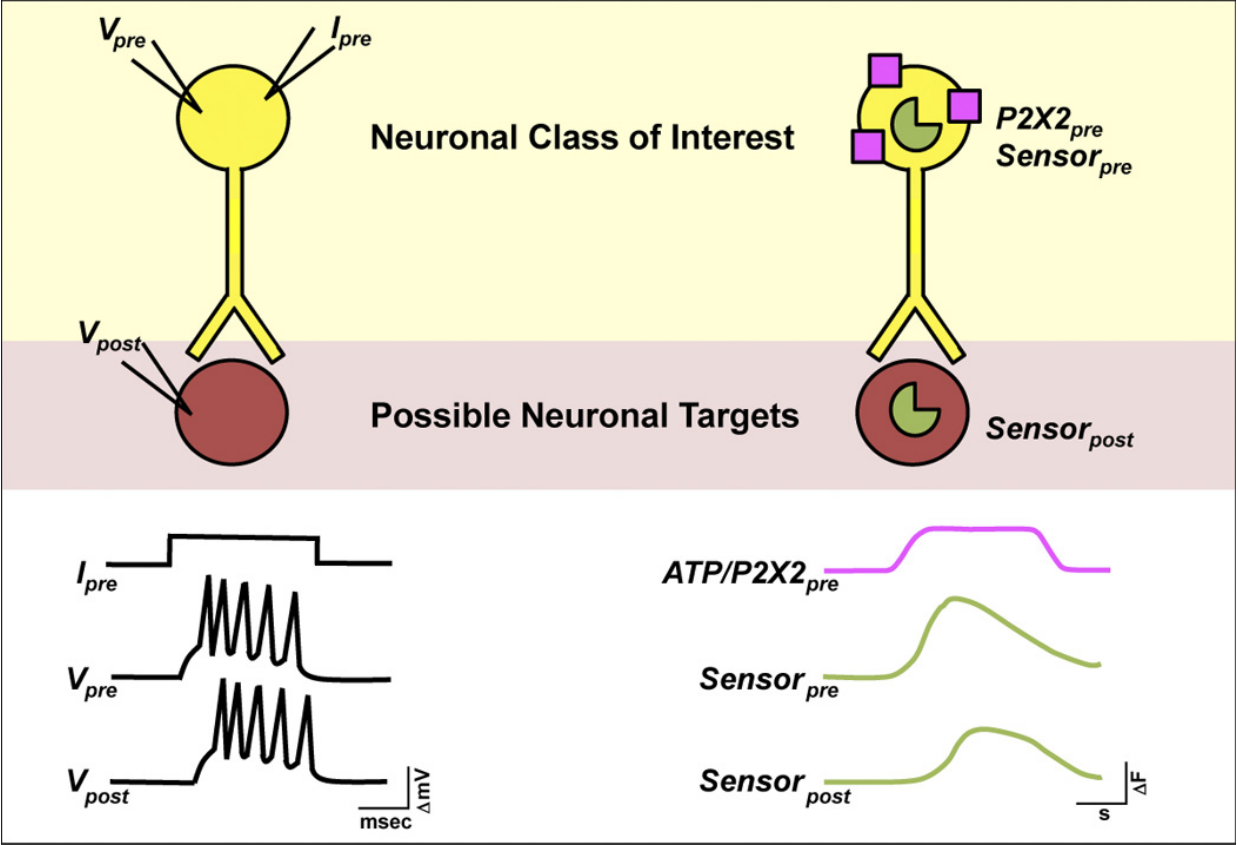
Figure 2.1. Schematic of dual binary, ATP/P2X2 excitation approach to network interrogation (figure from Yao et al., 2012). Left: an electrophysiological approach to connectivity in invertebrate nervous systems. The investigator stimulates a neuron of interest with depolarizing current while simultaneously recording membrane voltage in putative follower neurons (Kandel et al., 1967). Right: a physiogenetic approach to connectivity in the *Drosophila* nervous system. Depolarizing current is induced in neuronal classes of interest through ATP gating of transgenic P2X2 receptors (shown in purple), whereas Ca^{2+} is simultaneously monitored in putative follower neurons using genetically encoded sensors (shown in green). Note the differing time scales between methods.

A Ca²⁺

Veh

B

1 mM ATP

C

2.5 mM ATP   100% ΔF/F₀   30s

D   100% ΔF/F₀

2.5 mM GTP   30s

E

F   2.5 mM ATP

0 s   40 s   100 s   2,000 1,000 0

Pdf-GAL4;UAS-GCaMP3.0/+;UAS-P2X2/+

G Ca²⁺

Veh

H   40% ΔF/F₀

2.5 mM ATP   30s

Pdf-GAL4;UAS-GCaMP3.0/+;

52
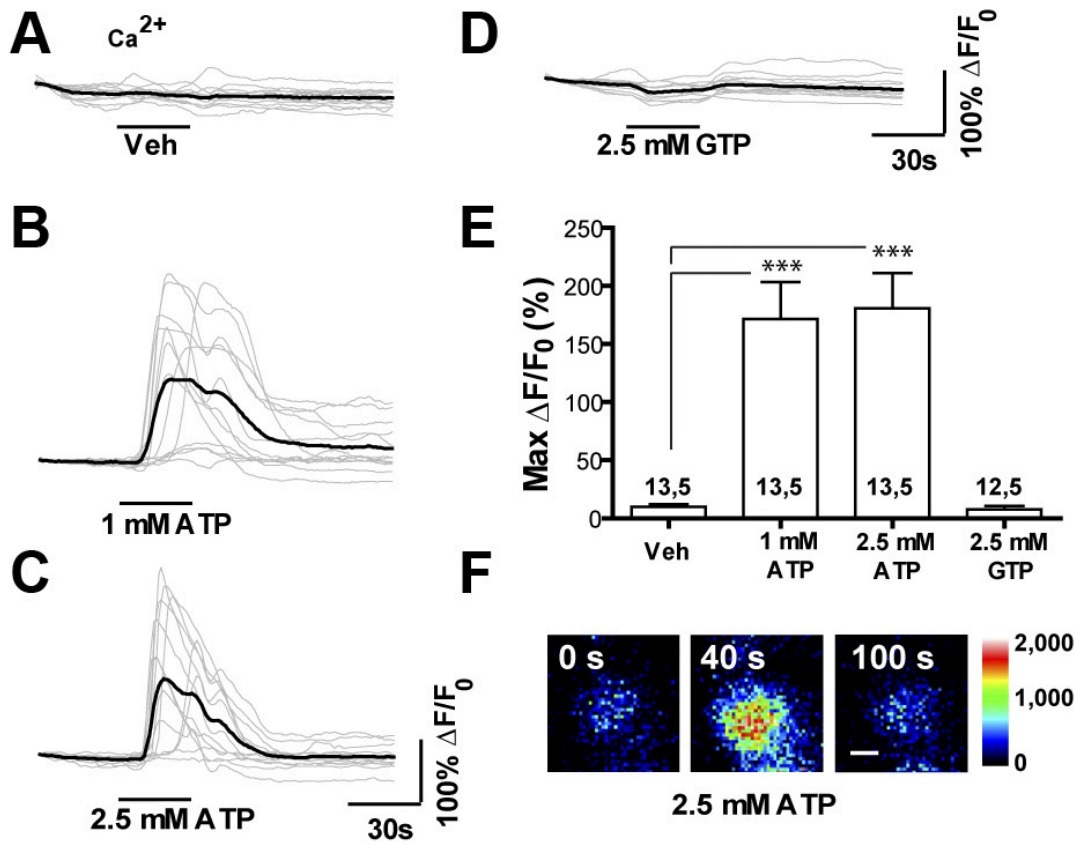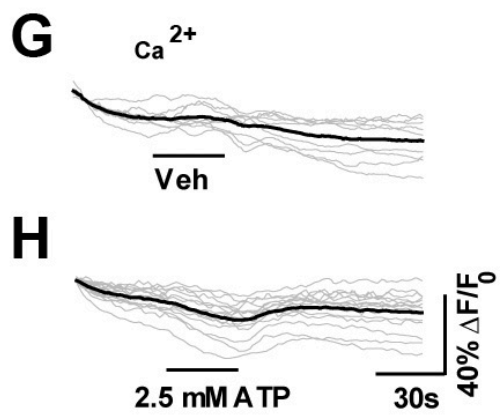
Figure 2.2. Bath application of ATP results in the excitation of P2X2-expressing deep brain neurons during live imaging experiments (figure from Yao et al., 2012). A–D: individual (gray) and mean (black) traces of Pdf(M)-Gal4;UAS-GCaMP3.0/+;UAS-P2X2/+ s-LN$_v$ responses to 30-s perfusion of (A) vehicle (N = 13 neurons from 5 brains [13,5]), (B) 1 mM ATP (N = 13,5) (C) 2.5 mM ATP (N = 13,5), and (D) 2.5 mM GTP (N = 12,5). Test compounds were perfused after a 35-s baseline interval and responses were recorded for a total of 150 s. E: histogram summarizing the mean maximum percentage increase in GCaMP3.0 fluorescence displayed by the neurons plotted in A-D. Perfusion of 1 and 2.5 mM ATP caused fluorescence increases that were significantly greater than vehicle control (P = 0.0001, by Mann-Whitney U test). The perfusion of 2.5 mM GTP did not cause significant fluorescence increases relative to the vehicle control (P = 0.6302 by Mann-Whitney U test). The two numbers displayed within or above each bar of the histogram indicate the number of neurons and the number of brains examined, respectively. F: representative intensity mapped micrographs of a single Pdf(M)-Gal4;UAS GCaMP3.0/+;UASP2X2/+ s-LN$_v$ before (0 s), during (40 s), and after (100 s) its response to bath-applied 2.5 mM ATP. The scale bar in F = 2.5 µm. G and H: characterization of ATP's P2X2-independent effects on GCaMP3.0 fluorescence: unlike vehicle perfusion (G), 30-s 2.5 mM ATP perfusion (H) caused a slight but consistent decrease in GCaMP3.0 fluorescence.
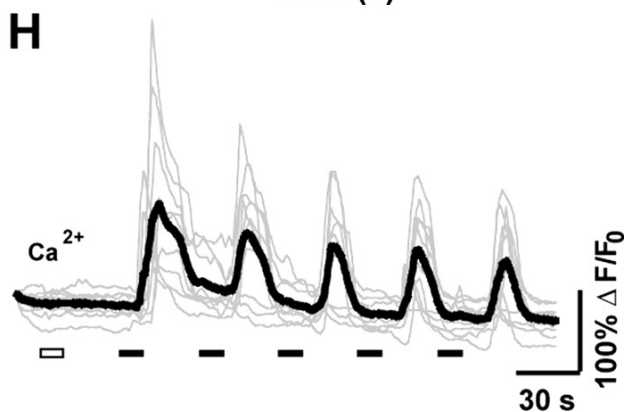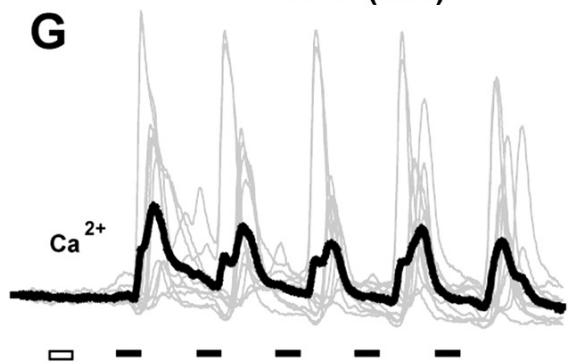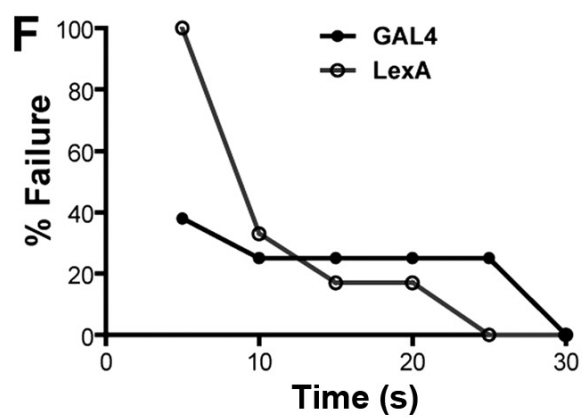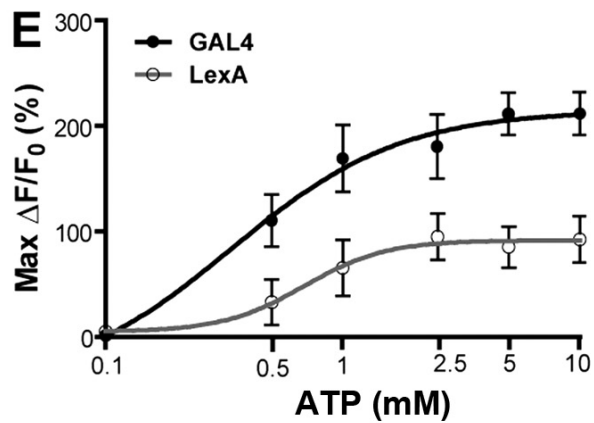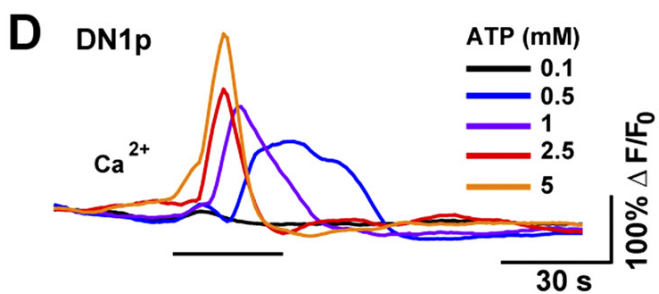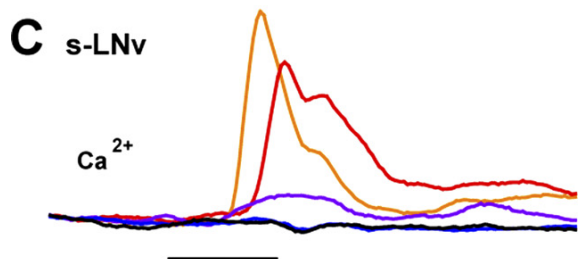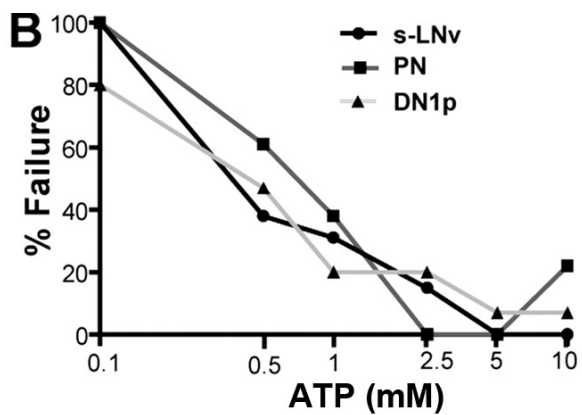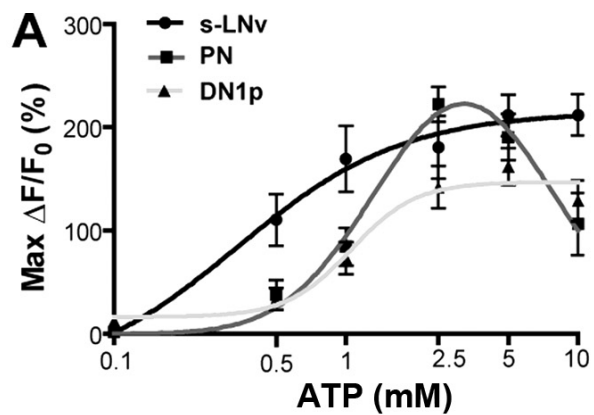
Figure 2.3. Bath-applied ATP reliably and repeatedly activates deeply situated P2X2-expressing neurons in the explanted adult brain (figure from Yao et al., 2012). A: dose-response curves for the excitation of P2X2-expressing s-LN$_v$s (N = 13,5), DN1ps (N = 15,5), and olfactory projection neurons (PN, N = 18,5)) by 30-s perfusions of ATP. The genotypes used for each neuronal class where Pdf-Gal4;UAS-GCaMP3.0/+;UAS-P2X2/+ for s-LN$_v$s,;UAS-GCaMP3.0/+; Clock(4.1M)-Gal4/UAS-P2X2 for DN1ps, and ;Cha(7.4)-Gal4/UAS-GCaMP3.0;UAS-P2X2/+ for PNs. Values represent the mean maximum increase in GCaMP3.0 fluorescence ($\Delta F/F_0$) detected during the 150 s following ATP perfusion. B: failure rate curves for 30-s ATP perfusions over a range of concentrations for s-LN$_v$s, DN1ps, and PNs based on the data shown in A. A maximum GCaMP3.0 fluorescence increase of < 25% was considered a failure to excite. C and D: GCaMP3.0 responses of a single s-LN$_v$ (C) and DN1p (D) cell body to increasing ATP concentrations (0.1-5 mM), each delivered for 30 s. Single neurons displayed graded responses to increasingATP doses. E: dose-response curves for s-LN$_v$ excitation in response to 30-s ATP perfusions comparing s-LN$_v$s from Pdf-Gal4;UAS-GCaMP3.0/+;UAS-P2X2/+ (N = 13,5) and ;Pdf-LexA,LexAop-GCaMP3.0/LexAop-P2X2; (N = 10,5) brains. F: failure rates of s-LN$_v$ excitation by various durations of 2.5 mM ATP perfusions comparing s-LN$_v$s excited using the GAL4 (N = 8,5) and LexA (N = 10,5) systems. Genotypes were identical to those used in E. G: individual (gray) and mean (black) GCaMP3.0 traces for repeatedly activated s-LNvs from Pdf-Gal4;UAS-GCaMP3.0/+;UAS-P2X2 brains (N = 11,5). H: individual (gray) and mean (black) GCaMP3.0 traces for repeatedly activated s-LNvs from Pdf-LexA,LexAop-GCaMP3.0/LexAopP2X2 brains (N = 10,5). For G and H the

