Neuronal Activity-Dependent Development of the Nociceptive Circuit in *Drosophila*

by

Takuya Kaneko

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cell and Developmental Biology)
In the University of Michigan
2018

Doctoral Committee:

Professor Yukiko Yamashita, Chair
Associate Professor Catherine A. Collins
Professor John Y. Kuwada
Associate Professor Bing Ye

Takuya Kaneko

tkaneko@umich.edu

ORCID iD: 0000-0003-1543-1105

DEDICATION

To my parents

ACKNOWLEDGEMENT

I would like to thank my dissertation advisor, Dr. Bing Ye, for his inspiring guidance and supervision. Bing provided me with the best Ph.D. training and equipped me with necessary skills for being an independent researcher. I am grateful for his creative ideas, which always excited me and fueled my interest in neuroscience. I was fortunate that I found a great mentor for my dissertation research.

This dissertation would not have been possible without support and guidance from my committee members, Drs. Yukiko Yamashita, Cathy Collins, and John Kuwada. I appreciate all their feedbacks during committee meetings, which are always critical and helpful.

In addition, I would like to acknowledge people who contributed to my dissertation research. The project on topographic projections is a collaboration with Dr. Limin Yang, a former postdoc in Ye lab, who helped my analysis even after moving to another institution. The other project on developmental plasticity is a collaboration with Dr. Ann Marie Macara, a former graduate student in the lab. My collaboration with Ann Marie for paper publication was the most valuable and enjoyable opportunity I had in Ye lab, and I greatly appreciate all her effort, which led to our successful completion of the project.

The publication of our paper was made possible by additional support from other collaborators, to whom I would like to give my sincere gratitude.

I would like to thank past and present members in Ye lab for their kind support and valuable feedbacks, as well as for a comfortable research environment they provided to me. I would also like to give my thanks to all friends in Michigan, who made my stressful days happier. Many friendly people around me have made my Ph.D. life in Michigan the best period of my life.

I would like to thank my parents for all the opportunities they provided me throughout my life. Without their understanding and support, I would not have been able to come to this wonderful place in Michigan for a Ph.D. far away from my hometown in Japan. Finally I would like to give my special thanks to my wife, Chio, for her unwavering support and her smile.

TABLE OF CONTENTS

DEDICATION……ii

ACKNOWLEDGEMENT……iii

LIST OF FIGURES……vii

ABSTRACT……ix

CHAPTER 1 Introduction……....1

Introduction to neuronal-activity-dependent development……1

Sensory-input-induced plasticity

Activity-dependent topographic projections

***Drosophila* nervous system for understanding activity-dependent development** ..28

Experience-dependent plasticity in *Drosophila*

Activity-independent topographic projections in *Drosophila*

Drosophila nociceptive sensory system

CHAPTER 2 Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in *Drosophila*……..43

CHAPTER 3 **Neural Activity Instructs Fine-Scale Topography in a Developing Sensory Circuit in *Drosophila*** 86

CHAPTER 4 **Discussion and Future Directions** 103

BIBLIOGRAPHY 121

LIST OF FIGURES

Figure 1.1. Synaptic plasticity in *Aplysia* and the mammalian hippocampus……..39

Figure 1.2. Synapse-specific plasticity……40

Figure 1.3. Fine-scale topography in *Drosophila* and vertebrates……..42

Figure 2.1. Activation of Larval Nociceptors during Development Suppresses Nociceptive Behavior in Mature Larvae……66

Figure 2.2. Developmental stimulation of nociceptors suppresses nociceptive behavior.68

Figure 2.3. Developmental activation of C4da nociceptors with AITC does not change the size or targeting of their presynaptic terminals……70

Figure 2.4. A08n Neurons Are Specific Postsynaptic Targets of Nociceptors……71

Figure 2.5. Mosaic activation of A08n neurons……...73

Figure 2.6. The Sensory Input-Induced Plasticity of Larval Nociceptive Circuit Is Pathway Specific ……..……..74

Figure 2.7. High levels of nociceptive inputs during development suppress C4da-to-A08n synaptic transmission……76

Figure 2.8. Serotonergic Neurons Are Required to Establish Sensory Input-Induced Plasticity of Larval Nociceptive Behavior……77

Figure 2.9. Silencing serotonergic neurons does not affect the nociceptive behavioral responses if nociceptors are not stimulated during development……79

Figure 2.10. Feedback Inhibition of Nociceptor-to-SON Transmission through Serotonergic Neurons ……80

Figure 2.11. The terminals of serotonergic neurons intimately intertwine with C4da presynaptic terminals ……82

Figure 2.12. Developmental Stimulation of Nociceptors Enhances 5-HT Receptor-Mediated Inhibition of Nociceptor-to-SON Transmission ……83

Figure 2.13. Feedback Modulation Enables Pathway-Specific Plasticity in Developing Sensory Circuits in *Drosophila* ……85

Figure 3.1. Postsynaptic neurons support C4da topography possibly through retrograde BMP signaling …..……..99

Figure 3.2. C4da fine-scale topography requires BMP signaling……..101

Figure 4.1. Mechanisms underlying fine-scale topography……120

ABSTRACT

How nature and nurture interact to sculpt the nervous system, which underlies animal behaviors, has fascinated both scientists and the general public for generations. At the level of neural circuit assembly, the answer lies in the interplay between genetic programs and neural activity; the development of a functional nervous system is not just hard-wired by the genome, but depends on sensory experiences and neuronal activities. However, the mechanisms underlying the "activity-dependent development" of the nervous system are poorly understood, mostly due to the lack of a model system that is amenable to efficient gene manipulations and circuit analyses. My dissertation research aims to develop a *Drosophila* system that is suitable for identifying the mechanisms behind activity-dependent development of neural circuits from the molecular to the circuit and behavioral levels. I have largely achieved this through two projects.

First, I discovered that the functional development of *Drosophila* somatosensory circuits depends on the sensory inputs during animal development. Our behavior analysis demonstrated that larval escaping behavior in response to noxious stimulation is suppressed if a larva experiences enhanced levels of noxious stimulation during development, demonstrating sensory input-induced plasticity. Using imaging-based physiological analyses (calcium and cAMP imaging techniques and optogenetic stimulation of neurons), we found that enhanced noxious stimulation during

development reduces the synaptic transmission from nociceptors (i.e., sensory neurons detecting noxious stimuli) to the second-order neurons (SONs) in the pathway. Our study further revealed that this physiological change accounts for the suppressed behavioral outputs. Importantly, we showed that the enhanced noxious experience has no effect on other sensory modalities such as the mechanosensory pathway and elucidated the mechanism that underlies this sensory-pathway-specificity.

Second, my work facilitated the discovery that the activity levels of nociceptors regulate their axonal projections in the central nervous system (CNS). Through advanced techniques that combine single-cell labeling and computational analysis, we found that the spatial arrangement of nociceptor axon terminals in the CNS reflects the locations of territories occupied by nociceptor dendrites on the body wall, forming a topographic map. The formation of this map depends on the levels of their activities, and manipulation of neuronal activity at single-cell level disrupts the map formation. This activity-dependent topography in *Drosophila* is likely established through the interactions of nociceptor presynaptic terminals with their postsynaptic SONs, similar to topography in vertebrates. This work is the first report of an activity-dependent topographic map in *Drosophila*, and has allowed for mechanistic analyses of the role of neuronal activity in neural circuit wiring. My dissertation research contributes to our understanding of how neural activity interacts with genetic programs to shape the nervous system.

CHAPTER 1

Introduction

A part of this chapter includes excerpts from a review article in Journal of Comprehensive Physiology A, Fine-scale topography in sensory systems: insights from *Drosophila* and vertebrates, written by **Takuya Kaneko** and Bing Ye in 2016.

Introduction to neuronal activity-dependent development

Animals develop through the interplay between nature and nurture – genetic inheritance and the environment. Developmental processes such as embryogenesis and organogenesis are mostly determined by genes, so that the phenotype - the observable properties of animals - is largely determined by the genotype - genetic information. In this way, animals within a species, who share most of their genes, develop common traits. Yet, the contribution of environmental factors in animal development cannot be ignored. In fact, monozygotic twins who share the exact same genotype often exhibit distinct phenotypic traits. Additionally, some animals exhibit a capacity to develop

different anatomical structures according to their individual environments. For instance, larvae of Hokkaido salamander, *Hynobius retardatus*, develop a larger head, which is beneficial as a predator, when the environment contains a large amount of their preys such as tadpoles (Matsunami et al., 2015). On the other hand, salamander larvae exhibit larger external gills and tail fin for protection when presented with their predators, dragonfly nymphs. Thus, multiple phenotypes can arise from a single genotype. The capacity to develop variety of phenotypes from a single genotype provides variation within a species and increases the chance of animals to survive in the given environment. How environmental factors instruct animal development has long been a fundamental, yet unanswered, question in biology.