white rectangles indicate 30 s of vehicle perfusion and black rectangles indicate 30 s

of 2.5 mM ATP perfusion, with 90-s intervals between ATP perfusions.

CHAPTER 3

Sensory-input-specific plasticity in developing *Drosophila* nociceptive circuit is established through modulation of serotonergic feedforward inhibition

Abstract

Sensory experience shapes neural circuit development. How experience during development causes long-lasting changes in sensory circuits and impacts behaviors in the mature animal are poorly understood. Here we establish a novel system for mechanistic analyses of the developmental plasticity of neural circuits. We show that sensory experience during development alters nociceptive behavior and circuit physiology in *Drosophila melanogaster*. Despite the convergence of nociceptive and mechanosensory inputs on common target neurons, developmental noxious input only modifies the transmission from nociceptors to their postsynaptic targets, suggesting a striking input-specificity. Excessive stimulation of nociceptors during development activates serotonergic neurons to sensitize nociceptor presynaptic terminals to serotoninergic inhibition, leading to abated nociceptive behavior. This is the first demonstration that sensory experience modulates synaptic physiology and behavior in *Drosophila* to adapt to excessive noxious stimuli. The findings suggest a novel

mechanism that enables pathway-specific plasticity in primary sensory afferents by using a common neuromodulator.

Introduction

The genome directs the wiring of neural circuits so animals can deal with the common experience of the species. Yet, to deal with the uncertainties individual animals encounter, the nervous system maintains the capacity to modify its circuitry based on the sensory experience of the animal. This can be achieved in mature as well as in developing animals. In fact, sensory experience during the development of animals often has a unique and profound impact on behaviors of mature animals (Hubel and Wiesel, 1970). Classic work in vertebrates, particularly those on the visual and somatosensory systems (Hubel and Wiesel, 1970), have demonstrated that the experience-dependent plasticity of certain aspects of neural circuitry occurs only in developing, but not in mature, animals, suggesting that developmental plasticity involves unique regulatory programs.

Hebbian mechanisms, in which concurrent activation of pre- and post-synapses results in modification of only those synapses (Hebb, 1949), are important in establishing developmental plasticity, including the ocular dominance in the visual cortex (Hubel and Wiesel, 1970). A potential problem in Hebbian plasticity is that strengthened synapses are likely to cause unconstrained potentiation in the circuit, breaking down the specificity of the plasticity (Pozo and Goda, 2010; Turrigiano, 2008). This destabilizing force may be counteracted by homeostatic mechanisms, which restore baseline function of the circuit (Blankenship et al., 2009; Davis, 2013). In fact,

recent studies show that occular dominance plasticity involves a combination of

Hebbian and homeostatic mechanisms (Kaneko et al., 2008; Mrsic-Flogel et al., 2007).

Whether or not the interaction between Hebbian and homeostatic plasticity is a general

mechanism that underlies developmental plasticity remains to be determined.

Due to its relatively simple nervous system and the amenability to circuit

manipulations and genetic analyses, *Drosophila* is potentially a good model for

discovering principles in developmental plasticity. Work on neuromuscular junctions

(NMJ) of *Drosophila* larvae have provided critical insights into mechanisms that underlie

activity-dependent plasticity during development (Griffith and Budnik, 2006; Menon et

al., 2013; Ruiz-Canada and Budnik, 2006). However, while the NMJ is an excellent

model for synaptic development and plasticity, additional systems are needed for

understanding the plasticity of neural circuits in the central nervous system (CNS),

which involves interneurons and exhibit convergence and divergence in information

flow, and for elucidating the impact of developmental plasticity on behaviors. In the

CNS of developing *Drosophila*, changing neuronal activity affects the morphology and

projections of neurons. For example, changing activity levels of larval nociceptors alters

the topographic arrangement of the axon terminals of these sensory neurons (Yang et

al., 2014). However, whether or not these alterations in neuronal morphology and wiring

are important for behaviors is unknown. In the larval visual system, prolonged light

exposure results in shortened dendrites and reduced calcium responses in neurons

postsynaptic to photoreceptors, whereas reduced exposure results in the opposite

structural and functional modifications, suggesting input-dependent changes in the set

point in homeostasis of the circuit (Yuan 2011). Despite this progress in our understanding of the mechanisms underlying developmental plasticity of neural circuits, how experience-dependent changes in a circuit are formed, without affecting other functions of the nervous system, and how the synaptic changes lead to alterations in behaviors remain poorly understood. This knowledge gap has motivated us to develop a system that links synaptic physiology to behavior in *Drosophila* for studying the mechanism of developmental plasticity.

Since *Drosophila melanogaster* lives in a wide range of geographic locations and variable environments (Singh et al., 1982), we reasoned that their larvae might encounter noxious stimuli, such as chemicals from plants and pesticides, intense radiation and harsh mechanical stimuli from predators, during development. These noxious cues are detected by the nociceptors, called class IV dendritic arborization (C4da) neurons, and elicit in 3rd instar larvae a stereotypical rolling of the body along the rostrocaudal axis (Hwang et al., 2007a). Direct activation of the nociceptors with channelrhodopsin (ChR2) also elicits such nociceptive responses (Robertson et al., 2013). While the morphology and functions of C4da neurons have been well characterized, the downstream neurons of the larval nociceptive circuit have just begun to be identified. An elegant study has recently determined that the C4da nociceptors directly synapse on a group of neurons, termed Basin-2 and -4 neurons, which also receive inputs from mechanosensory neurons and are involved in the multi-modal integration of both nociceptive and mechanosensory information (Ohyama et al., 2015).

This advance has opened up the opportunity to analyze the development and physiology of the nociceptive circuit.

In this study we show that the functional development of the nociceptive circuit in *Drosophila melanogaster* is modulated by sensory input. In investigating the underlying mechanism, we first identified second-order neurons that receive inputs from nociceptors, but not from mechanosensors, revealing that the larval nociceptive circuit contains both multi-modality integration and modality-specific processing. Taking advantage of this feature of the circuit, we demonstrate that developmental plasticity of the nociceptive circuit exhibits a striking degree of input-specific functional adaptation to noxious inputs, which requires the co-activation of nociceptors and feedforward inhibition of the nociceptor-to-target synaptic transmission by serotonergic interneurons. This unique mechanism enables the nervous system to maintain other serotonin-dependent functions and sensory functions of other modalities while causing a long-lasting functional change in the nociceptive circuit.

Results

Excessive noxious experience during development suppresses larval nociceptive behavior

In larvae, stimulation of the nociceptors elicits a stereotypical series of behavioral responses, which starts as body curling and is often followed by rolling the body along the rostrocaudal axis (Robertson et al., 2013) (Figure 1A). Using the nociceptive circuit

and its robust behavioral output as a model, we investigated the functional consequences of exposure to noxious stimuli during development.

Plants have developed various noxious chemical compounds to repel insects. One such chemical found in cruciferous plants, allyl-isothiocyanate (AITC), which is used as a food flavor, preservative and—at high concentration—as an insecticide (Zhang, 2010), acts through TrpA1 channels to excite the C4da nociceptors in *Drosophila* larvae (Iwasaki et al., 2008). Consistently, AITC induced C4da-dependent nociceptive behavioral responses (Figure 1B) and activated C4da neurons (Figures 1C and 1D). To test the consequences of exposure to AITC during development, we reared larvae in an environment containing 2.5 mM AITC, a concentration comparable to those in plants (Sultana et al., 2003). The nociceptive behavioral responses of mature larvae (late third-instar) were tested with optogenetic activation of C4da neurons, as described previously (Honjo et al., 2012; Robertson et al., 2013). Larvae raised on AITC exhibited a two-fold suppression in nociceptive rolling behavior (Figure 1E), suggesting that excessive noxious stimulation during development results in a suppression of nociceptive behavior.

To determine whether developmental activation of the nociceptors is sufficient for inducing the suppression of nociceptive behavior, we specifically activated C4da neurons during development using an optogenetic approach. ChR2 was specifically expressed in the C4da neurons and activated in developing larvae with brief pulses of blue light (5 sec ON followed by 5 min OFF). Red light, which does not activate ChR2,

was used as negative control. After larval development is completed 5 days after egg laying (AEL), the nociceptive behavior was tested with exposure to blue light after at least 1 hr in the dark. Rearing these larvae under pulses of blue light dramatically suppressed nociceptive response, including rolling, curling, and the overall response as indicated by changes in body angle (Figures 1F, S1C, and S1D). The ChR2-mediated suppression of nociceptive behavior is not due to the bleaching of all-trans-retinal, a key component in ChR2 function (Figures S1E and S1F). Moreover, developmental activation of the nociceptors did not change the size and targeting of their presynaptic terminals (Figure S1G). These results suggest that activating the nociceptors during development suppresses nociceptive behavior without altering nociceptor morphology.

The activity-dependent suppression of nociceptive behavior is mediated through a developmental program

This form of activity-dependent suppression of nociceptive behavior could be due to either developmental or acute changes. Moreover, it might be a result of chronic activation of nociceptors rather than a change through a developmental program.

To consider the consequences of acute stimulation, larvae were reared under constant darkness for 5 days before being illuminated with pulsed red or blue light for an hour. No significant difference was observed between the control and experimental groups (Figure 1G), suggesting that the behavioral suppression shown in our previous experiments is not due to acute changes. Next, we determined whether the activity-dependent suppression of nociceptive behavior is caused by chronic stimulation in any period of the larvae's life or by stimulation within a specific time window of the larval

development. Larvae expressing ChR2 in their nociceptors were reared in 48-hr

intervals of pulsed blue or red light on days 1 to 2, days 2 to 3, or days 3 to 4 of

development and kept in darkness otherwise (Figure 1H). Stimulating nociceptors on

days 3 and 4 suppressed the nociceptive response in mature larvae tested on day 5.

Further, nociceptor stimulation on day 4 caused a more severe reduction in nociceptive

responses than that on day 5. Specifically, the nociceptive rolling was significantly

reduced by stimulation on day 4, but not day 5, though stimulation on day 5 also led to

reduced curling (Figure 1I). These results indicate a time-window that the nociceptive

behavior is most susceptible to activity-dependent modifications during development,

and suggest that this form of plasticity is a developmental program.

The A08n neurons are specific postsynaptic targets of nociceptors

In order to identify the mechanism that underlies the developmental plasticity of larval

nociceptive behavior, it is necessary to analyze the neurons downstream of the

nociceptors. Recent advances in delineating the larval nociceptive circuit have

identified two groups of segmentally repeated neurons, termed Basin-2 and -4, as

postsynaptic targets of both nociceptors and NompC-expressing mechanosensory

neurons (Ohyama et al., 2015). In addition, a pair of neurons, called A08n, has been

identified as potentially postsynaptic to C4da neurons (Vogelstein et al., 2014) (Figure

2A). Using an improved GRASP technique (Macpherson et al., 2015), we found that

A08n dendrites are synaptic partners of C4da axon terminals but not those of

mechanosensory neurons (Figures 2B and 2C), raising the possibility the C4da-to-A08n

synaptic transmission is dedicated to the nociceptive circuit. To test this possibility, we

used calcium imaging to record nociceptor-evoked responses of A08n and Basin-4 neurons. Activation of nociceptors by AITC elicited robust responses in A08n neurons (Figure D). These AITC-elicited responses are nociceptor-dependent because no response was observed when C4da neurons were ablated or when the PNS was disconnected from the CNS. In contrast, activation of mechanosensors did not elicit any response in A08n neurons (Figure 2E), suggesting that the C4da-to-A08n transmission is dedicated to the nociceptive circuit. Consistent with the previous report that Basin-4 is postsynaptic to both nociceptors and mechanosensors (Ohyama et al., 2015), Basin-4 responded to both activation of nociceptors and that of mechanosensors (Figure 2F and 2G).

The role of A08n neurons in larval behavior has not been determined, although optogenetic stimulation of GMR82E12-GAL4-expressing neurons, which includes A08n neurons, leads to a behavior probability distribution that resembles the behavioral output caused by nociceptor activation (Vogelstein et al., 2014). Indeed, optogenetic stimulation of GMR82E12-Gal4-expressing neurons by CsChrimson dramatically enhanced curling and rolling, resembling the responses elicited by C4da activation (Figure 2H). In addition to the A08n neurons in the VNC, the GMR82E12-GAL4 driver marks some neurons in the central brain. To confirm the role of A08n neurons in nociceptive behavior, we took advantage of the FLP-out mosaic technique to express CsChrimson in both, one, or none of the two A08n neurons (Gordon and Scott, 2009; Struhl and Basler, 1993; Yang et al., 2014) (Figures 2I and S2). With no expression of CsChrimson in A08n neurons, larvae rarely exhibited nociceptive response despite

CsChrimson expression in the central brain. On the other hand, larvae expressing

CsChrimson in both A08n neurons exhibited nociceptive behavioral response at the

same level as larvae expressing CsChrimson in all GMR82E12-GAL4 neurons; larvae

expressing CsChrimson in one of the two A08n neurons responded at about half of the

level as those expressing in both neurons. Therefore, activating A08n neurons is

sufficient to elicit nociceptive behavior. We then electrically silenced the A08n neurons

by expressing the inwardly rectifying potassium channel Kir2.1 in these neurons (Baines

et al., 2001; Hodge, 2009; Johns et al., 1999) while simultaneously stimulating ChR2-

expressing C4da neurons, and recorded behavioral responses. Larvae with inhibited

A08n neurons showed a reduction in nociceptive behavior in response to C4da

activation (Figure 2J). The incomplete suppression of nociceptive behavior in the

absence of A08n activity is likely because the nociceptive circuit consists of multiple

pathways downstream of C4da nociceptors, such as the Basin-Goro pathway (Ohyama

et al., 2015). These results demonstrate that A08n neurons mediate nociceptive

behavior.

The developmental plasticity of larval nociceptive circuit is input-specific
We took advantage of the multi-modal inputs to Basin-4 and the mono-modal input to

A08n to study the specificity of the developmental plasticity of larval nociceptive circuit.

Nociceptors were stimulated by either ChR2-mediated optogenetics in developing

larvae, followed by acute AITC stimulation in mature larval preparations for calcium

imaging (Figure 3A), or AITC during development, followed by acute ATP-induced $P2X_2$

activation (Yao, 2012) for calcium imaging (Figures 3B, 3C and 3D). Responses to

C4da stimulation were significantly reduced in both A08n and Basin-4 neurons (Figures

3A, 3B, 3C, S3A, and S3B). In contrast, the Basin-4 response to mechanosensor

stimulation was not affected by enhanced noxious inputs during development (Figure

3D), suggesting that nociceptive inputs during development specifically altered the

nociceptive, but not mechanosensory, circuit.

Because of the input-specificity, we further investigated the mechanism that

underlies the experience-dependent plasticity by focusing on the synaptic transmission

from nociceptors to the A08n neurons, which is separated from mechanosensory inputs.

The A08n response to nociceptor stimulation in mature larvae was significantly reduced

by the C4da activation on days 3 and 4 of development, but not by that on days 1 and 2

(Figures 3E and 3F), which is consistent with the behavioral output we observed

(Figures 1H and 1I). Furthermore, the suppression of C4da-to-A08n transmission by

developmental stimulation was not caused by changes in sensory transduction in the

nociceptors, because no change in calcium response in either C4da somata or their

axon terminals was observed (Figures S3C and S3D). The results presented thus far

suggest that excessive activation of nociceptors during development selectively

suppresses the synaptic transmission from the nociceptors to their target neurons

(Figure 3G).

Serotonergic neurons are required for the establishment of the developmental plasticity

of larval nociceptive behavior

Next, we set out to determine how the developmental experience-dependent

suppression of nociceptive behavior is mediated. The serotonergic system is involved

in synaptic and behavioral plasticity of the mature nervous system in various species

including mammals (Lesch and Waider, 2012), *Aplysia* (Kandel, 2001)*, and *C. elegans*

(Zhang et al., 2005). In *Drosophila* larvae, the processes of serotonergic neurons,

which specifically expresses tryptophan hydroxylase (TRH) (Huser et al., 2012), are

near the C4da axon terminals (Figure 4A), raising the possibility that serotonin

modulates synaptic transmission from C4da neurons to their targets. Stimulating

nociceptors activates the serotonergic neurons in the larval VNC (Figures 4B and 4C),

suggesting that developmental stimulation of nociceptors likely causes enhanced

serotonergic activities. Electrically silencing serotonergic neurons dis-inhibited the

nociceptive behavioral responses in larvae whose nociceptors were stimulated during

development (Figures 4D and 4E). These results suggest that serotonergic neurons are

required for establishing the developmental plasticity of the larval nociceptive circuit.

Serotonergic neurons modulate the nociceptor-to-target transmission in mature larvae

by providing feedforward inhibition

In order to understand how serotonergic neurons contribute to the developmental

plasticity, we first investigated how serotonin modulates the nociceptive circuit in mature

larvae. We found that ipsapirone, an agonist of the vertebrate 5-HT1a receptor (Glaser

and Traber, 1985; Maj et al., 1987; Traber et al., 1984), inhibited C4da-to-A08n

transmission (Figures 5A and 5B). 5-HT1a receptor is a G-protein-coupled receptor

(GPCR) that downregulates cyclic AMP (cAMP) levels in cells (Raymond et al., 1999).

We observed that ipsapirone reduced cAMP levels in C4da presynaptic terminals (Figure 5C), suggesting the presence of serotonergic modulation of these terminals. There are three known families of serotonin receptors in *Drosophila*: 5-HT1 (including 5-HT1a and 1b), 5-HT2, and 5-HT7. The specificity of ipsapirone to these receptors is unknown. Because the 5-HT1a and 1b are similarly homologous to the mammalian 5-HT1a receptors (Saudou et al., 1992), ipsapirone may act as an agonist to both 5-HT1a and 5-HT1b. Thus, these results suggest that in mature larvae, activating 5-HT1 receptors (5-HT1R) reduces sensory-input-elicited cAMP responses in the nociceptor presynaptic terminals and inhibits the nociceptor-to-target transmission. Together with the finding that stimulating nociceptors activates serotonergic neurons (Figures 4B and 4C), these results suggest that in mature larvae serotonergic neurons modulate the nociceptor-to-target transmission by providing feedforward inhibition (Figure 5D).

To determine the type and location of the 5-HT1R involved, we used RNAi to knock down either 5-HT1a or 5-HT1b in C4da neurons and examined the effect on the ipsapirone-induced inhibition of the C4da-to-A08n transmission. Knockdown of 5-HT1b, but not 5HT-1a, in the C4da neurons reduced the inhibition of C4da-to-A08n transmission by ipsapirone (Figure 5E). In contrast, knockdown of either 5-HT1a or 5-HT1b in the A08n neurons had no effect (Figure 5F). These results suggest that 5-HT1b in C4da axon terminals mediates the inhibition of nociceptor-to-A08n transmission by 5-HT.

Developmental stimulation of nociceptors sensitizes nociceptor presynaptic terminals to

5-HT receptor-mediated inhibition

Next, we investigated how developmental stimulation of nociceptors affect the

serotonergic modulation of C4da-to-target transmission. The C4da-elicited responses

of serotonergic neurons were unchanged by developmental stimulation of the

nociceptors (Figure 6A). We thus tested the hypothesis that developmental stimulation

of nociceptors enhances the 5-HT1R agonist-induced inhibition of the C4da-to-A08n

transmission. One µM ipsapirone, which only partially inhibited C4da-to-A08n

transmission when nociceptors were not activated during development, reached the

maximum inhibition after developmental stimulation of nociceptors (Figure 6B),

indicating enhanced sensitivity of the C4da-to-A08n synaptic transmission to ipsapirone.

Furthermore, knocking down 5-HT1b, but not 5-HT1a, in C4da neurons reduced the

inhibition of C4da-to-A08n transmission by developmental stimulation of nociceptors

(Figure 6C). Taken together, these results suggest that excessive activity of the

nociceptors during development enhanced 5-HT1R-mediated inhibition of C4da-to-A08

transmission. Consistent with this notion, developmental stimulation of nociceptors

significantly reduced the sensory-induced increase in cAMP levels in C4da axon

terminals (Figures 6D and 6E) without affecting the basal cAMP levels (Figure 6F).

Discussion

We define here a mechanistic model that explains the experience-dependent

developmental plasticity (Figure 6G). In mature larvae, 5-HT inhibits cAMP levels in

nociceptor presynaptic terminals and suppresses the nociceptor-to-target synaptic

transmission. During a specific period in development, excessive stimulation of the

nociceptors activates serotonergic neurons to sensitize the presynaptic terminals of

these sensory neurons to serotonin. This developmental modification enhances the

responsiveness of nociceptor presynaptic terminals to 5-HT modulation in mature

larvae, leading to further suppression of cAMP production and abated nociceptive

behavioral responses. This study therefore reveals a striking input-specificity in the

developmental plasticity of larval nociceptive circuit and a role for serotonergic

interneurons in establishing this unique form of plasticity.

A novel mechanism that underlies input-specificity in developmental plasticity

Our results demonstrate an input-specificity in the plasticity of the *Drosophila*

nociceptive circuit. Although the mechanosensory and nociceptive pathways converge

on shared target neurons (e.g., Basin-4) to integrate inputs from two different sensory

modalities (Ohyama et al., 2015), developmental noxious input only modifies the

nociceptor-to-target transmission. Our results further suggest that the serotonergic

system plays a role in both the establishment and expression of this form of plasticity.

Considering the broad distribution of serotonergic neurites (Figure 4A) and the wide

diffusion range of serotonin (Bunin and Wightman, 1999), it is conceivable that 5-HT

reaches both the presynaptic terminals of mechanosensors and those of nociceptors,

which are immediately adjacent to each other (Grueber et al., 2007). Yet, no change

was observed in the mechanosensor-to-target transmission after developmental

stimulation of nociceptors (Figure 3D). These findings suggest that both serotonergic

neurons and nociceptors need to be activated to enhance the inhibition of nociceptor

presynaptic terminals and establish the developmental plasticity of the nociceptive

circuit. This is consistent with Hebb's postulate on synaptic plasticity, which proposes

that concurrent activation of pre- and post-synapses results in modification of only those

synapses (Hebb, 1949). In this case, Hebbian plasticity strengthens the connection

between serotonergic neurons and nociceptor presynaptic terminals, resulting in

enhanced inhibition of the nociceptor-to-target transmission.

A unique aspect of this form of plasticity is that it is mediated through a group of

interneurons (i.e., the serotonergic neurons) that—like the second-order neurons in the

circuit—also receive inputs from the nociceptors. This is different from the serotonergic

facilitation that occurs during the sensitization of gill-withdrawal reflex in *Aplysia*, where

the serotonergic neurons are activated by another modality (Kandel, 2001). This makes

sense because the developmental plasticity described here is not a form of associative

learning. The co-activation of the second-order neurons in the sensory circuit and the

modulatory interneurons—which provide a feedforward control—forms a circuit motif for

experience-dependent plasticity, which allows the establishment of long-lasting changes

in the nociceptive circuit while maintaining normal functions of other sensory modalities.

Moreover, the nociceptive circuit establishes the plasticity by sensitizing the nociceptors

to serotonin, rather than enhancing the activity of serotonergic neurons (Figure 6A),

which allows the nervous system to maintain other serotonin-dependent functions.