One of the developmental processes most susceptible to the environment is the wiring of neurons. In the nervous system, environmental factors are directly detected as sensory stimuli, which appear as neuronal activity of sensory neurons. The impact of sensory inputs on a developing brain was first demonstrated by a classic study in which visual inputs were deprived from developing cats (Hubel and Wiesel, 1970). Hubel and Wiesel sutured one eyelid of new born kittens, and opened the eye several months later. They identified that the sutured eye had defective neuronal circuits and lacked visual functions, whereas the spared eye was functional. This classic study illustrates that genetic programs alone are insufficient for shaping a functional brain, and experiencing sensory inputs during development is a necessary step.

The levels of sensory inputs during development determine the functional levels of a mature neural circuit. For example, more neuronal connections develop in the visual system of rats reared in a group with wooden and plastic objects, or “toys”, that

are changed and rearranged daily, compared with rats reared in a pair without any toy (Turner and Greenough, 1985; Volkmar and Greenough, 1972). Rats reared in isolation develop a less complex visual system with fewer neuronal connections than the above two groups. Thus, the nervous system possesses a capacity to modify developing neural circuits based on animals' sensory experience during development. Through this process, individual animals within the same species develop functionally different neuronal circuits, and adapt to their own environments.

How does neuronal activity during development shape the nervous system? This remains a mystery, mostly due to the lack of a model system that is amenable to efficient gene manipulations and circuit analyses. One of the most suitable model systems for such analyses is the fruit fly, *Drosophila melanogaster*; therefore, in my dissertation research, I aim to develop a *Drosophila* system for studying activity-dependent development of neural circuits. My dissertation research focuses on two major developmental processes that are dependent on neuronal activity: sensory-input induced plasticity and activity-dependent topographic projections.

Sensory-input-induced plasticity

The environment presents animals with a wide variety of sensory inputs such as visual, auditory, tactile, and noxious stimuli. Upon detection of individual types of stimuli, the nervous system elicits unique motor outputs in animals. Behavioral outputs in response to the same type of sensory inputs are typically stereotyped. When presented with food, animals tend to approach the food; when presented with predators, animals try to

escape. Yet, at the same time, animals possess a capacity to modify their responses in order to adapt to their individual environments.

In particular, animals from distinct environments often develop different sensitivity to sensory stimuli. For example, the worm nematode, *Caenorhabditis elegans*, exhibits larger responses to tactile inputs when raised in a group, compared to those raised in isolation (Rose et al., 2005). The sensitization to tactile inputs seems to arise from physical contacts during development, which prepares them for future contacts. In another situation, sensory inputs during development may desensitize animals. For instance, animals raised in a noisy environment may not react to a loud noise that otherwise startles animals.

This type of animal adaptation is attributable to the plasticity of neural circuits; the nervous system is plastic and capable of modulating its circuitry based on animals' experience of sensory information from the environment. This form of plasticity, or sensory-input-induced plasticity, is observed in both developing and mature animals. Studies of changes in both developing and mature neuronal circuits have provided profound insights into the molecular mechanism behind the sensory-input-induced plasticity.

Presynaptic mechanism underlying sensory-input-induced plasticity: habituation and sensitization of the Aplysia gill withdrawal reflex

The cellular and molecular bases of neural plasticity have mainly been explored in studies of the marine mollusk, *Aplysia californica* (Kandel, 2001; Kandel, 2013). This invertebrate animal has been an excellent system for neurobiology since 1960s

because of several advantages (Kandel et al., 1967). First, *Aplysia* has only 2000 neurons; as a comparison, mouse has about 10^8 neurons. Second, neurons of this species are typically large and identifiable, which allowed electrophysiological recordings that would not have been possible in other systems in early studies. Third, *Aplysia* exhibits stereotyped and quantifiable behavioral outputs in response to sensory inputs. The best studied behavioral response is the gill withdrawal reflex in which the mollusk withdraws the gill into the mantle shelf for protection upon a tactile stimulus to the siphon. Finally, and importantly, this behavioral output exhibits sensory-input-induced plasticity. After the siphon is touched several times, *Aplysia* exhibits smaller levels of responses against siphon stimulation, which is referred to as "habituation" (Carew et al., 1981; Castellucci et al., 1970). By contrast, the gill withdrawal reflex is enhanced when a noxious electric shock is applied to the tail prior to a tactile input to the siphon, a change called "sensitization" (Kandel, 2001).

Habituation and sensitization of the gill withdrawal reflex can be long-lasting in response to enhanced levels of sensory experience. When a single session of 10 tactile inputs is applied to the siphon, *Aplysia* exhibits suppressed, or habituated, withdrawal reflexes for a few minutes (Carew et al., 1981; Castellucci et al., 1970). On the other hand, when *Aplysia* experiences the same session four times with intervals of several hours, the mollusk shows a long-term habituation that lasts for a few weeks. A long-lasting effect is similarly observed when *Aplysia* experiences electrical inputs to the tail several times (Kandel, 2001). Whereas a single tail shock produces a sensitized response for a few hours, the effect of four shocks lasts over 24 hours. Moreover, four sessions of four electric shocks result in long-term sensitization that lasts over a week.

The ease of electrophysiological recording in *Aplysia* has facilitated identification of the neural circuits responsible for the gill withdrawal (Kandel, 2001). Tactile inputs to the siphon activate a group of mechanosensory neurons that innervate the siphon. These sensory neurons directly form synapses on motor neurons that control the gill muscle (Figure 1.1a). Upon siphon stimulation, the presynaptic sensory neurons release glutamate, a neurotransmitter, to activate the postsynaptic motor neurons, leading to the gill withdrawal. The strength of transmission in this synapse changes in response to sensory experience, and this circuit change accounts for behavioral modification.

For instance, one session of continuous siphon stimulation reduces the transmission from the sensory neurons to the motor neurons, which causes short-term habituation (Carew et al., 1981; Castellucci et al., 1970). The suppressed transmission is attributable to decreases in the amount of glutamate released from the presynaptic terminals of the sensory neurons. The same level of siphon stimulation does not affect the postsynaptic motor neurons; the sensitivity of glutamate receptors on the postsynaptic terminals is unaltered. In addition to the reduction in the levels of glutamate release, enhanced levels of siphon stimulation decreases the number of synaptic contacts between pre and postsynaptic neurons when establishing long-term habituation. This structural change is mainly caused through pruning of the presynaptic terminals. Thus, both short-term and long-term habituation in *Aplysia* is established by a reduction in the strength of synaptic transmission, or synaptic efficacy, and primarily through presynaptic modification.

Similar to habituation, sensitization of the *Aplysia* gill withdrawal reflex is caused by modification of the same sensorimotor synapse (Kandel, 2001). An electronic noxious input from the tail facilitates glutamate release from the presynaptic terminals of the siphon mechanosensory neurons. How is synaptic efficacy of a sensory circuit modulated by an input for another sensory circuit? In *Aplysia*, this is achieved by a group of modulatory interneurons that secrete serotonin, a neuromodulator conserved across species. The serotonergic neurons receive the tail-shock signals from sensory neurons innervating the tail, and in turn send the signals to the siphon sensory neurons by secreting serotonin onto their axon terminals (Figure 1.1a). This circuit motif allows a noxious input to the tail to impact glutamate release from the siphon mechanosensory neurons. In other words, serotonergic neurons serve as a bridge between two sensory neurons responsible for different sensory modalities (i.e. tactile and noxious).

Serotonin facilitates neurotransmitter release by modulating the resting membrane potential of the presynaptic terminals of the siphon sensory neurons through the following steps (Kandel, 2001). First, serotonin secreted from serotonergic neurons binds to G-protein coupled receptors on the presynaptic terminals. Then, this binding activates adenylate cyclase and, as a result, increases the intracellular concentration of cAMP, a second messenger that activates proteinase kinase A (PKA). Finally, activated PKA closes a particular type of K^+ channels on the axon terminals, raising the membrane potential. When the resting membrane potential is high, action potentials induced by sensory inputs easily lead to neurotransmitter release through the opening of voltage-gated Ca^{2+} channel at the presynaptic terminals. This mechanism allows

tactile stimulation to the siphon preceded by a noxious shock to the tail to elicit an enhanced gill withdrawal response, establishing short-term sensitization.

Long-term sensitization is achieved by a similar mechanism, but involves a protein synthesis (Kandel, 2001). Multiple tail shocks enhance serotonin secretion onto the presynaptic terminals of the siphon sensory neurons. Whereas a transient serotonin increase activates PKA locally at the presynaptic terminals, continuous serotonin elevation results in translocation of PKA into the nucleus. The nuclear translocation allows PKA to activate nuclear substrates that include CREB, a transcriptional factor. Active CREB binds to CRE (cAMP response element) sequence and initiates transcription of target genes. This process ultimately results in the growth of the presynaptic terminals, which increases their synaptic contacts with postsynaptic motor neurons and results in long-term sensitization (Bailey and Chen, 1989). Thus, both short-term and long-term sensitization is established primarily through serotonin-mediated presynaptic modification.