Interestingly, inhibitory local interneurons have also been suggested to play a

role in establishing activity-dependent plasticity in the olfactory system in adult

Drosophila (Sachse et al., 2007). The present study has raised the possibility that

feedforward inhibition from those interneurons generate input-specific plasticity in the

olfactory system.

Use-dependent strengthening of serotonergic modulation in developmental plasticity

At the core of the circuit and behavioral plasticity observed in this study is the

use-dependent strengthening of serotonergic modulation. Noxious input during

development likely enhances serotonin release by activating serotonergic neurons and

leads to excessive activation of serotonin receptors in the nociceptor presynaptic

terminals, which in turn sensitizes these presynaptic terminals to serotonergic

modulation. The enhanced sensitivity can be achieved by: a) an increase in the

expression level of serotonin receptors in the presynaptic terminals, b) modification of a

signaling transduction pathway leading to reduced presynaptic neurotransmitter

release—which would be a novel form of synaptic scaling, or c) modification of the

function of the serotonin transporter in the presynaptic terminals (Fabre et al., 2000).

The present study has laid the foundation for future studies on the molecular pathways

underlying the developmental plasticity.

A sensitive period in activity-dependent plasticity of developing nociceptive circuit in the
larva

The full-blown nociceptive responses gradually emerge during development

(Sulkowski et al., 2011). Although such responses are less frequent in 2nd instar larvae

and absent in 1st instar larvae, partial responses start to occur in 1st instar larvae. We

show that the larval experience during the 2nd and early 3rd instar larval stages has the larger impact on the nociceptor function than any other developmental periods. Coincidentally, this is when the topographic projections of the nociceptors are established in an activity-dependent manner (Yang et al., 2014). This suggests that the developmental noxious stimulation becomes more effective when the presynaptic terminals complete their projections and reach their ultimate destinations.

The development of the serotonergic system might be essential for opening the sensitive period for plasticity. The 2nd instar larval stage is when serotonin starts to have an auto-regulatory effect on serotonergic innervation (Sykes and Condron, 2005). In rat visual cortex, a decrease in LTP during critical period correlates with a transient increase in 5-HT levels (Edagawa et al., 2001; Kim et al., 2006). Thus, it is possible that the sensitive period in *Drosophila* larvae is the time when nociceptors efficiently activate serotonergic neurons to enhance serotonin secretion. The larval nociceptive circuit offers a new system for investigating the mechanism that underlies the sensitive period of developmental plasticity.

Ethological significance of developmental plasticity in the nociceptive circuit

Developmental plasticity of the nervous system allows animals to adjust their behaviors for fitness to a unique, yet relatively stable, environment. As nociceptive behavior is disruptive to baseline functions of the nervous system, it is conceivable that developing in an environment in which there is excessive noxious sensory input requires the animals to suppress nociceptive circuit function. The identification of a

nociceptive circuit-specific developmental plasticity suggests a neural mechanism for survivability in the presence of noxious environmental factors, such as noxious heat and plant-derived chemicals. This type of behavioral modulation may be useful in the natural environment as *Drosophila melanogaster* lives in a wide range of geographic locations and variable environments (Singh et al., 1982). Moreover, some of noxious chemicals have anti-microbial properties (Zhang, 2010) and could potentially protect *Drosophila* from bacteria or deleterious fungi growing in rotten fruits. Finally, our data show that exposure to low levels of the insecticide, AITC, results in modulation of insect behavior, indicating potential unintentional ecological consequences of insecticide off-target effects.

Methods

Fly strains.   GAL4/LexA stocks: GMR82E12-GAL4 (Bloomington stock center, stock number B-40153) (Vogelstein et al., 2014), GMR82E12-lexA (B-54417) (Vogelstein et al., 2014), *ppk*-GAL4 (Grueber et al., 2007), *ppk*-LexA (Gou et al., 2014), *nompC*-GAL4 (B-36369), *nompC*-LexA (B-52241), GMR57F07-GAL4 (B-46389) (Ohyama et al., 2015), *TRH*-GAL4 (B-38389), *TRH*-LexA (B-52248).

UAS/LexAop stocks: UAS-CD4-GFP (BL-35836), UAS-GCaMP6f (Chen, 2013), LexAop-GCaMP6f (Chen, 2013) (B-44277), UAS-Epac1-camps (Shafer et al., 2008), UAS-ChR2::YFP (Honjo et al., 2012), UAS-CsChrimson::Venus (B-55139), LexAop-P2X2 (Yao, 2012), UAS-DenMark (B-33062) (Nicolai et al., 2010), UAS-syt-HA(Robinson et al., 2002), UAS-Kir2.1(Baines et al., 2001; Nitabach et al., 2002),

LexAop-kir2.1 (Prieto-Godino et al., 2012), UAS-syb::spGFP[1-10] (Macpherson et al., 2015), LexAop-CD4::spGFP[11](Macpherson et al., 2015), UAS-RNAi-5-HT1b (B-33418), UAS-RNAi-5-HT1a (25834).

The FLP-out Gal80 technique was used to express CsChrimson::Venus, DenMark, and Syt-HA in single neurons (Gordon and Scott, 2009), and a multicolor FLP-out technique was used to label single C4da, A08n, or serotonergic neurons with fluorescent proteins (Yang et al., 2014).

To make the *ppk*-ChR2::YFP transgenic flies, ChR2-YFP cDNA was amplified from UAS-ChR2::YFP transgenic flies by PCR, cloned into the pBluscript-ENTR-Topo vector, and inserted via Gateway cloning into the pDEST-APPHIH vector in between the attR1 and attR2 sites, which are downstream of the 1-Kb promoter of *ppk* gene. The resultant plasmid, pDEST-APPHIH-ChR2 were then used for transgenesis with the ϕC31 system.

Ppk-reaper was used to ablate C4da neurons (Xiang et al., 2010). TRH-Gal4 and UAS-Kir2.1 were used to electrically silence serotonergic neurons. GMR82E12-LexA and LexAop-kir2.1 were used to silence A08n neurons.

Behavioral tests. Embryos were collected for 6-12 hours at 25 °C on basic fly food laced with 100 mM ATR and allowed to develop. Larvae were raised on food throughout development and were placed either under red/blue lights, constant

darkness, or with additional compounds within the food (2.5 mM AITC). Control and experimental groups are paired in all behavioral tests. Mature (wandering 3rd instar) larvae were transferred to room temperature, removed from food, rinsed, and left in dark for an hour before being tested for behavioral responses. Behavioral tests were done on $\varnothing$35 mm grape-agar plates covered with 1 ml water in a dark room.

For optogenetic stimulations, a light intensity of 26 µW/mm^2 were used. LED stimulation was for 5 seconds. Nociceptors were optogenetically activated by *ppk*-GAL4/UAS-ChR2::YFP, *ppk*-ChR2::YFP, or *ppk*-GAL4/UAS-CsChrimson::Venus, as indicated in the figures. For AITC assays, 1 ml of 100 mM AITC (Tocris) solution was placed on the grape-agar plate, and larvae were then submerged into the solution. AITC stimulation was for 1 minute.

Automatic optogenetic stimulation (26 µW/mm^2) of nociceptors during development was achieved by a custom-made array of LEDs. The illumination duration and frequency were controlled by a BASIC Stamp microcontroller and the intensity by a PWM Dimmer. The LEDs (CREE XP-E Blue 3W LED, CREE XP-E Red LED) were mounted on aluminum heatsinks (RapidLED). For even distribution of light on the agar dish for rearing larvae, the light emitted by LED passes through an 80° CREE XP-E/XP-G lens. The developmental optogenetic stimulation was performed in a 25°C incubator. LEDs were programmed to turn on for 5 seconds every 5 minutes throughout the appropriate experimental duration. This setting was chosen because it caused the most dramatic suppression of nociceptive behavior among the settings that we tested.

Quantification of the nociceptive behavior. Rolling is counted only when a larva

exhibited at least one complete (360˚) rotation of the body along rostrocaudal axis

(Hwang et al., 2007b). Curling is defined as an acute decrease in body angle formed by

simultaneous movements of the head and tail immediately upon noxious stimulation.

The numbers of rolling and curling responses were manually counted over the 5-second

stimulation interval for optogenetic assays or over the 1-minute period of AITC

stimulation. Percent rolling and curling were the total number of rolling and curling

events that took place divided by the total number of larvae counted (Rolling x

100/Total).

Body angle was calculated as the angle between the two lines that connect the

middle point of the skeleton and each of the two end points of the skeleton. The angle

ranges between 0 and 180 with a smaller angle indicating a more curled larva. To

obtain the skeleton as well as its middle and end points, a custom ImageJ plugin was

developed to first detect and track the larvae at each frame. The contours of the

detected larvae were then smoothed using a 3x3 mean filter. Skeletonization algorithm

was applied to obtain the centerlines of the detected larvae. A longest path search was

then performed to remove the small spurs and result in a clean centerline of the animal.

The middle point of the skeleton is the point on the skeleton line with equal distances to

the two end points along the skeleton. The positions of the middle point and two end

points of the skeleton line were used for the body angle calculation as described earlier.

Calcium imaging. Calcium levels were measured by imaging with the GCaMP6f

indicator (Chen, 2013). Live-imaging was conducted on a Leica SP5 confocal

microscope equipped with a resonance scanner, an acousto-optical beam splitter, and a

HC Fluotar L 25x/0.95 W VISIR immersion objective (Leica). Larvae were dissected as

previously described (Matsunaga et al., 2013). Dissection and imaging were performed

in a modified hemolymph-like 3 (HL3) saline (70 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 20

mM $MgCl_2$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.2) (Stewart,

1994). 7 mM glutamate was added to the HL3 solution to block muscle contractions and

eliminate motor feedback to the sensory circuits by saturating glutamate receptors at

the neuromuscular junction (Macleod et al., 2004; Reiff et al., 2005; Reiff et al., 2002). A

low concentration of calcium was additionally used to reduce muscle contraction and

nerve damage during dissections (Caldwell et al., 2013).

To specifically activate the nociceptors and mechanosensors, we used the

ATP/$P2X_2$ technique (Yao, 2012). Rat ATP-sensitive cation channel $P2X_2$ was

specifically expressed in either the nociceptors or mechanosensors by using *ppk*-LexA

and *nompC*-LexA, respectively. Because there is no ATP-sensitive channels in

Drosophila, ATP application excites neurons expressing $P2X_2$ (Yao, 2012). Unless

otherwise stated, 1 mM ATP was used to activate neurons expressing $P2X_2$ and 2 mM

AITC was used as a natural stimulant to activate the C4da neurons. We used the

ValveBank®4 Pinch Valve Perfusion System (Automate Scientific) for controlling

perfusion. AITC, ipsapirone (Tocris) were bath-applied onto the specimen. Calcium

responses were recorded in xyzt mode such that an image stack was taken every 2 seconds over the course of the experiment.

After projecting the xyzt image file along the z-axis, the ImageJ software (NIH) was used to analyze the movie using the Time Series Analyzer Plugin. The average intensity of a selected Region of Interest (ROI) was measured over the course of the experiment. The change in fluorescence was calculated as follows: $\Delta F/F0 = (Ft - F0)/F0$, where Ft is the fluorescent mean value of a ROI in a given frame. F0 is the baseline 30-second interval before stimulation. Max ΔF is the maximum value during the stimulation period of the recording.

cAMP imaging. cAMP levels were measured by imaging with the Epac1-camps indicator (Shafer et al., 2008; Yao, 2012). The procedure for cAMP imaging was the same as that for Ca^{2+} imaging described above except for the following. The level of cAMP response was quantified as changes in the inverse FRET ratio, which is CFP intensity divided by YFP intensity (ΔCFP/YFP) scanned with a 458-nm confocal laser. The basal level of cAMP was quantified as YFP intensity scanned with a 458-nm normalized by that scanned with a 515-nm laser (YFP^{458}/YFP^{515}). A Z-stack was taken every 3 seconds over the course of the experiment.

Immunostaining. Third instar larvae were dissected and stained as described previously (Kim et al., 2013; Wang et al., 2013). Primary antibodies used are: mouse anti-GFP (1:100, Sigma), chicken anti-GFP (1:2500, Aves Laboratories), rabbit anti-RFP

(1:5000, Rockland), and rat anti-HA (1:250, Roche). Secondary antibodies used are

(1:500, Jackson ImmunoResearch): donkey anti-mouse Alexa Fluor 488, anti-chicken

Alexa Fluor 488, anti-chicken Alexa Fluor 647, anti-rabbit Rhodamine RX, and anti-rat

Alexa Fluor 647.

Synaptobrevin-GRASP. Synaptobrevin (syb)-GRASP was performed as described

previously (Macpherson et al., 2015). The UAS-syb::spGFP$^{1-10}$ transgene was

expressed in C4da or NompC-expressing neurons with *ppk*-Gal4 or *nompC*-Gal4,

respectively. The *LexAop*-CD4::spGFP^{11} was expressed in A08n neurons with

GMR82E12-LexA. Reconstituted GFP (i.e., GRASP) signals were detected by anti-

mouse monoclonal GFP antibody (1:100, Sigma G6539, referred to as anti-GRASP).

Syb::spGFP$^{1-10}$ was preferentially detected by anti-chicken polyclonal antibody (1:2500,

Aves Laboratories GFP-1010, referred to as anti-spGFP$^{1-10}$ (Macpherson et al., 2015)).

Statistical analysis. Statistical analysis was performed using Prism software. The

fractions of rolling and curling behavior under stimulation was analyzed using the Chi-

squared test. Calcium and cAMP imaging, and axon terminal length of single C4da

neurons was analyzed using the Mann-Whitney nonparametric t-test. For multi-group

comparison, one-way ANOVA test was used. In all figures, n.s., *, **, and *** represent

P >0.05, P < 0.05, P < 0.01, and P<0.001 respectively. Error bars are standard error of

the mean.

Figure 3.1
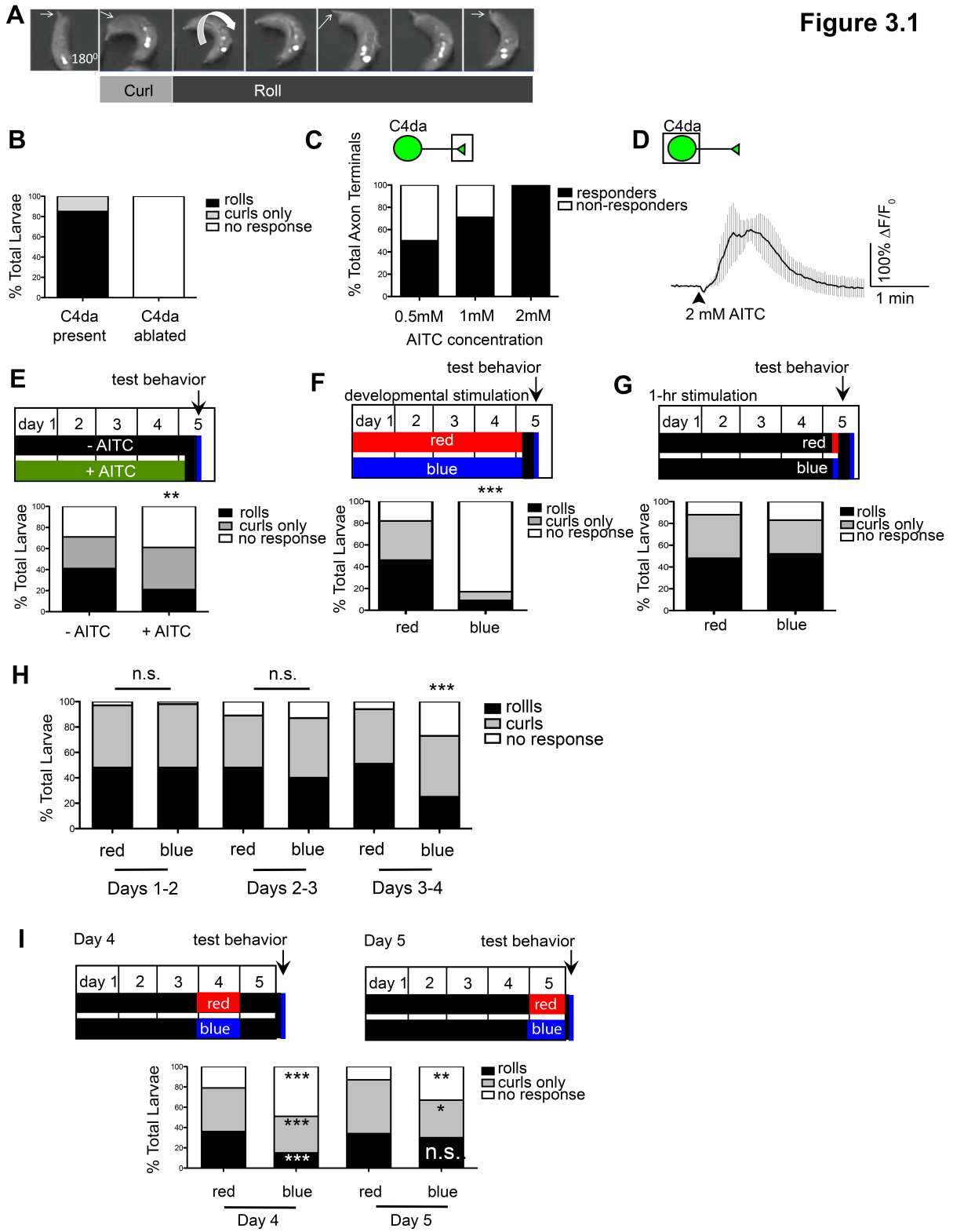
Figure 3.1. Activation of larval nociceptors during development suppresses the nociceptive behavior in mature larvae.

(**A**) A montage showing stereotypical nociceptive behavior responses, including curling and rolling, in a 3rd instar larva. ChR2 was expressed specifically in C4da nociceptors and activated by illumination of blue light. Small arrows point to the openings of dorsal trachea and the large arrow indicates the moment of rolling.

(**B**) Larvae exhibit nociceptive responses to AITC, which is dependent on C4da nociceptors. Bars represent the percentage of total larvae that performed rolling, curling only, and no response. Note that every roll starts with curling. (n = 20 larvae)

(**C** and **D**) AITC activates C4da neurons. AITC enhances calcium levels in C4da axon terminals (**C**, n=7) and cell bodies (**D**, n=3). A schematic C4da neuron is shown in each panel. The boxed areas indicate the parts imaged for calcium signals. The trace in (D) shows the average of responses. Error bars are SEM.

(**E**) Larvae raised on AITC exhibit reduced nociceptive behavior. Top panel: a diagram showing the experimental scheme. The developmental timeline of larvae is shown as days 1-5 with day 1 being the first day of development after egg laying (AEL) and day 5 being late 3rd instar. Developmental exposure to AITC was followed by acute optogenetic activation of C4da neurons to test behavioral responses. Control larvae were reared in regular food throughout development (day 1-5) (black bar), whereas experimental larvae were reared in food containing 2.5 mM AITC (green bar). The vertical blue bar indicates the blue light used in optogenetic stimulation and the vertical black bar indicates the one hour darkness prior to the behavioral tests. Throughout the paper the following color coding is used: green for AITC, black for darkness or no AITC,

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red for red light, blue for blue light, and gray for ATP. (n = 150 larvae totaling both groups)

(**F** and **G**) While developmental stimulation led to a dramatic suppression of nociceptive behavior, acute stimulation (1 hr) had no effect. **(F)** (n = 85 larvae total) **(G)** (n = 120 larvae total)

(**H**) The 2nd instar and early 3rd instar larval stages (days 3-4 AEL) are the sensitive period for activity-dependent modification of larval nociceptive behavior. Larvae were tested for nociceptive response after ChR2-mediated activation of C4da neurons during days 1-2 (left), days 2-3 (middle), or days 3-4 (right).

(**I**) The nociceptive behavior is more susceptible to activity-dependent modifications on day 4 AEL than on day 5 AEL. (n = 135 total larvae day 4, n = 120 total larvae day 5)

Figure 3.2

A

VNC

A

P

● nociceptor axon terminals
● A08n neurons

B

CD4-GFP   DenMark, Syt

C

Basin-4 > CD4::spGFP^{11}

| GRASP signals | UAS-syb::spGFP$^{1-10}$ | merge |

ppk-Gal4

NompC-Gal4

A08n > CD4::spGFP^{11}

| GRASP signals | UAS-syb::spGFP$^{1-10}$ | merge |

ppk-Gal4

NompC-Gal4

D

C4da → A08n

30% $\Delta F/F_0$

30 s

AITC

— C4da present
— C4da ablated
— PNS severed

E

C4da → Basin 4

60% $\Delta F/F_0$

1 min

AITC

F

NompC → A08n

40% $\Delta F/F_0$

1 min

vehicle   ATP

G

NompC → Basin 4

40% $\Delta F/F_0$

1 min

ATP

H

% Total larvae

no GAL4   C4da   A08n

■ rolls
■ curls only
□ no response

I

VNC

% Larvae showing nociceptive responses

n = 1/59   n = 23/61   n = 28/35

0   1   2
A08n expressing CsChrimson

J

test behavior

| day 1 | 2 | 3 | 4 | 5 |

+ A08n
− A08n

% Total larvae

**

+ A08n   − A08n

■ rolls
■ curls only
□ no response

Figure 3.2. The A08n neurons are specific postsynaptic targets of nociceptors.

(**A**) Schematic of the axon projections of nociceptors and the neurites of A08n within the VNC. A: anterior; P: posterior.

(**B**) The majority of A08n neurites are dendrites. A single A08n neuron labeled by the FLP-out technique. The soma (arrowhead) of the A08n neuron is located near the posterior end of the VNC. The A08n neurites in close proximity with the C4da axon terminals are mostly positive for the dendrite marker, DenMark, and contain scattered spots that are positive for the axonal marker, Synaptotagmin (Syt). Scale bar: 10 µm.

(**C**) A08n neurons are synaptic partners of C4da, but not NompC-expressing mechanosensory, neurons. The synaptobrevin (syb)-GRASP technique was used to detect synaptic connections between neurons. SpGFP[11] expressed in Basin-4 neurons produces GRASP signals (green) with spGFP[1-10] (magenta) expressed in either nociceptors (*ppk*-GAL4) or mechanosensors (*NompC*-GAL4). In contrast, spGFP[11] expressed in A08n neurons only produces GRASP signals with spGFP[1-10] in nociceptors. Scale bar: 10 µm.

(**D**) AITC activates A08n neurons specifically through C4da nociceptors, as shown by calcium imaging. A schematic is included to show the pre- and post-synaptic neurons. The neuron that was recorded by GCaMP calcium imaging is shown in green. The traces show the averages of responses. Error bars are SEM. No response was observed in the absence of C4da neurons ("C4da ablated") or with the severing of nerves connecting the VNC with the PNS ("PNS severed"). (n = 3)

(**E**) Stimulating NompC-expressing mechanosensors does not activate A08n neurons. Mechanosensors expressing P2X$_2$ were stimulated by 1 mM ATP for Ca^{2+} imaging. (n = 10 neurons, 5 brains)

(**F**) Stimulating C4da nociceptors activates Basin-4 neurons. Note that AITC does not activate NompC-expressing mechanosensory neurons (data not shown). (n = 9 neurons, 5 brains)

(**G**) Stimulating NompC-expressing mechanosensors activates Basin-4 neurons. (n = 8 neurons, 4 brains)

(**H**) Activation of C4da or A08n neurons by CsChrimson elicits nociceptive response. (n > 60)

(**I**) Larvae expressing CsChrimson in one or two A08n neurons exhibit an increase in nociceptive behavior, which includes both curling and rolling behavior, as compared to larvae that do not express CsChrimson in either A08n neurons. (n > 60)

(**J**) Larvae with silenced A08n neurons show a significant reduction in nociceptive response. (n > 60)

Figure 3.3
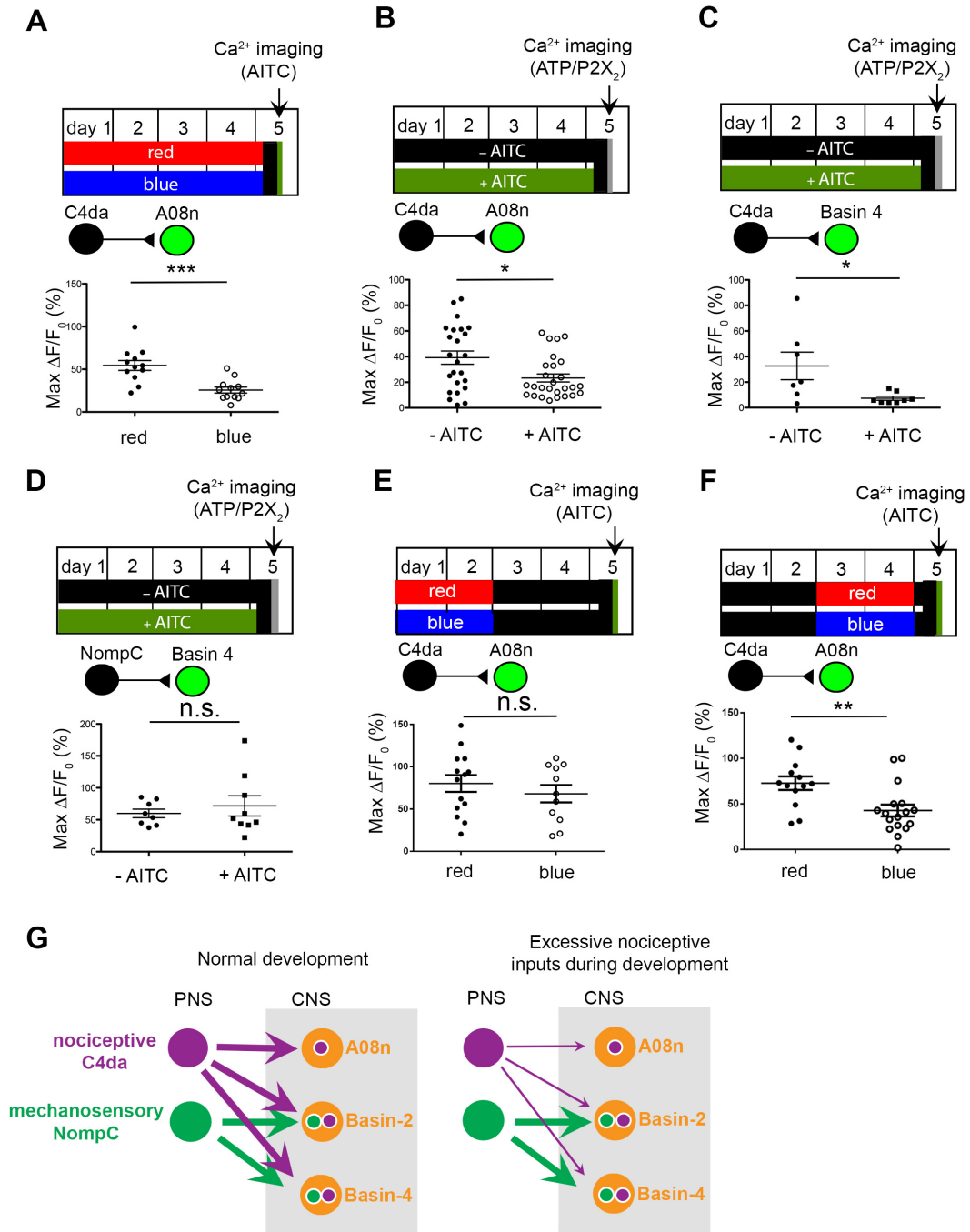
Figure 3.3. The developmental plasticity of larval nociceptive circuit is input-specific.