In summary, sensory-input-induced plasticity in the *Aplysia* withdrawal reflex is based on synaptic plasticity - the ability of synapses to change their strength. Studies of *Aplysia* have identified presynaptic mechanisms underlying synaptic plasticity. However, presynaptic regulation is not the only way for manipulating synaptic efficacy. The next section discusses postsynaptic modification that enables long-lasting changes in neural connections by reviewing the hippocampus in the mammalian brain.

Postsynaptic mechanism: LTP and LTD in the mammalian hippocampus

For the past several decades, the hippocampus has received considerable attraction ever since its major contribution for memory acquisition was identified. Of particular interest is the question of how this brain region stores and updates our memory according to our experience. Accumulating evidence suggests that our memory is based on the ability of hippocampal neurons to change the strength of neural connections in response to experience. In fact, the hippocampus changes synaptic strength when presynaptic neurons are directly stimulated in a certain pattern by an inserted electrode. These studies mainly apply electrophysiological neural stimulation, rather than natural sensory inputs, yet principles identified in the hippocampus generally underlie sensory-input-induced plasticity in the mammalian brain as briefly discussed later in this section.

One of the best studied synapses in the hippocampus is that formed by presynaptic CA3 neurons and postsynaptic CA1 neurons (CA1 and CA3 indicate particular regions within the hippocampus) (Bliss and Collingridge, 1993; Luo, 2015; Malenka and Bear, 2004). When the presynaptic CA3 neurons receive a brief high-frequency trains of stimuli, the strength of their synapses with postsynaptic CA1 neurons increases (Figure 1.1b). This effect is long-lasting and called long-term potentiation (LTP). LTP lasts for tens of minutes to hours when induced in slices of hippocampus. This effect continues up to 24 hours if four or more trains of high-frequency stimulation is given to the presynaptic neurons. In addition to an increase in synaptic efficacy, synapses in the hippocampus are capable of decreasing their strength, an effect termed long-term depression (LTD). LTD in the CA3-CA1 synapse

can be induced by a long period of low-frequency stimulation to presynaptic CA3 neurons. Thus, as seen in *Aplysia*, the mammalian hippocampus exhibits synaptic plasticity; hippocampal neurons change synaptic efficacy bidirectionally in response to particular types of stimulation.

Both LTP and LTD are established primarily through postsynaptic regulation in the CA3-CA1 synapse. The key molecules for the two events are NMDA receptors on the postsynaptic terminals, whose activation triggers cascades for postsynaptic modification (Bliss and Collingridge, 1993; Huganir and Nicoll, 2013). NMDA receptors are ion channels and require glutamate binding to open the pore of the channels. But, glutamate release from presynaptic terminals is insufficient to open and activate the receptor-channel. This is because the pore of NMDA receptors is blocked by extracellular Mg^{2+}, which can be removed by depolarization of the postsynaptic terminals. Therefore, NMDA receptors become active only when the following two events occur simultaneously: 1) activation of presynaptic neurons to release glutamate, and 2) activation of postsynaptic neurons to depolarize the membrane potential. That is, NMDA receptors serve as detectors of co-activity of pre- and postsynaptic neurons.

One brief train of high-frequency stimulation to CA3 neurons for LTP expression successfully activates NMDA receptors on CA1 neurons through the following steps (Bliss and Collingridge, 1993). First, action potentials induced by stimulation early in the train induce glutamate release from the CA3 presynaptic terminals, which activates another type of glutamate receptor, AMPA ion channels, on the CA1 postsynaptic surface. Second, activated AMPA channels depolarize the postsynaptic terminals and

remove Mg^{2+} from NMDA receptors. Finally, subsequent glutamate release induced by presynaptic stimulation later in the train activates and opens NMDA receptors.

How do activated NMDA receptors then express LTP? In the CA3-CA1 synapse, this is established through NMDA-mediated increase in the number of AMPA receptors on the postsynaptic surface (Huganir and Nicoll, 2013). When high-frequency stimulation activates NMDA receptors, the intracellular levels of Ca^{2+} dramatically increase in the postsynaptic neurons. This is due to a high conductance of NMDA receptors for Ca^{2+}. Elevated $Ca2^{+}$ in turn triggers multiple signaling pathways that include activation of calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). Activation of these signaling molecules leads to phosphorylation of one of the subunit of AMPA receptors, GluA1. This phosphorylation, then, leads to exocytosis of intracellular vesicles that contain AMPA receptors, inserting more glutamate receptors on the postsynaptic membrane. The more glutamate receptors postsynaptic neurons possess, the more sensitive to glutamate they become, contributing to LTP expression.

LTD relies on a similar mechanism to express the opposite outcome from LTP (Malenka and Bear, 2004). Compared with high-frequency stimulation for LTP, long low-frequency stimulation for LTD results in lower levels of NMDA activation, leading to a smaller Ca^{2+} increase in the postsynaptic terminals. Low levels of Ca^{2+} increase is insufficient to activate CaMKII pathway, but activates another pathway mediated by the calcium-dependent phosphatase, calcineurin. One function of activated calcineurin is to dephosphorylate GluA1, which facilitates the endocytosis of AMPA receptors from the postsynaptic surface. Through this process, postsynaptic neurons become less sensitive to signals from presynaptic neurons, expressing LTD.

As is the case in *Aplysia*, expression of longer LTP in response to repeated trains of high-frequency stimulation involves an increase in the number of synaptic contacts (Engert and Bonhoeffer, 1999; Luscher and Malenka, 2012). This additional phase of LTP, termed late LTP, is initiated by a split of existing postsynaptic terminals. Then, the growth of the postsynaptic terminals likely induces duplication of the presynaptic terminals. This process ultimately increases the number of synaptic contacts between the same two neurons, drastically enhancing synaptic efficacy. Similar to the earlier phase of LTP, or early LTP, this additional step for late LTP requires NMDA receptors, emphasizing the major contribution of the postsynaptic side. Despite this similarity, late LTP depends on gene transcription, whereas early LTP does not. Prolonged Ca^{2+} elevation induced by NMDA receptors is possibly the key step to initiate gene transcription for late LTP.

In summary, hippocampal neurons are capable of producing long-lasting changes on synaptic efficacy in response to a particular pattern of stimulation. These changes are primarily based on postsynaptic mechanism. This type of synaptic modification is not limited to the hippocampus. Indeed, many other regions of the mammalian brain similarly express LTP and LTD in response to electrical stimulation. Yet, how does this kind of modification happen inside the brain of living animals in response to sensory experience, which may not necessarily produce certain patterns of stimulation? It is believed that, in reality, interconnected neurons create specific patterns of action potentials to induce LTP or LTD according to experience.

Evidence suggesting that LTP and LTD indeed occur in response to natural sensory inputs comes from studies on other areas of the mammalian brain, which

include the visual cortex and the somatosensory cortex (Foeller and Feldman, 2004; Kirkwood et al., 1996). An example is a region of the primary somatosensory cortex that is responsible for receiving signals of sensory inputs from whiskers. When a subset of whiskers are trimmed to deprive sensory inputs, the strength of synapses downstream of the trimmed whiskers decreases (Allen et al., 2003). This decrease possibly represents LTD expression because of the following observation: whisker deprivation provides presynaptic neurons of the synapses with a particular pattern of action potentials, which is, when given by electrode stimulation, indeed capable of inducing LTD. Moreover, another experiment has demonstrated that sensory inputs through the remaining whiskers increase synaptic efficacy downstream of these intact whiskers (Takahashi et al., 2003). This change is likely caused by LTP expression because of the two characteristics. First, the increase in synaptic efficacy is dependent on the function of AMPA receptors. Second, GFP-fused AMPA receptors are in fact inserted in the postsynaptic terminals *in vivo* as synaptic efficacy increases. Taken together, these lines of evidence support that mechanisms underlying LTP and LTD discussed in this section generally underlie synaptic plasticity induced by natural sensory inputs.

As discussed so far, studies of mature neuronal circuits have provided important insights into presynaptic and postsynaptic mechanisms that create long-lasting changes in synaptic efficacy. The interplay of these two mechanisms establishes sensory-input-induced plasticity across species. Yet, how the nervous system induces these stable changes in a sensory-pathway-specific manner remains an open question. The

following sections first review recent studies on this process, and then discuss possible mechanisms by coming back to the *Aplysia* and hippocampus systems.

Sensory-pathway-specificity of neural plasticity

Animals are capable of modifying their responses to one particular type of sensory stimuli, without disrupting those to other sensory stimuli. A noisy environment, for instance, may desensitize animals' responses to sound, but not to touch or light. This form of specificity, or sensory-pathway-specificity, can be straightforward if individual sensory circuits are distinctly separated from other pathways. However, the nervous system is not that simple. In fact, whereas distinct types of sensory stimuli are usually detected by different groups of sensory neurons, these different sensory neurons often share common downstream neuronal circuits. This means that neurons within shared circuits receive signals of multiple sensory modalities. One possible function of these multimodal neurons is to integrate multiple sensory inputs in order to elicit behavioral outputs that are most appropriate for individual situations. Despite this advantage, highly interconnected sensory pathways raise the following question: how does the nervous system modulate single sensory circuits without disrupting other sensory modalities that share the same circuits? This is a fundamental question in neurobiology that remains to be addressed.