(**A**) Optogenetic activation of C4da neurons during development inhibits the C4da-to-A08n transmission. C4da neurons in mature larvae were stimulated by AITC for Ca^{2+} imaging. (n = 12 neurons, 6 brains per group)

(**B**) Stimulation of C4da neurons by AITC during development inhibits the C4da-to-A08n transmission. C4da neurons in mature larvae were stimulated by $P2X_2$ for Ca^{2+} imaging. (n = 24, 27 neurons, 13 brains per group)

(**C**) Stimulation of C4da neurons by AITC during development inhibits the C4da-to-Basin4 transmission. (n = 7, 8 neurons, 4 brains per group)

(**D**) Treating larvae with AITC during development does not alter the mechanosensor-to-Basin4 transmission. (n = 8, 9 neurons, 5 brains per group)

(**E & F**) C4da activation during day 3-4 AEL of larval development results in reduction in C4da-to-A08n transmission (**F**) (n = 13, 17 neurons, 9 brains) while that during day 1-2 AEL has no effect (**E**) (n = 14, 11 neurons, 7 brains per group).

(**G**) Schematics showing the input-specificity of developmental plasticity in the synaptic connections between larval nociceptive and the second-order neurons in the circuit. Left: circuit diagram of connections under normal developmental conditions; right: excessive nociceptive input specifically suppresses the synaptic transmission from the nociceptors to the second-order neurons.

Figure 3.4

A

Red & Green: TRH; Blue: C4da axon terminals

B

C4da   TRH

30% ΔF/F$_0$

4 min

0.5   1   2

ATP concentration (mM)

C

Peak frequency (Hz)

ATP concentration (mM)

D

test behavior

| day 1 | 2 | 3 | 4 | 5 |

TRH

TRH + Kir2.1

% Total Larvae

- rolls
- curls only
- no response

TRH   TRH + Kir2.1

E

Δ Body Angle (Degrees)

TRH   TRH + Kir2.1
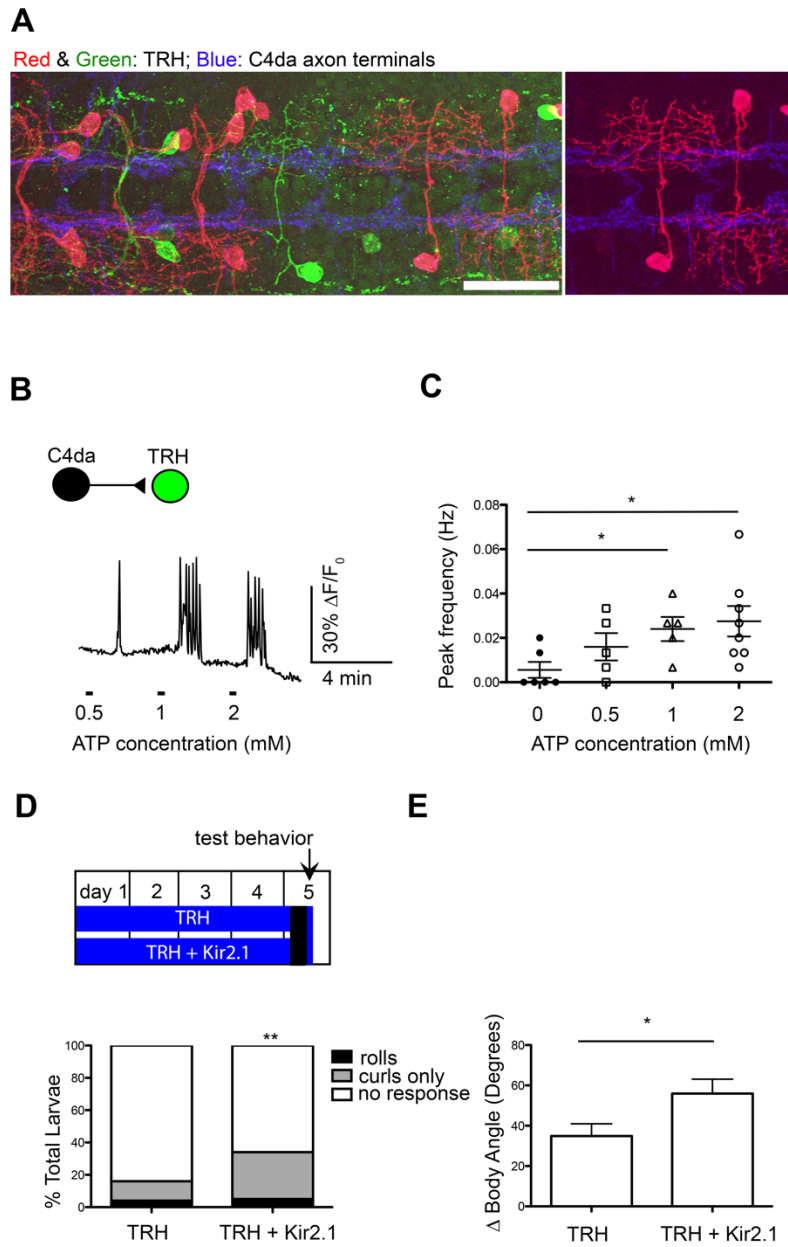
Figure 3.4. Serotonergic neurons are required for establishing the developmental plasticity of larval nociceptive circuit.

(**A**) Projections of serotonergic neurons overlap with C4da axon terminals. Serotonergic neurons (TRH), which were labeled by a multicolor FLP-out technique, are shown in red and green, and C4da axon terminals are shown in blue. Scale bar: 25 μm.

(**B** & **C**) Stimulating C4da neurons activates serotonergic neurons. (**B**) A representative trace showing that enhancing C4da activity levels via $P2X_2$ (by increasing ATP concentrations) increases the frequency of calcium peaks in TRH neurons. (**C**) Quantification of calcium peak frequency in TRH neurons. (n = 5-7 neurons, 4 brains per group).

(**D** & **E**) Electrically silencing TRH neurons significantly dis-inhibited the nociceptive behavioral responses in larvae whose nociceptors were stimulated during development. (n = 240 larvae total)

Figure 3.5
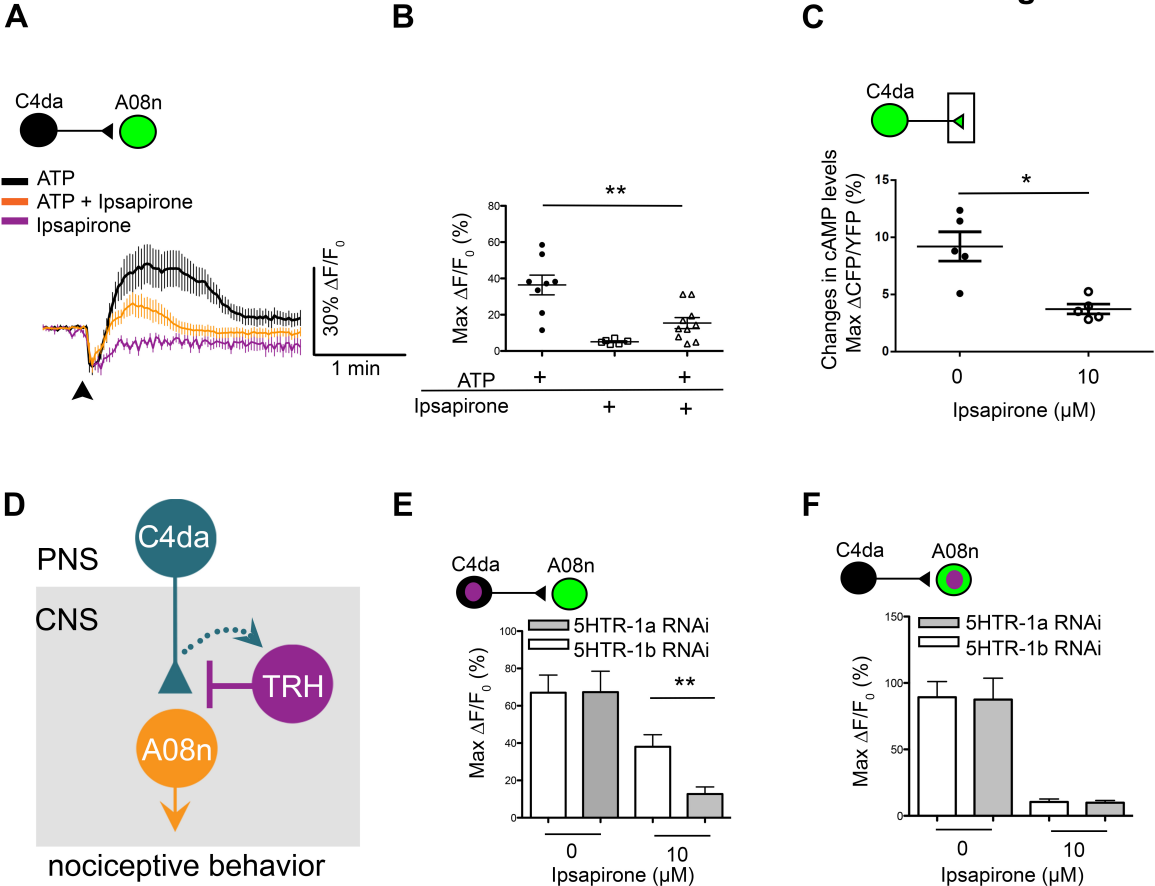
Figure 3.5. Serotoninergic neurons modulate the nociceptor-to-target transmission in mature larvae by providing feedforward inhibition

(**A** & **B**) Ipsapirone (100 μM) inhibits C4da-to-A08n synaptic transmission. ATP/P2X$_2$ was used to activate C4da neurons. (**A**) The traces show the averages of responses. Error bars are SEM. (**B**) Quantification and statistical analysis. (n = 9, 6, 10 neurons, 5 brains per group)

(**C**) Ipsapirone diminishes AITC-elicited increase in cAMP levels in C4da axon terminals. The levels of cAMP were quantified as changes in the inverse FRET ratio, which is CFP intensity divided by YFP intensity (ΔCFP/YFP) scanned with a 458-nm confocal laser.

(**D**) Schematic model of the serotonergic modulation of the nociceptor-to-target transmission.

(**E**) Knockdown of 5-HT1b in C4da neurons significantly disinhibits ipsapirone-induced suppression of A08n responses to AITC (2 mM), as compared to knockdown of 5-HT1a. (n = 26, 12, 25, 11 neurons, 13 brains for 5-HTR-1b knockdown, 7 brains for 5-HTR-1a knockdown)

(**F**) Knockdown of either 5-HT1a or 1b in A08n neurons has no effect on ipsapirone-induced inhibition of A08n responses to AITC. (n = 10, 12, 12, 9 neurons, 6 brains per group)

Figure 6

94
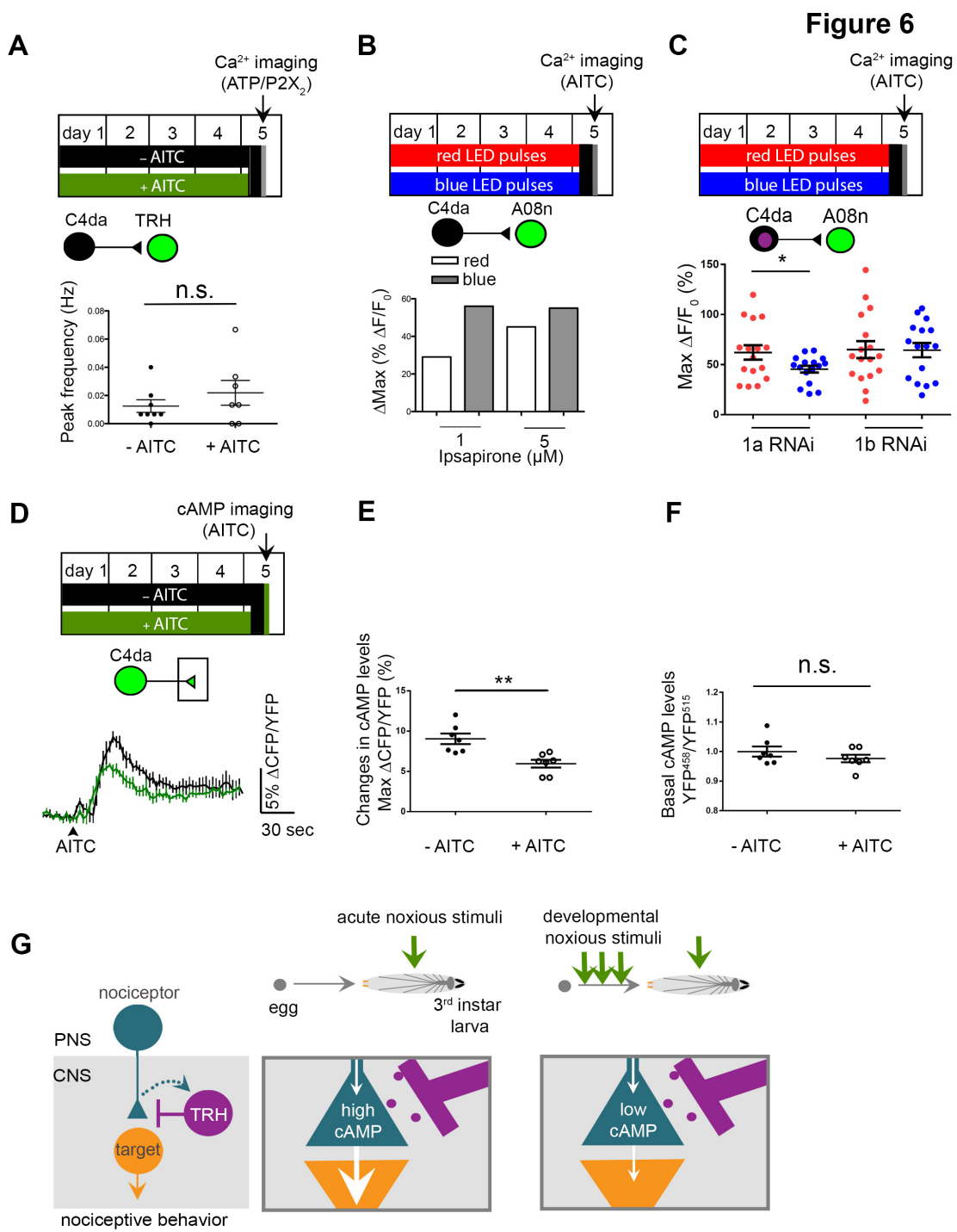
Figure 3.6. Developmental stimulation of nociceptors enhances 5-HT receptor-mediated inhibition of nociceptor-to-target transmission.

(**A**) Noxious stimulation during development does not affect nociceptor-induced responses in serotonergic neurons. (n = 8, 7 neurons, 4 brains per group)

(**B**) Developmental stimulation of C4da neurons results in a 2-fold increase in C4da-to-A08n synaptic transmission sensitivity under low ipsapirone concentrations as compared to control. The barchart depicts the change in % maximum fluorescence between AITC-alone and AITC-ipsapirone co-application. (n = 12 neurons, 6 brains per group)

(**C**) Knockdown of 5-HT1b, but not 5-HT1a, in C4da neurons rescues the inhibition of C4da-to-A08n transmission by developmental stimulation of nociceptors. n > 6 for each group

(**D** & **E**) Noxious stimulation during development significantly diminishes AITC-elicited increase in cAMP levels in C4da axon terminals without affecting the basal cAMP levels. (**D**) Average traces of cAMP responses. (**E**) Quantification and statistical analysis of cAMP levels. n > 6 for each group

(**F**) Quantification and statistical analysis of the basal cAMP levels. The value, YFP^{458}/YFP^{515}, was normalized by the average of the "-AITC" group. N=7 for each group.

(**G**) Proposed model of the experience-dependent developmental plasticity in the nociceptive circuit. Left: circuit diagram; middle: mechanism underlying acute noxious stimulation in the mature larva; right: mechanism underlying developmental noxious stimulation.

Supplemental Figure 3.1
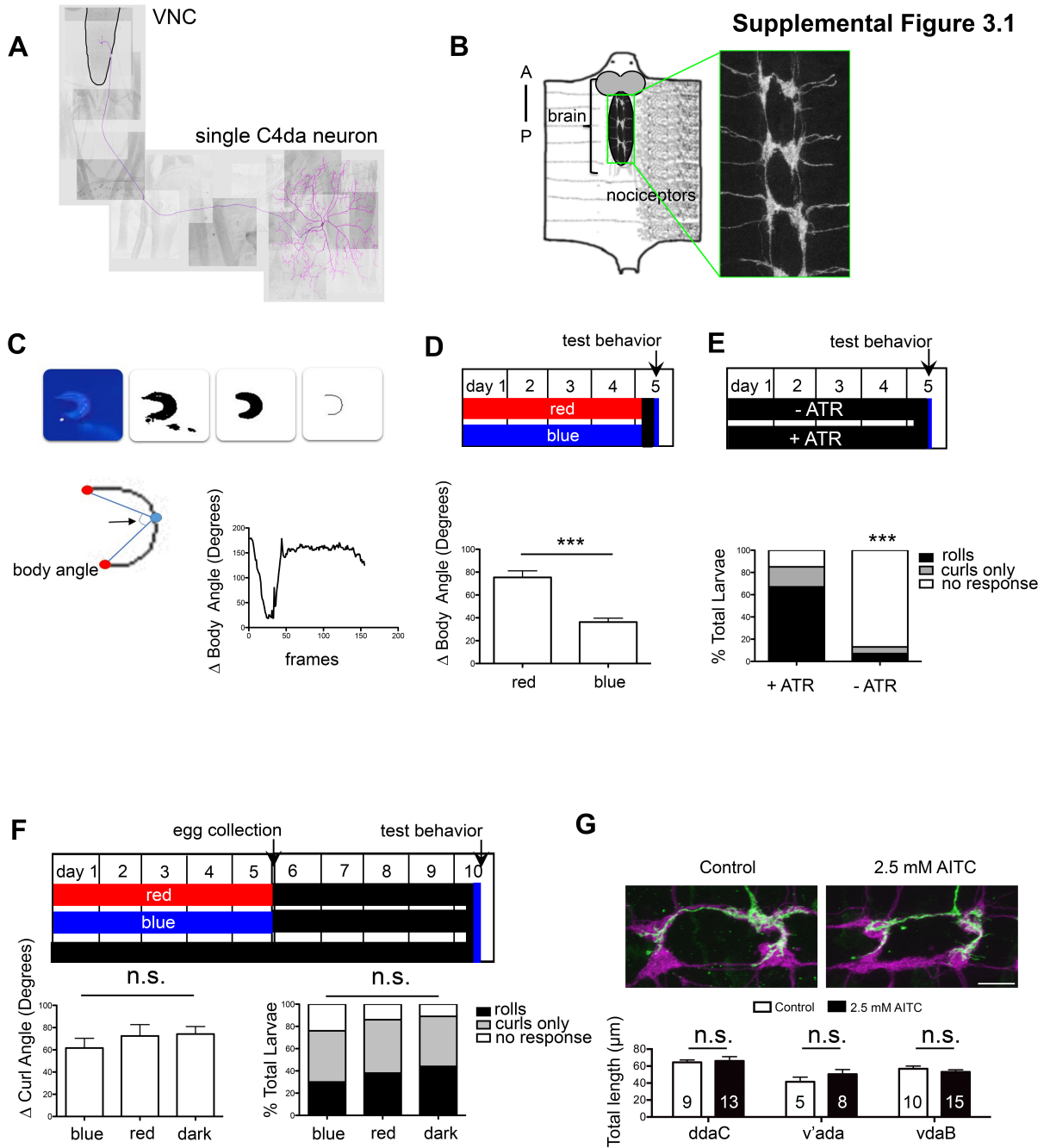
Supplemental Figure 3.1. Developmental activation of larval nociceptors suppresses nociceptive behavior without affecting the morphological development of nociceptors.

(**A**) Micrograph of a single C4da neuron labeled by a genetic mosaic technique in the 'fillet' preparation of a 3rd instar larva. C4da neurons elaborate their dendrites on the larval body wall and extend the axon into the ventral nerve chord (VNC). The micrograph is a composite of multiple images of parts of the neuron. The outline of the VNC is highlighted.

(**B**) Schematic of a larval fillet used for live-imaging studies. Compass indicates anterior (A) and posterior (P) of the larval fillet. The axon terminals of C4da neurons in several segments are magnified.

(**C**) Diagram illustrating the automatic measurement of larval body angle for quantifying curling behavior. A single larva was extracted from the video recording to obtain its skeleton using automatic image processing. The two red dots indicate the end points of the skeleton line while the blue dot is the middle point of the line. Graph indicates body angles of larvae during curling in response to ChR2-mediated stimulation. The speed is 30 frames per second.

(**D**) Optogenetic activation of C4da neurons during development resulted in a significant reduction in curling. (n = 140 larvae total)

(**E**) All-trans-retinal (ATR) is required for ChR2 activation of C4da neurons. (n = 230 larvae total)

(**F**) The ChR2-mediated suppression of nociceptive behavior is not due to the bleaching of ATR. 100 mM ATR-containing food was kept under blue or red light or constant

darkness for 5 days. Larvae were then allowed to develop on food in constant darkness

from day 6 to10 before being tested for behavior. No change in nociceptive behavior

was observed between the tested conditions analyzed using one-way ANOVA. (n = 80

larvae for curling analyses, 165 larvae for percent nociceptive behavior)

(**G**) Developmental activation of C4da nociceptors with AITC did not change the size

and targeting of their presynaptic terminals. The micrographs show the presynaptic

terminal of a representative ddaC neuron (green) from each group. The presynaptic

terminals of the other C4da neurons in the same body segment were labeled by a

different fluorescent protein (magenta). The quantification for each subtype of C4da

neurons (ddaC, v'ada, and vdaB) is shown in the barchart.

A08n expressing CsChrimson
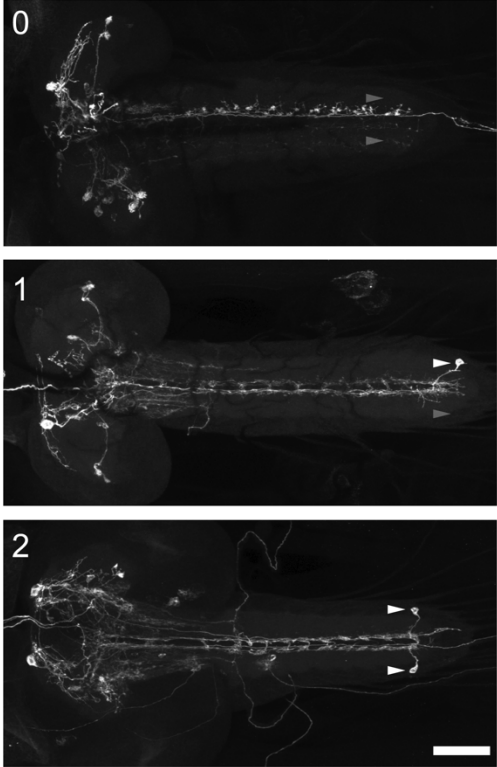
Supplemental Figure 3.2. Mosaic activation of A08n neurons.

CsChrimson::Venus was expressed in none ("0"), one ("1"), or both ("2") A08n neurons,

in addition to several neurons in the brain. The FLP-out mosaic technique with

GMR82E12-Gal4 driver was used for the mosaic expression of CsChrimson.

Arrowheads point to the cell bodies of A08n. Scale bar: 50 µm.

Supplemental Figure 3.3. Excessive nociceptive input during development suppresses C4da-A08n synaptic transmission
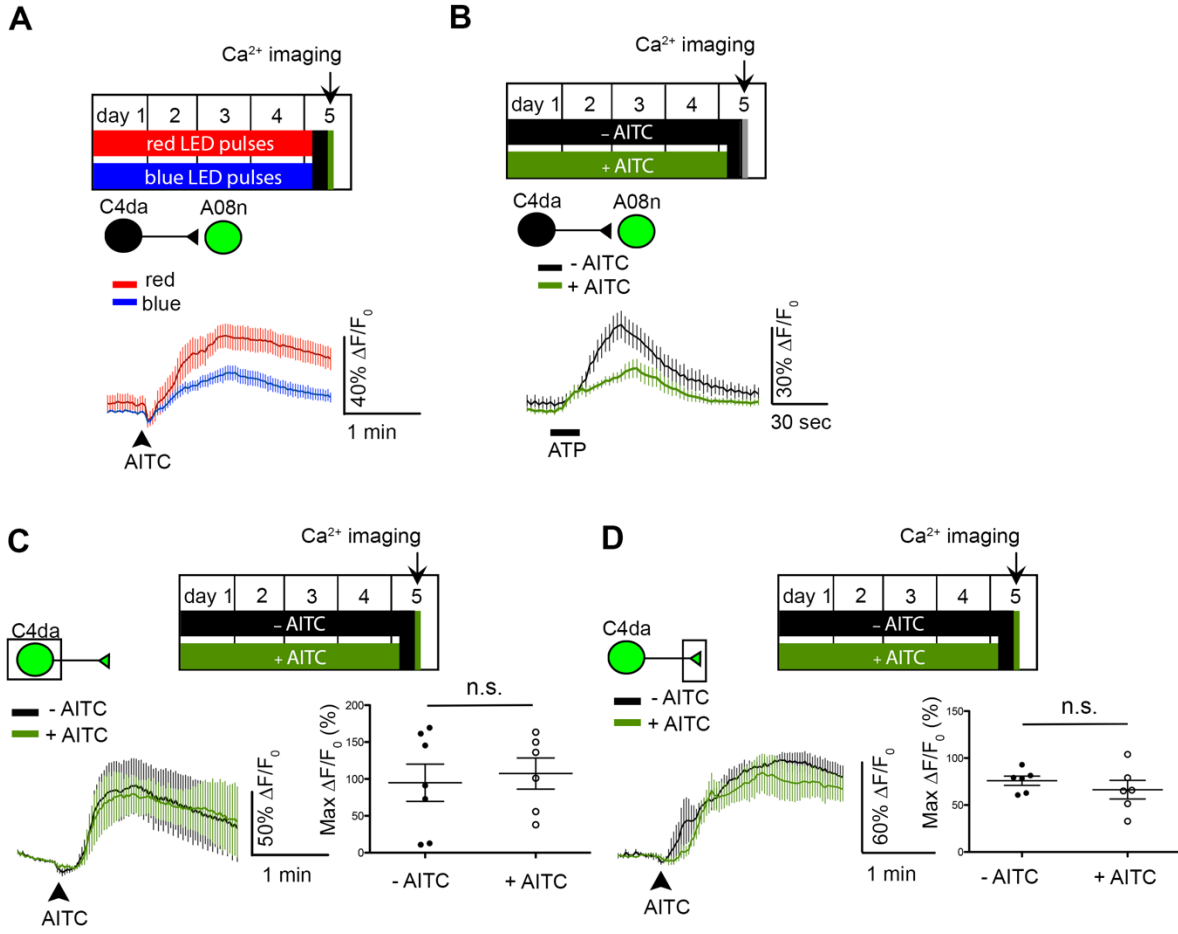
A) Larvae raised under developmental activation of C4da neurons show reduction in C4da-to-A08n synaptic transmission upon AITC stimulation. (n = 12 neurons, 6 brains per group)

(**B**) Larvae raised under developmental noxious stimulation show reduction in C4da-to-A08n synaptic transmission upon $P2X_2$-mediated activation of C4da neurons. (n = 24, 27 neurons, 13 brains per group)

(**C** & **D**) Developmental noxious stimulation does not change the sensory transduction in C4da somas (**C**) (n = 6, 7 neurons, 4 brains per group) or axon terminals (**D**) (n = 6 neurons, 3 brains per group) upon noxious stimulation

CHAPTER 4

Discussion of neural circuit assembly and future directions

Discussion

This collaborative body of work has established a calcium live-imaging technique to physiologically measure neural dynamics within larval brains and demonstrated that excessive noxious experience during a sensitive period in development modifies nociceptive behavior through an intrinsic program of circuit development. This is the first demonstration, to our knowledge, that noxious sensory experience shapes the development of *Drosophila*, and reveals a neural mechanism involved in offsetting harmful environmental stimuli.