Evidence from recent studies of developing nervous systems suggests that sensory-pathway-specificity occurs in multimodal neurons at the synaptic level. For example, neurons in the optic tectum of the frog, *Xenopus laevis*, respond to two different sensory stimuli: visual and mechanosensory inputs (Deeg and Aizenman,

2011). In this system, visual inputs are transmitted to the optic tectum through the optic nerve, while mechanosensory signals are derived from neurons in the hindbrain. These two types of neurons of different origins converge on the same postsynaptic neurons in the optic tectum by forming synapses on different dendritic branches of these shared postsynaptic neurons (Figure 1.2a). In spite of this signal convergence, the two different types of synapses, visual synapses and mechanosensory synapses, individually change their strength, or synaptic efficacy, in response to sensory inputs.

When *Xenopus* tadpoles experience enhanced levels of mechanosensory stimulation by a continuous vibration for 48 hours, the efficacy of mechanosensory synapses decreases (Deeg and Aizenman, 2011)(Figure 1.2a). This same condition has no effect on visual synapses formed by the same postsynaptic neurons in the tectum. This observation indicates that elevated mechanosenory inputs specifically modulate the efficacy for mechanosesory transduction without affecting responses of the optic tectum to visual inputs. Similarly, when *Xenopus* tadpoles are placed in darkness to deprive visual inputs for 48 hours, the efficacy of visual synapses increases without changing that of mechanosensory synapses of the same postsynaptic neurons. One hypothesis is that this pathway-specific change is caused by modification of individual presynaptic neurons. However, visual deprivation has little, if any, effect on neurotransmitter release probability of the presynaptic optic nerve. This observation supports the other possibility that postsynaptic multimodal neurons in the optic tectum are capable of modulating the efficacy of one type of synapses without disrupting the functions of the others, exhibiting pathway-specificity at the synaptic level.

Sensory-pathway-specificity is similarly observed in the worm, *C. elegans*. As mentioned earlier, *C. elegans* worms raised in groups exhibit larger responses to tactile inputs after development is complete, compared to those raised in isolation (Rose et al., 2005). This increase in sensitivity is likely caused by enhanced levels of mechanosensory inputs during development through physical contacts in groups. Indeed, additional mechanosensory stimuli to isolated worms sufficiently enhance their responses to tactile stimuli. Motor outputs induced by tactile inputs is similarly observed when worms encounter a heat probe; essentially worms swim backward away from stimuli. Despite this similarity, worms from the two different groups (i.e. isolation vs. groups) exhibit comparable levels of responses to a heated probe. That is, the effect of mechanosensory stimuli during development is specific to mechanosensory pathways.

What circuit modification underlies the change in behavior? In *C. elegans*, tactile and heat stimuli are detected by different sensory neurons, yet they converge on shared postsynaptic interneurons for common motor outputs. Three lines of evidence suggest that mechanosensory synapses of the shared interneurons become more effective as worms experience mechanosensory inputs during development (Rose et al., 2005). First, in colony-raised worms, the presynaptic terminals of mechanosensory neurons contain higher levels of GFP-tagged synaptobrevin, a protein that regulates neurotransmitter release. Second, colony-raised worms express more GFP-tagged glutamate receptors in the postsynaptic interneurons. Third, a brief mechanosensory stimulation on isolate-raised worms during development sufficiently increases the levels of glutamate receptors in the postsynaptic neurons. Thus, sensory-pathway-specificity

in behavioral modification in worms is likely based on synapse-specific regulation in multimodal interneurons, where multiple sensory modalities first converge (Figure 1.2b).

The two examples above highlight the contribution of synapse-specific modulation for establishing sensory-pathway-specificity. Although the mechanism behind synapse-specific plasticity is largely unknown, profound insights have been gained, again, from the *Aplysia* and hippocampal systems.

Presynaptic mechanism underlying synapse-specific plasticity

In *Aplysia*, serotonin-mediated sensitization of synapses occurs in a synapse specific manner. This insight is provided from a culture system that contains an isolated *Aplysia* sensory neuron (neuron A1) and two motor neurons (neurons B1 and B2), where A sensory neuron has a bifurcated axon that forms separate synaptic contacts with motor neurons B1 and B2 (Kandel, 2001; Kandel, 2013). When one pulse of serotonin was applied locally onto the synapse formed between neuron A1 and neuron B1, the efficacy of this synapse increased for a short period of time, indicating the expression of short-term sensitization. In another set of experiments, five pulses of serotonin were locally given to the A1-B1 synapses, which enhanced synaptic efficacy for as long as 24 hours with increased synaptic contacts between neurons A1 and B1, an indication of long-term sensitization. In each situation, the change in synaptic efficacy was synapse-specific, and the other synapse, A1-B2 synapse, exhibited no change in its efficacy (Figure 1.2c).

How does one synapse change its efficacy in response to serotonin without affecting other synapses of the same presynaptic neuron? The expression of short-

term sensitization seems straightforward because this process is mediated by serotonin-induced local PKA activation, as discussed earlier, which has no influence on other parts of the presynaptic neuron. In the case of the expression of long-term sensitization, on the other hand, the mechanism is more complicated. In response to continuous serotonin elevation, activated PKA translocate to the nucleus and initiate gene transcription for subsequent structural changes. Therefore, synapse-specific regulation requires a mechanism that allows new gene products to selectively function only at the serotonin-stimulated synapse.

A clue for understanding the mechanism has been gained from a unique property of cultured *Aplysia* neurons (Kandel, 2013; Martin, 2002). When a single pulse of serotonin, which usually induces only short-term sensitization, was given to the A1-B2 synapses in combination with five pulses of serotonin on the A1-B1 synapses, both the A1-B1 and A1-B2 synapses exhibited long-term sensitization with increased synaptic contacts (Figure 1.2c). This remarkable observation indicates that a single pulse of serotonin, although insufficient to induce gene expression, is actually sufficient to allow the A1-B2 synapse to capture gene products generated by active PKA from the A1-B1 synapse. This serotonin-mediated process - marking a synapse for capturing new gene products - is called synaptic tagging. Similar to other serotonin-mediated steps, synaptic tagging depends on the activity of PKA. When a PKA inhibitor is applied locally at a synapse, a single pulse of serotonin prevents this synapse from using gene products derived from multiple serotonin pulses at another synapse. Thus, PKA-mediated short-term sensitization seems to be a prerequisite for synaptic tagging and subsequent long-term sensitization. Tagging a particular synapse by the serotonin-PKA

pathway underlies specific modulation of the particular synapse with other synapses of the same presynaptic neuron unaltered; however, detailed molecular mechanisms remain unexplored.

Postsynaptic mechanism underlying synapse-specific plasticity

Synaptic specificity is similarly a critical property of LTP expression in the hippocampus (Bliss and Collingridge, 1993; Luo, 2015). When high-frequency stimulation is given to one CA3 neuron (neuron A1), the efficacy of its synapse with a postsynaptic CA1 neuron (neuron B1) increases by expressing LTP. Yet, stimulation to neuron A1 does not affect the synapse of neuron B1 formed by another CA3 presynaptic neuron (neuron A2) (Figure 1.2d). This synapse-specific change is largely dependent on the function of NMDA receptors on the postsynaptic surface. Activation of NMDA-type glutamate receptors, which is necessary for LTP expression, requires neural activity of both presynaptic and postsynaptic neurons. Given this unique property, NMDA receptors are active only at the A1-B1 synapse, but not at the A2-B1 synapse, which lacks neural activity of the presynaptic side. Thus, NMDA receptors allow LTP expression only at synapses formed by co-active neurons. This function of NMDA receptors reflects a long-established hypothesis in neuroscience stated by Hebb (Hebb, 1949): coactivity of pre and postsynaptic neurons strengthens their synaptic connections. Therefore, NMDA receptors are the molecular nature underlying Hebb's postulate, a key for establishing synapse-specific regulation.

Synaptic specificity in the hippocampus is maintained even during the late phase of LTP, which involves gene expression. How do gene products generated in

postsynaptic neuron B1 specifically modulate the “stimulated” A1-B1 synapse without altering the “unstimulated” A2-B1 synapse? As in *Aplysia*, the specificity seems to attributable to synaptic tagging in the hippocampus. Indeed, the presence of a tag at a stimulated synapse is supported by multiple lines of evidence (Frey and Morris, 1997); however, the molecular property of the synaptic tag is largely a mystery.

The *Aplysia* and hippocampus systems have laid a basis for understanding the presynaptic and postsynaptic mechanisms behind synapse-specific plasticity. Yet, at the same time, these studies have left behind additional mysteries in neurobiology. Is serotonin-mediated synaptic tagging a general strategy conserved across species? How is synaptic specificity established in postsynaptic neurons that lack NMDA glutamate receptors – e.g. in an insect brain where the primary neurotransmitter is acetylcholine, rather than glutamate? An ideal system for elucidating further mechanisms is the fruit fly, *Drosophila melanogaster*. However, in this powerful model organism, pathway-specific plasticity has not been well documented, which is the motivation for my dissertation research described in Chapter 2.

This chapter has so far discussed one major role of neural activity in a developing nervous system. Sensory inputs during development, which induce neuronal activity in sensory neurons, largely contribute for shaping the final pattern and strength of synaptic connections. However, neuronal activity is not limited to that evoked by sensory experience. In fact, without sensory inputs, neurons exhibit spontaneous activity. Unlike evoked activity, spontaneous neuronal firing usually depends on the genome, rather than the environment. For instance, the pattern of

spontaneous firing depends on the number or the type of ion channels neurons possess. The contribution of spontaneous activity in a developing nervous system has been identified by studies of topographic map formation. These studies have provided general insights into activity-dependent development.

Activity-dependent topographic projections

This part of the chapter (pp. 21-28) uses excerpts from a review article (Kaneko and Ye, 2016, J. Comp. Physiol. A).

The neural circuits of sensory systems are typically organized into two-dimensional neural maps to encode features of sensory stimuli. Depending on what features are encoded, each neural map can be either discrete or continuous (Luo and Flanagan 2007). For example, the olfactory maps encoding odorant types are discrete. In a discrete map, neurons detecting different types of stimuli are anatomically well separated. On the other hand, the retinotopic maps encoding the positions of visual stimuli are continuous. A continuous map in the nervous system usually reconstructs quantitative features of sensory stimuli, such as the locations of visual or somatosensory stimuli and the frequencies of sound; thus, these maps are also called topographic maps (Luo and Flanagan 2007). Two features of topographic maps make them distinct from discrete neural maps. First, neurons in a topographic map are of the same type. This is true not only for the specific sensory features they detect but also for the fact that the cell-fate of individual neurons in the map is determined by the same genetic program. Second, the axon terminals of neighboring afferent neurons in a

topographic map are adjacent to each other, forming a “continuous” map. A unique issue in continuous maps is the establishment of the synaptic connections that are specific enough to distinguish adjacent presynaptic terminals, which may initially be intermingled with each other and may be separated by only a few microns once development is completed. Studies in the vertebrate visual system have revealed that the mechanisms underlying regional topography (“coarse”) and the mechanisms specifying the topographic locations of neighboring neurons (“fine-scale”) are different.

Fine-scale topography in the vertebrate visual system

A large number of studies on the development of vertebrate retinotopic maps have provided profound insights into the development, cell biology, and plasticity of neural circuits. In the vertebrate visual system, the retinal ganglion cells (RGCs) project their axons to the midbrain visual centers: the optic tectum in amphibians and birds, and the lateral geniculate nucleus and superior colliculus in mammals (Ruthazer and Cline 2004; Huberman et al. 2008; Cang and Feldheim 2013). RGC axon terminals in these target structures form topographic maps that represent the two-dimensional arrangements of RGC cell bodies in the retina. For example, in frogs, RGCs in the nasal part of retina project their axons to the posterior side of the tectum whereas temporal RGCs terminate at the anterior side (Figure 1.3b) (Ruthazer and Cline 2004). In these maps, neighboring RGCs project their axons to neighboring synaptic targets without intermingling with each other. This sorting of the terminals establishes point-to-point specificity in the synaptic connections, or fine-scale topography. Here, the

establishment of fine-scale retinotopic maps requires neural activity (Debski and Cline 2002; Ruthazer and Cline 2004; Ackman and Crair 2014).

Neural activity-dependent regulation of fine-scale retinotopy

Sperry proposed that each RGC possesses a distinct molecular tag, allowing it to find the topographically correct target. As it is hard to conceive the existence of such molecular tags for thousands of RGCs in each retina, he later revised this model by suggesting that concentration gradients of a few molecules may serve as such molecular tags (Sperry 1963). Studies by Bonhoeffer and Flanagan groups subsequently discovered that the concentration gradients of cell-surface proteins Ephs and ephrins in RGCs and their targets direct the formation of topographic maps (McLaughlin and O'Leary 2005; Flanagan 2006). Thus, the concentration of membrane proteins (ligands and receptors in this case) serves as a unique molecular tag for each pair of pre- and post-synaptic neurons. Although it is conceivable that fine-scale topography might be established with the same mechanism, a number of studies have demonstrated that there are important differences between the mechanism underlying fine-scale topography and that underlying coarse topography.

One important aspect of fine-scale topography in vertebrates is its requirement of neural activity during development. For example, in chick embryos, blocking neural activity in the eye by the injections of tetrodotoxin (TTX) results in defective retinotopic maps that contain enlarged synaptic terminals. As a result, neighboring RGCs have overlapping terminals, abolishing topography despite the presence of Ephs and ephrins (Figure 1.3b) (Kobayashi et al. 1990). Neural activity during development is also

essential for the segregation of the axons from the two eyes (Ruthazer and Cline 2004). However, because synaptic connections in retinotopy involve point-to-point accuracy while eye-specific segregation does not, the molecular mechanisms underlying these two activity-dependent processes in development are likely to be different.

A number of studies support the “co-activity” hypothesis for establishing the retinotopic maps and eye-specific segregation (Cline 1991). This hypothesis states that neighboring RGCs exhibit similar activity (i.e. are co-active), and are thus connected to the same target neurons. This model is in principle a special case of Hebb’s postulate of synaptic connection, which states that correlated pre- and post-synaptic activity results in the selective strengthening of synapses (Hebb 1949). There are two key tenets of the co-activity hypothesis: 1) neighboring presynaptic neurons are co-active. As discussed below, patterned retinal RGC activity supports this in both topographic maps and eye-specific segregation (Ruthazer and Cline 2004); 2) co-active presynaptic terminals form synapses with postsynaptic targets that exhibit the same co-activity (e.g. same postsynaptic neurons or different neurons with the same activity pattern). While this is supported in eye-specific segregation (Zhang et al. 2012), its involvement in the establishment of topographic maps remains hypothetical.

An important line of evidence supporting the co-activity hypothesis is the presence of coordinated spontaneous activity among neighboring RGCs that sweeps across the entire retina in waves prior to the eye-opening (Meister et al. 1991; Wong et al. 1993; Feller et al. 1996; Ackman et al. 2012). In mice lacking the β2 subunit of the nicotinic acetylcholine receptor (i.e. β2 knockout mice, or β2KO) (Grubb et al. 2003; McLaughlin et al. 2003; Mrsic-Flogel et al. 2005; Chandrasekaran et al.

2005; Pfeiffenberger et al. 2006; Willshaw et al. 2014), normal patterns of RGC activity are disrupted, which consequently affects the co-activity of neighboring RGCs. In these mutants, both topography and eye-specific segregation are defective. The RGCs in the mutant mice possess enlarged terminals and, as a result, defects in fine-scale connectivity in retinotopic maps.

The role of retinal waves in establishing topography has been controversial as a result of inconsistent phenotypes observed in β2KO mice (Sun et al. 2008; Chalupa 2009; Feller 2009; Stafford et al. 2009), which might be due to the fact that β2 deletion is not specific to the retina in β2KO mice. To address this issue, β2 gene was expressed in the ganglion cell layer, leading to a restoration of retinal waves in β2KO mice (Xu et al. 2011). These waves are smaller in size than wild-type, and individual waves occur locally without propagating across the entire retina. Despite this abnormality, RGCs project the axons to the topographically appropriate locations. The abnormally enlarged terminals in β2KO are rescued in these mice, implicating the successful establishment of fine-scale topography. The smaller waves only produce co-activity in local areas, and hence, co-activity in local areas is sufficient for establishing point-to-point connections. These findings suggest an instructive role of the retinal waves in fine-scale topography.

How might the co-activity of neighboring presynaptic neurons contribute to fine-scale topography? In regard to the original Hebbian hypothesis, the co-activity model is based on the assumption that the activity of the target neurons synchronizes with the patterned retinal RGC activity. The synchronous activity between pre- and post-synaptic neurons would produce stable synapses through Hebbian plasticity. An in-vivo

calcium imaging study revealed the presence of waves in the postsynaptic neurons in mouse (Ackman et al. 2012). The postsynaptic waves emerge at the same frequency and at the same speed as the presynaptic RGC waves. Moreover, the postsynaptic wave disappears upon the inhibition of the waves in the retina, indicating that the postsynaptic wave is driven by the activity of RGC presynaptic terminals. This would ensure that the afferents and target neurons are co-active, which might lead to specific connections between the afferents and their appropriate targets. Indeed, coincidence in the activities of RGCs and their target neurons strengthens the synaptic connections between these neurons in mouse (Butts et al. 2007; Shah and Crair 2008). Thus, "neurons that fire together, wire together" to establish synaptic specificity in the establishment of fine-scale topographic maps.