The advancement of imaging neural dynamics

The tools and technologies available to probe *Drosophila* neural circuitry are rapidly expanding. Since publishing our P2X2/calcium live-imaging method (Chapter 2), there have been several advances in both optogenetics and calcium sensors. Although there are continually new tools available, our methodology is flexible and can readily integrate newer activators and sensors. Genetically encoded calcium indicators are continuously modified and improved upon, a new version coming out almost each year. Recent

advances of calcium sensors include the further optimization of GCaMP and the

expanded spectral options such as the red-shifted RCaMP.

The newest iteration of GCaMP is the GCaMP6 line. This new series of GCaMP

provides drastic improvements in sensitivity and kinetics in vivo. These high sensitivity

GECI variants were named based on decay kinetics - GCaMP6s, 6m, 6f (slow, medium,

fast). GCaMP6m is the most sensitive to low levels of activity, followed by GCaMP6s.

Both GCaMP6m and GCaMP6s have slow decay kinetics, but the combination of

sensitivity and slow decay may be advantageous for specific types of experiments.

GCaMP6f offers the best compromise of increased sensitivity and improved kinetics,

making it a good choice for the widest range functional imaging applications (Chen et

al., 2013). Additionally, recent progress in live-imaging technology (Chhetri et al., 2015;

Dunn et al., 2016; et al., 2015; Randlett et al., 2015) has enabled researchers to

address questions previously unavailable due to lack of methods. However, GCaMP6

still has not surpassed the sensitivity or speed needed to detect subtle physiological

dynamics compared to synthetic calcium indicators or electrophysiology.

The red, single-wavelength calcium sensor RCaMP is based on the design of

GCaMP. To create this red sensor, the EGFP molecule was replaced with the circularly

permuted red fluorescent protein, mRuby (Akerboom et al., 2012). Since 2013, RCaMP

has been further improved upon resulting in the creation of RCaMP-2 (Inoue et al.,

2015) and most recently jRCaMP1a, b. This newest iteration of RCaMP is comparable

to that of the GCaMP6 line (Dana et al., 2016). To this date both jRCaMP and GCaMP6

are the most optimized genetically encoded calcium sensors available. With the improvements to RCaMP, red calcium sensors now allow for the successful combination of channelrhodopsin-2 (ChR2) and calcium live-imaging.

Due to recent advances in optogenetic tools, the GFP-based GCaMP6 calcium sensor can now be successfully in combination with red-shifted opsins such as CsChrimson. CsChrimson is red-shifted by 45 nm more than previously described channelrhodopsins (Klapoetke et al., 2014). The activation range for CsChrimson is far enough in the red spectrum that there is no crosstalk with the blue-light excitation needed for GCaMP6. Simultaneous GCaMP6 imaging and CsChrimson stimulation has been successfully demonstrated in uncovering the pathway for processing courtship song in *Drosophila* (Zhou et al., 2015).

These advances in imaging-based recordings have radically changed the field of neurobiology by allowing us to record the responses of a large number of neurons simultaneously. However, whole-brain activity recordings are currently limited by the ability to analyze and quantify the large amount of imaging data in order to address biologically relevant questions.

Establishment and expression of plasticity

Previous work in the central nervous system of *Drosophila* have described the homeostatic regulation of enhanced sensory-input through structural and functional modifications. In the visual system, central neurons postsynaptic to the photoreceptors

displayed structural and functional modifications resulting in bidirectional adaptations invoked by different sensory exposure (Yuan et al., 2011). This type of plasticity potentially results from the change in the set point in homeostasis of the circuit based on alterations in sensory input.

In contrast to the homeostatic plasticity observed in the visual circuit, our results demonstrate that the key to the nociceptive plasticity observed is the use-dependent potentiation of the 5-HT connection between serotonergic neurons and the presynaptic terminals of the nociceptors, resembling that of the Hebbian postulate of synaptic plasticity. Similar to the learning and memory paradigm in which activation of the NMDA receptor is required for the establishment of the LTP, during the development of the nociceptive circuit the activity of the nociceptors is required for the establishment of synaptic plasticity by enhancing the sensitivity of the nociceptor presynaptic terminals to 5-HT-induced modulation. In the mature animal, this plasticity is expressed through the enhanced sensitivity of the nociceptor presynaptic terminals to serotonergic feedforward inhibition much like how AMPA recruitment is the expression of synaptic strengthening.

The Hebbian theory requires the concurrent activation of pre- and postsynapses (Hebb, 1949). Coactivation of dopaminergic interneurons and olfactory sensory neurons resulted in a functional modification of the postsynaptic targets in the *Drosophila* mushroom body. Dopamine acts as part of a molecular coincidence detector underlying learning such that postsynaptic potentiation requires both calcium influx and enhanced sensitivity to dopaminergic input. Activated postsynaptic targets that respond to

dopamine have synergistically elevated cAMP levels. This increase in cAMP signaling is suggested to strengthen odor learning (Boto et al., 2014). Populations of postsynaptic neurons in the mushroom body display a heterogeneous sensitivity to dopamine. This requirement for co-activation of sensory neurons and sensitivity to neuromodulation may provide an explanation for the specificity of the circuit plasticity. However, this Hebbian feedforward potentiation of the olfactory circuit alone would destabilize the nervous system and result in the loss of sensory information (as discussed in Chapter 1). Hence, this circuit would also require a homeostatic mechanism to maintain circuit stability.

Homeostasis helps to specify which circuits are potentiated, such that homeostasis brings the synapses not involved in the activity-dependent change back to baseline (Turrigiano, 2008) (as discussed in Chapter 1). The mechanism that we proposed in Chapter 3 achieves this specificity without the need of homeostasis. The plasticity we observed is not homeostasis because excessive inputs onto the nociceptors led to enhanced responsiveness of the nociceptor synapse to 5-HT. Moreover, the input specificity and the requirement of co-activation between nociceptors and serotonergic neurons are typical of Hebbian plasticity but not homeostatic plasticity.

Interneuron-mediated plasticity

Interneuron-mediated plasticity is a general feature of activity-dependent circuit modification. In mature *Aplysia*, serotonergic interneurons presynaptically facilitate the

siphon sensory neuron to gill motor neuron synaptic transmission where the

serotonergic neurons are activated by a completely different modality (Kandel, 2001). In

the developing olfactory system of *Drosophila* the olfactory sensory neuron to projection

neuron synaptic transmission is modified through GABAergic interneurons (Sachse et

al., 2007). Additionally, activation of dopaminergic interneurons modified the function of

postsynaptic neurons in the mature *Drosophila* mushroom bodies through feedforward

excitation (Boto et al., 2014). Our study found that serotonergic interneurons modify the

presynaptic terminals of nociceptors through feedforward inhibition. However, unlike the

previous studies, the plasticity is achieved through the sensitization of the nociceptors to

serotonin rather than the enhancement of serotonergic neuron activity. Considering the

wide-range effects of serotonin modulation, it is likely that serotonergic neurons are

capable of modulating somatosensory neurons other than the nociceptors. The specific

sensitization of the nociceptors to serotonin allows the nervous system to maintain other

serotonin-dependent functions.

Future Directions

Here, I will expand on previously discussed conclusions and introduce hypotheses that

will hopefully provide new opportunities to increase our knowledge of the

neurobiological basis of behavior.

Whole-brain activity mapping in Drosophila

   Genetically encoded calcium indicators are used to non-invasively record the activity

across populations of neurons as well as activity patterns across entire central nervous

systems. A major technical challenge that remains is to accurately and simultaneously image whole-brain activity patterns in combination with animal behavior. Technological advance in imaging, including the development of three-dimensional light-sheet microscopy, has seen some success such as whole-brain imaging of 'fictive' locomotion in the *Drosophila* larval brain. So far, whole-brain imaging in *Drosophila* larvae utilizes brain explants and therefore inducing behavior through stimulating sensory systems has yet to be developed (Chhetri et al., 2015; Lemon et al., 2015). I have developed new methods that allow recording environmental sensory responses in the sensorimotor region of the larval brain with behavior. However, these experiments were performed in a semi-intact larval preparation. Due to the opacity of the larval cuticle, imaging an intact animal is currently unfeasible. Additionally, new methods of analysis need to be developed in order to process this imaging data in order to interpret any biological meaning from these recordings. There has been some effort in developing new analytic tools both in our lab and in others. For example, computational methods for whole-brain imaging analysis include automated mapping of whole-CNS activity patterns as well as identifying regions and neurons across samples (Lemon et al., 2015). The Ye lab, in collaboration with Dr. Jie Zhou at the Northern Illinois University, has developed preliminary algorithms and software to quantitatively analyze the activity waves. The major challenge to whole-brain activity mapping is to be able to address a biologically relevant question. Thus far, only descriptive studies mapping fictive locomotion have been reported (Lemon et al., 2015). With the advances in neuronal activators such as optogenetics, it may be possible to begin addressing how individual or populations of sensory neurons contribute to the activity patterns observed within the brain.

Parallel evolution of escape reflex circuitry

Zebrafish larvae exhibit an escape reflex common to both fish and amphibians known as a C-start that is regulated by a pair of large neurons called the Mauthner cells (M-cells). These two neurons are located in the hindbrain of fish. A single M-cell sends an axon down the spinal cord on the side opposite of the soma, resembling a morphology very similar to the pair of *Drosophila* larval A08n neurons (Fetcho, 1991). The A08n neurons not only share morphological similarities with the M-cells, but they also appear to be homologous in function. Ablation of the M-cells eliminates the fast escape response but does not eliminate slower escape responses because the latter are mediated by other reticulospinal interneurons (Eaton et al., 2001; Nissanov et al., 1990). Additionally, the caridoid escape response in crustaceans are mediated by multiple neurons; the medial and lateral giants mediate a fast escape response while another group of neurons mediate slower escape responses (Wiersma, 1947; Wiersma and Ikeda, 1964).

In *Drosophila*, genetic silencing of the A08n neurons does not completely inhibit the nociceptive response and activation of the A08n neurons is sufficient to evoke nociceptive behavior, although further analysis is needed to observe changes in latency of response. M-cells have directionality. The C-start moves the animal rapidly away from the stimulus. Activation of one M-cell excites the ipsilateral motor neurons to contract muscles forming the C-bend, while the opposing side is inhibited through interneurons (Fetcho, 1991). It would be interesting to further characterize the A08n

neurons in their role in the nociceptive circuit, in particular observing how the A08n

neurons potentially regulate nociceptive latency, intensity and directionality.

Unintended ecological consequences of insecticides

I have shown in Chapter 3 that in the presence of chronic noxious stimuli there is

suppression in circuit function and subsequent behavioral response. The natural

noxious stimulus used was AITC. This plant-derived compound is used as a food flavor,

preservative and, at high concentration, as an insecticide. AITC acts through TrpA1

channels to excite the nociceptors to elicit an aversive response. Therefore, I reason

that other insecticides acting through TrpA1 may also influence brain development of

insects when encountered at sublethal concentrations.

In fact, a variety of natural insecticides and deterrents act through TrpA1

including; AITC, methyl salicylate, cinnamonaldehyde, and allicin (Talavera et al., 2009).

These natural compounds are often the basis of synthetic insecticides. For example, the

most widely used class of synthetic insecticide, the neonicitinoids, are based off

nicotine. Neonicotinoids are known to cause drastic behavioral changes in bees as well

as other non-target organisms (Williams et al., 2015). Adult *Drosophila* exposed to

chronic sublethal levels of neonicotinoids were observed to have altered behavior

(Charpentier et al., 2014). *Drosophila* larval nociceptor neurons help larvae escape from

parasitoid wasps that are ubiquitously present in the natural environment of *Drosophil*a

(Robertson et al., 2013). Additionally, it has been recently demonstrated that animals

that have undergone an anesthetic treatment have a higher risk of predation (Crook et

al., 2014). Thus, sublethal levels of neonicotinoid insecticides present during larval brain development may shape the nociceptive circuit to suppress pain response through activating dTrpA1. Suppression in nociceptive response may then increase the risk of predation, reducing the survival rate of *Drosophil*a larva. Therefore, I reason that neonicotinoid insecticides suppress the nociceptive circuit and subsequent behavior thereby increasing the risk of predation, indicating potential unintentional ecological consequences of insecticide off-target effects.

Conclusion

The results presented in this collaborative dissertation demonstrate a unique form of plasticity that occurs during the development of *Drosophila* and offer a mechanistic model that will inspire further investigation of neural plasticity in both developing and mature animals. To summarize: (1) We have shown that noxious sensory experience modifies the development of the nociceptive circuit, (2) We have identified second-order neurons dedicated to the nociceptive circuit, and finally, (3) We have demonstrated that the developmental plasticity of the nociceptive circuit exhibits a striking input-specificity established through requirement of co-activation of nociceptors and feedforward inhibition by serotonergic interneurons. This dissertation has laid the foundation for future studies on the molecular pathways underlying the developmental plasticity.

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