The connections between co-active pre- and post-synaptic neurons rely on NMDA-type glutamate receptors (Constantine-Paton et al. 1990; Cline 1991). RGCs are glutamatergic neurons, and NMDA receptors are localized on the dendrites of the postsynaptic neurons of RGCs. It is postulated that co-activation of multiple neighboring RGCs leads to sufficient depolarization to activate postsynaptic NMDA receptors (Cline 1991). As a result, the synaptic connections between these RGCs and the postsynaptic partner(s) are strengthened. Therefore, NMDA receptors serve as a detector of co-activity of neighboring afferents. The molecular mechanism downstream of NMDA receptors in establishing topography remains to be determined.

The co-activity model explains how neighboring afferents converge on shared targets, but does not explain how different neighboring afferents might connect with different postsynaptic targets. Recent studies on retinal waves suggest a role of the

sequence of RGC activation for synaptic segregation among different neighboring afferents (i.e. the point-to-point connectivity). Interestingly, the propagation of retinal waves exhibits a directional bias (Stafford et al. 2009; Ackman et al. 2012). The waves frequently start from the ventral-temporal retina and sweep toward the dorsal-nasal pole. The propagation at a fixed direction provides two neighboring neurons along the propagation axis with less correlated activity than those perpendicular to the axis (Stafford et al. 2009). Importantly, RGC terminals with defective retinal waves (as in β2KO mice) exhibit enlargement along the axis of wave propagation (Grubb et al. 2003; Mrsic-Flogel et al. 2005; Ackman and Crair 2014), indicating that the topography along this axis is particularly dependent on the retinal wave. This finding highlights the importance of sequential activation among adjacent RGCs in establishing fine-scale topography.

The sequential activation in the form of a wave might cause intrinsic differences, such as biochemical activities, in neighboring neurons along the axis of wave propagation. This possibility is supported by the findings that neural activity dynamically regulates the activity of cell adhesion molecules. For example, in a cultured hippocampal neuron, N-Cadherin stabilizes and localizes to the cell surface in response to neural activity, enhancing its adhesive activity (Itoh et al. 1997; Tanaka et al. 2000; Brigidi et al. 2014). The retinal wave is also known to produce oscillations in the intrinsic cAMP level of individual RGCs (Dunn et al. 2006). The cAMP oscillations in RGCs then modify the intrinsic ephrin signaling to control its repellent action in vitro (Nicol et al. 2007). The molecular differences among neighboring afferents along the

axis of wave propagation may modulate the axon-axon interactions to establish the point-to-point topography.

In summary, studies on the vertebrate visual system have shaped our current understanding of activity-dependent topographic projections. The formation of topographic maps is a general strategy conserved across species; therefore, studies on the *Drosophila* sensory system are expected to provide detailed insights into how neural activity and molecular signaling interplay to instruct fine-scale axonal projections, which is the main subject of Chapter 3.

Drosophila nervous system for understanding activity-dependent development

Drosophila melanogaster is one of the best model systems in neurobiology research. This is due in part to its short life cycle and simple nervous system. Most important is that powerful genetic techniques have accumulated through a long history of *Drosophila* research. These powerful techniques allow genetic manipulation of subsets of neurons despite massive numbers of neurons in the nervous system. Gene manipulation includes loss/gain of gene functions, neural visualization, activity manipulation, and activity recording. Given this advantage, the *Drosophila* nervous system has been extensively explored for elucidating mechanisms behind activity-dependent development, such as neural plasticity.

Experience-dependent plasticity in *Drosophila*

The *Drosophila* system best studied for neural plasticity is the larval neuromuscular junction (NMJ). The larval NMJ consists of synapses formed by motor neurons on muscle cells on the larval body wall. In this system, synaptic efficacy increases as larvae crawl during development (Schuster, 2006; Sigrist et al., 2003). Enhanced larval locomotion (when driven by a warm environment for instance) involves elevated neural activity in the motor system, and yields long-term strengthening of synaptic transmission. This plasticity is established by both presynaptic modification - enhanced neurotransmitter release - and postsynaptic modification - increased neurotransmitter receptors. Moreover, a long period of vigorous locomotion leads to increases in the number of synaptic contacts, which requires coordination of both pre- and postsynaptic growth. Importantly, studies of the NMJ have well characterized a signaling pathway that allows the coordinated development across synapses. In addition to neurotransmitter-mediated signaling from pre- to postsynaptic terminals, postsynaptic muscles signal presynaptic motoneurons by secreting the BMP ligand, Gbb, to activate BMP signaling in the presynaptic terminals (Marques and Zhang, 2006). This type of "retrograde" signaling contributes to mutual communication across synapses for coordinated pre- and postsynaptic growth.

Similar to the motor system, the *Drosophila* sensory system changes synaptic efficacy in response to experience. For example, levels of visual experience during development impact synaptic transmission in the visual system (Yuan et al., 2011). When fly larvae are exposed to constant light throughout development, the transmission of synapses between photoreceptors and their postsynaptic neurons becomes less

effective. This change is associated with the shortened dendrites of the postsynaptic neurons and reduced synaptic contacts. In contrast, constant darkness throughout development lengthens the postsynaptic dendrites, increases synaptic contacts, and enhances synaptic transmission from photoreceptors to the postsynaptic neurons. The functional and morphological modification depends on the cAMP-PKA pathway in the postsynaptic neurons. This phenomenon highlights the conserved role of this pathway in synaptic plasticity across species. Besides the visual system, sensory-input-induced plasticity is similarly identified in the olfactory system (Sachse et al., 2007); however, it remains an open question whether the *Drosophila* nervous system is capable of modifying one particular sensory circuit without affecting other modalities.

Activity-independent topographic projections in *Drosophila*

Drosophila has been an excellent system for studying fine-scale topographic projections due to its amenability to single-neuron analysis. Studies of topography in this organism have been primarily focused on the visual system. This work demonstrated that topographic projections of photoreceptors are independent from neural activity, which will be discussed below. Despite this unique aspect, the *Drosophila* visual system has provided critical principles that are potentially conserved even in activity-dependent topographic projections of other sensory pathways.

The following section (pp. 31-36) interpolates material from a review article (Kaneko and Ye, 2016, J. Comp. Physiol. A).

Fine-scale topographic maps in the Drosophila visual system

The compound eye in *Drosophila* consists of approximately 750 ommatidia, each of which contains 8 photoreceptors, known as R1-R8 (Clandinin and Zipursky 2002; Hadjieconomou et al. 2011). These R cells are categorized into three groups based on the second-order neuropils in which the axons of these neurons terminate (Harris et al. 1976). The six outer photoreceptors (R1-R6), which detect green light, project their axons to the lamina. R7 and R8 sense ultraviolet and blue light, respectively, and terminate in two distinct layers in the medulla. Topographic maps representing the visual world are formed in each of these three target regions.

The retinotopic map formed by R1-R6 cells in the lamina has been extensively studied for fine-scale specificity in synaptic connections. In the lamina, the terminals of R1-R6 cells form an array of anatomical units called cartridges, each of which is responsible for passing along the visual excitation elicited by a light point in the visual space (Clandinin and Zipursky 2002; Hadjieconomou et al. 2011). The array of the cartridges reconstructs the visual space by forming a topographic map; neighboring cartridges are responsible for neighboring points in space. Because R1-R6 photoreceptors in the same ommatidium see distinct light points, the six axons terminate in different cartridges. At the same time, the light from a point is received by six photoreceptors (R1-R6) that are distributed in six neighboring ommatidia, and the axons of these six neurons converge on the same laminar cartridge. As a result, every cartridge possesses the terminals of R1-R6 cells from six different ommatidia. The R1-R6 photoreceptors in the eye each form a topographic map (Figure 1.3a). For instance, R4 photoreceptors from adjacent ommatidia select cartridges that are next to each

other. The formation of these topographic maps exhibits remarkably high fidelity with less than two photoreceptors terminating at inappropriate cartridges in each eye (Schwabe et al. 2013).

The R1-R6 topographic maps are established through two steps. First, the photoreceptor axons from the same ommatidium are bundled together prior to reaching the lamina (Clandinin and Zipursky 2000). The bundles do not intermingle with each other and thus preserve the spatial arrangement of the ommatidia, contributing to the coarse topography. Second, once the axon bundles enter the lamina, R1-R6 axons segregate away from the bundles. The extensions of the six axons out of the bundles are stereotypic in such a way that their terminals reconstruct the visual space. R4 axons, for example, exhibit stereotypic extensions with a consistent angle from the bundles, and hence different R4 axons from neighboring bundles do not mix with one another (Figure 1.3a). Thus, such precise projections out of the axon bundles ensure the fine-scale connectivity in the fly visual system.

Genetically hardwired fine-scale topography in the Drosophila visual system

The molecular mechanisms underlying the fine-scale topography in the fly visual system has been studied at single-neuron resolution. One approach employed by Clandinin and colleagues is to label a single ommatidium with the fluorescent lipophilic dye DiI, so that the locations of the R1-R6 axon terminals from the ommatidium can be assessed (Clandinin and Zipursky 2000). Through this technique, they found that the extension of the photoreceptor axons out of the bundle is defective in mutants lacking particular subsets of R1-R6 cells. For instance, in mutants lacking R3 and R4, the

axons of the remaining photoreceptors successfully defasciculate from the axon bundle, but fail to select the proper cartridges. This indicates that interactions among axons in the same ommatidium play a role in their fine-scale projections from the bundle.

Screening of candidate molecules responsible for the axon-axon interactions was facilitated by genetic techniques for eye-specific gene manipulation (Lee et al. 2001; Lee et al. 2003). Moreover, fly genetic mosaic techniques, such as flip-out and mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo 1999), made it possible to visualize and genetically manipulate single photoreceptors within an ommatidium (Lee and Luo 1999; Lee et al. 2001; Clandinin and Zipursky 2002). Using these techniques, Clandinin, Zipursky, and colleagues identified two cadherin superfamily proteins, Flamingo (Fmi) and N-cadherin (Ncad), as responsible for the axon-axon interactions underlying fine-scale topography (Lee et al. 2001; Lee et al. 2003). When all photoreceptors are homozygous for Fmi or Ncad mutation, these neurons fail to connect with the appropriate targets.

Later studies revealed that Fmi and Ncad act redundantly in individual photoreceptor axons. Inhibition of either Fmi or Ncad (through mutation or RNAi) in single photoreceptors does not cause any error in the target selection of the mutants (Chen and Clandinin 2008; Schwabe et al. 2013). The projections become defective only when Fmi and Ncad genes are simultaneously knocked down in R1, R2, R5 and R6 through RNAi expression (Schwabe et al. 2013). The axons frequently choose inappropriate targets in spite of the successful segregation from their original bundles. Most of the defective axons terminate close to their proper cartridges, suggesting that Fmi and Ncad regulate fine-scale topography, but not coarse topography.

Several lines of evidence suggest that the axon-axon interactions underlying fine-scale topography rely on the relative, rather than absolute, levels of cadherin proteins among neighboring axons. First, R4-specific overexpression of Fmi causes targeting errors in neighboring neurons despite the normal expression of Fmi in these neighbors (Chen and Clandinin 2008). Defective targeting of wild-type photoreceptors also appears when Ncad is overexpressed in R4 specifically (Schwabe et al. 2013). The R4-specific overexpression of Ncad involves a targeting error of the axons from adjacent ommatidia that have a direct contact with the R4 axons. Second, Fmi is differentially expressed in the axons of the six different photoreceptors in a lamina cartridge (Lee et al. 2003; Schwabe et al. 2013). Its protein level is high in R2 and R5, intermediate in R1 and R6, and low in R3 and R4. Therefore, the axon-axon interactions are precisely regulated by the intrinsic differences in the activity of cell-adhesion proteins.

The axon-axon interactions direct the fine-scale projections of photoreceptors by polarizing the growth cones of extending axons (Schwabe et al. 2013). The distribution of filopodia in the growth cones (i.e. growth cone polarity) correlates with the direction of axon terminal extension, which in turn points toward the specific targets. Thus, it is likely that the polarity of a photoreceptor growth cone directs the axon terminal to the topographically correct target. The regulation of the growth cone polarity requires the cadherin-mediated axon-axon interactions. When both Fmi and Ncad are knocked down in R1, R2, R5 and R6, the growth cones exhibit variable polarity and do not point toward their correct target cartridges. This abnormal polarity directs the axon toward improper direction, disrupting topographic orientation of neighboring axon terminals.

Together, these findings suggest that the axon-axon interactions, which are based on differentially regulated intrinsic cadherin activity, instruct the growth cone polarity of individual photoreceptors in an ommatidium, which then directs the axon terminals to their correct targets for establishing fine-scale topography.

In addition to axon-axon interactions, axon-target interactions are necessary for the establishment of fine-scale topography of photoreceptors, although their roles are likely to be permissive. While Fmi is specifically expressed in photoreceptors (Lee et al. 2003), Ncad is expressed in both photoreceptors and their target neurons (Lee et al. 2001). When Ncad expression is removed from some of the target neurons in the lamina, photoreceptor axons show defective projections (Prakash et al. 2005). Consequently, cartridges that harbor Ncad mutant postsynaptic neurons do not contain six photoreceptor presynaptic terminals. This result suggests that Ncad expression in the target neurons is essential for photoreceptor cells to terminate at the appropriate cartridges. Therefore, Ncad mediates attractive interactions between pre- and post-synaptic terminals. This is different from Fmi, which is neither expressed nor required in the target neurons (Chen and Clandinin 2008). These results point to distinct roles for adhesion molecules, despite the redundancy in the presynaptic terminals as mentioned above.

Remarkably, the topographic maps in the fly visual system are formed independently of neuronal activity (Hiesinger et al. 2006). Electron microscopy of the lamina shows no morphological abnormality of cartridges in several mutants that have defective neural activity. The cellular composition of each cartridge is normal despite a defect in the generation of electrical potentials or in the release of neurotransmitter.

Moreover, DiI injection of single ommatidia in *trp;trpl* mutants, which are defective in light-elicited response, shows normal projections of photoreceptor axons. R4-specific labeling of the mutants further confirms the distribution of a single R4 terminal to each cartridge. Therefore, genetically programmed mechanisms are sufficient to instruct individual photoreceptors to their own targets by differentially regulating the intrinsic levels of cadherin activity between neighboring neurons.

In summary, studies of the *Drosophila* visual system have revealed important molecular principles behind fine-scale topography, which potentially underlie even activity-dependent topographic projections. Detailed investigation of molecular pathways that indeed interplay with neural activity, however, awaits identification of activity-dependent topographic projections, ideally in another *Drosophila* sensory system.

Drosophila Nociceptive sensory system

My dissertation research focuses on the larval nociceptive circuit in *Drosophila*, which seems suitable for further investigating activity-dependent development. The nociceptive system is one of the somatosensory systems responsible for detecting noxious stimuli from the environment, such as intense radiation and heat, chemicals from plants and pesticides, and harsh mechanical stimuli from predators (Hwang et al., 2007). In larvae, these noxious stimuli are detected by nociceptive sensory neurons, which are called class IV dendritic arborization (C4da) neurons (Kim et al., 2013). C4da neurons elaborate their dendrites on the body wall, where they detect noxious inputs, and project their axons to the ventral nerve cord (VNC), which is a CNS structure

equivalent to the vertebrate spinal cord (Grueber et al., 2007). To date, studies of the nociceptive system have primarily focused on C4da neurons and have yielded critical insights into neural physiology and development (Emoto et al., 2004; Grueber et al., 2003; Ye et al., 2007). Despite the profound work on C4da neurons, however, the nociceptive system has not been extensively explored at the circuit level. Given this opportunity, I aim to investigate this system to elucidate principles underlying activity-dependent development. In particular, my dissertation research addresses the following two questions:

1) **How do noxious inputs during development influence the development of the nociceptive circuit?** The nociceptive system seems particularly suitable for addressing this question regarding neural plasticity for the following reasons. First, functional levels of the nociceptive circuit can be reliably assessed through analysis of behavioral outputs. Upon detection of noxious inputs, the nociceptive system elicits a series of stereotyped behavioral responses in larvae: an abrupt curling of the body that is frequently followed by a rolling of the body. This type of behavior, or nociceptive behavior, likely allows larvae to escape from the source of noxious stimuli (Hwang et al., 2007). The levels of the nociceptive behavior, which are reliably quantifiable, reflect possible circuit modulation in response to experience of noxious inputs. Second, due to recent identification of interneurons postsynaptic to C4da neurons, it has become possible to study the levels of synaptic transmission from C4da neurons, which reflect potential synaptic modification (Ohyama et al., 2015; Vogelstein et al., 2014). This advance is expected to allow detailed investigation of the linkage between two levels of

plasticity, the synaptic and the behavioral levels. Finally and most importantly, some of the newly identified postsynaptic neurons receive mechanosensory inputs besides nociceptive inputs (Ohyama et al., 2015). Given these multimodal interneurons, the nociceptive circuit is conceivably equipped with a mechanism that enables pathway-specific-plasticity.

2) **How is topography in the nociceptive system developed?** Similar to the *Drosophila* visual system, where photoreceptors are grouped into anatomical units of ommatidia, in the nociceptive system, C4da neurons are organized into another type of anatomical unit, namely, body segments. Reflecting this anatomical aspect, C4da neurons from adjacent body segments project the axons into neighboring, but spatially distinct, synaptic areas in the VNC, called C4da neuropils (Grueber et al., 2007). C4da neurons from more anterior body segments, for instance, terminate their axons in more anterior C4da neuropils in the VNC. Despite this anatomical separation across body segments, C4da neurons from the same hemi-segment terminate their axons within the same C4da neuropil. Each body hemi-segment possesses the dendrites of three C4da neurons on the body wall, which are arranged along the dorso-ventral (D-V) body axis. As a result, each C4da neuropil in the VNC is packed with the axon terminals of three C4da neurons, which are expected to align along the D-V axis to establish topography. Topographic projections have not been explored in any of the *Drosophila* somatosensory systems, and identification of activity-dependent topography in this organism has been desired for detailed mechanistic analysis of the role of neuronal activity during circuit development.

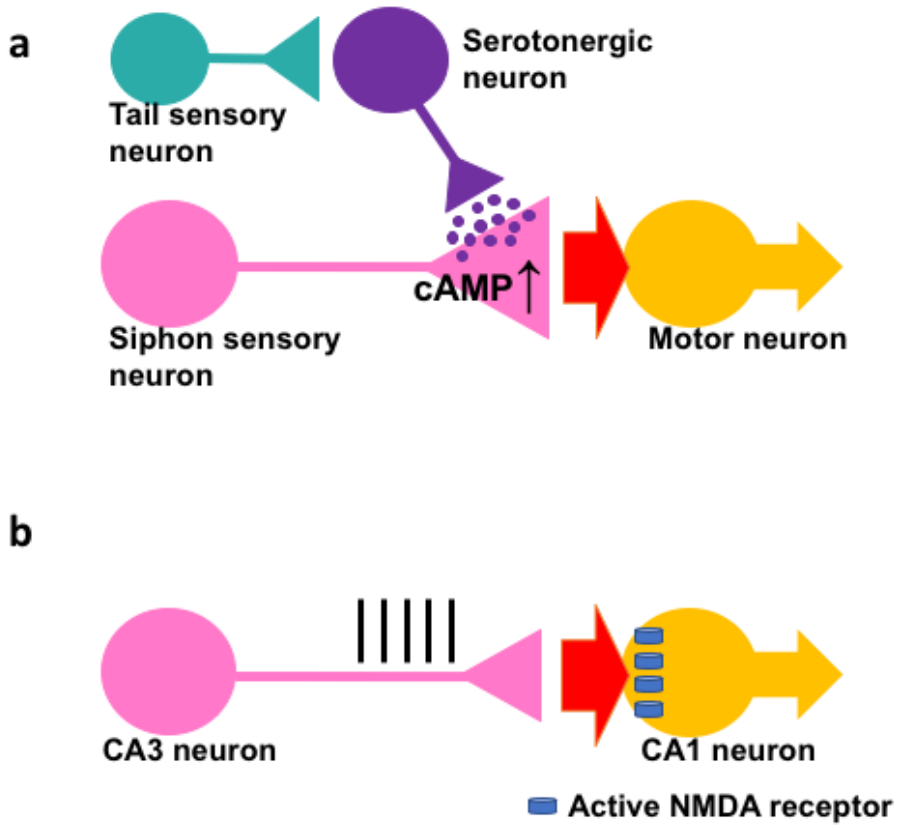


Figure 1.1. Synaptic plasticity in *Aplysia* and the mammalian hippocampus

(a) Synaptic plasticity that underlies sensitization of the gill withdrawal reflex in *Aplysia*. Serotonergic neurons, which are activated by tail sensory neurons, increase the efficacy of synaptic transmission from siphon sensory neurons to motor neurons.

(b) Long-term potentiation in the mammalian hippocampus. High-frequency stimulation to presynaptic CA3 neurons activates NMDA receptors on the postsynaptic terminals of CA1 neurons and consequently elevates synaptic efficacy between CA3 neurons and CA1 neurons.

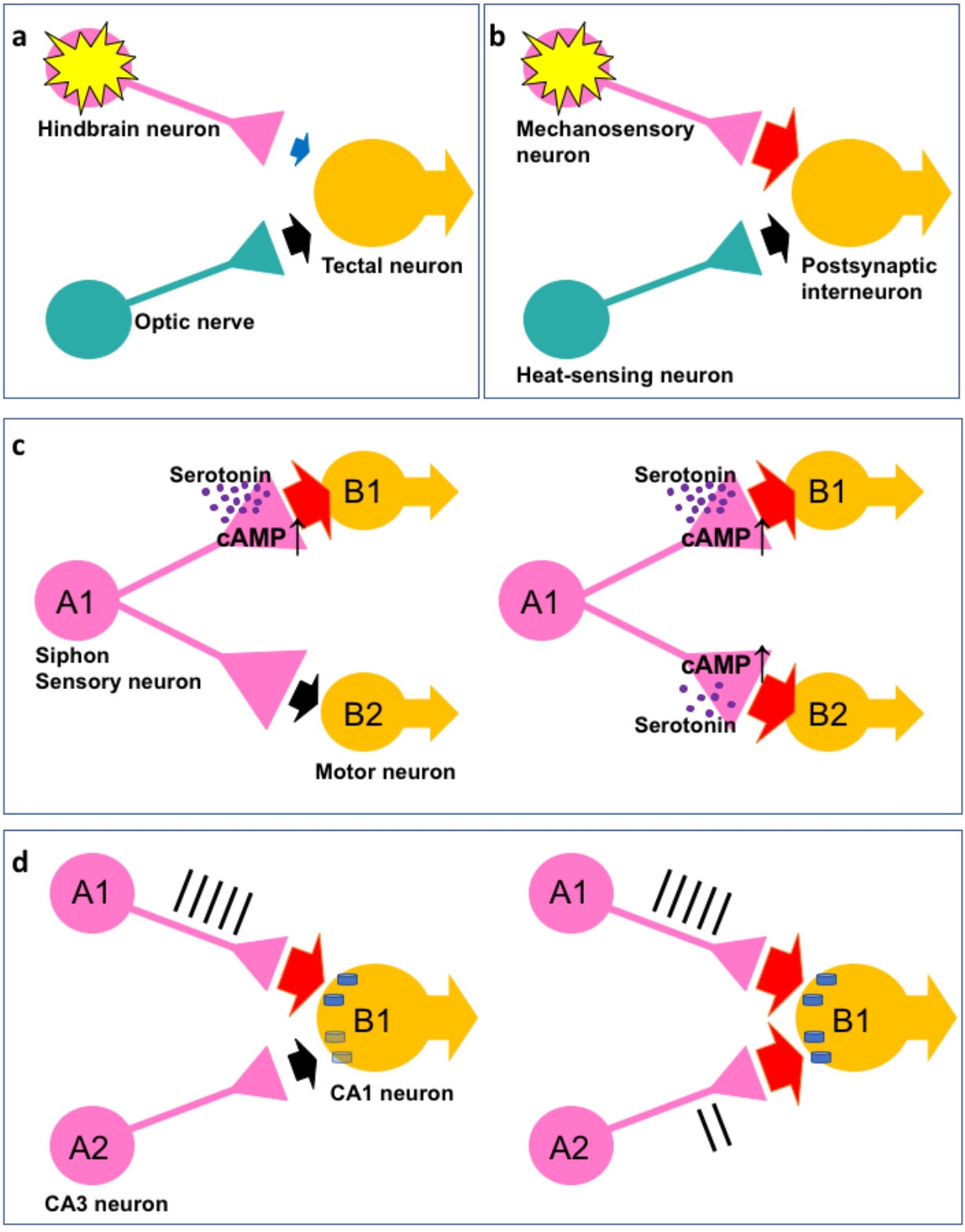

a
Hindbrain neuron
Tectal neuron
Optic nerve
b
Mechanosensory neuron
Postsynaptic interneuron
Heat-sensing neuron
c
Serotonin
B1
cAMP
A1
Siphon Sensory neuron
B2
Motor neuron
Serotonin
B1
cAMP
A1
cAMP
Serotonin
B2
d
A1
B1
CA1 neuron
A2
CA3 neuron
A1
B1
A2

Figure 1.2. Synapse-specific plasticity

(a) Sensory-pathway-specific plasticity in *Xenopus* tadpoles. Neurons in the optic tectum (tectal neurons) receive visual inputs and mechanosensory inputs from two different types of neurons. In response to repeated inputs from one type of presynaptic neurons, tectal neurons specifically suppress the transmission from these presynaptic neurons without disrupting the transmission from the other type of presynaptic neurons.

(b) Sensory-pathway-specific plasticity in *C. elegans*. Mechanosensory stimulation during development enhances synaptic transmission from mechanosensory neurons to multimodal interneurons without affecting the responses of these multimodal interneurons to inputs from heat-sensing neurons.

(c) Synapse-specific plasticity in *Aplysia*. The serotonin-receiving synapse (i.e. A1-B1) is specifically modified, and the other synapse of the same presynaptic sensory neuron (i.e. A1-B2) is unaltered. When a low level of serotonin, which usually induces only short-term sensitization, is given to the A1-B2 synapse in combination with a high level of serotonin on the A1-B1 synapse, both of the A1-B1 and A1-B2 synapses exhibit long-term sensitization – a process called synaptic tagging (Martin, 2002).

(d) Synapse-specific LTP in the mammalian hippocampus. High frequency stimulation to neuron A1 specifically modulates the A1-B1 synapse without changing the A2-B1 synapse. This specificity is attributable to NMDA receptors, which locate on the postsynaptic terminals and become active only when pre- and postsynaptic neurons are coactive. When high frequency stimulation to neuron A1 activates both neuron A1 and neuron B1, a low level of electrode stimulation to neuron A2 sufficiently activates NMDA receptors on the A2-B1 synapse. As a result, both of the A1-B1 and A2-B1 synapses express LTP (Luo, 2015).

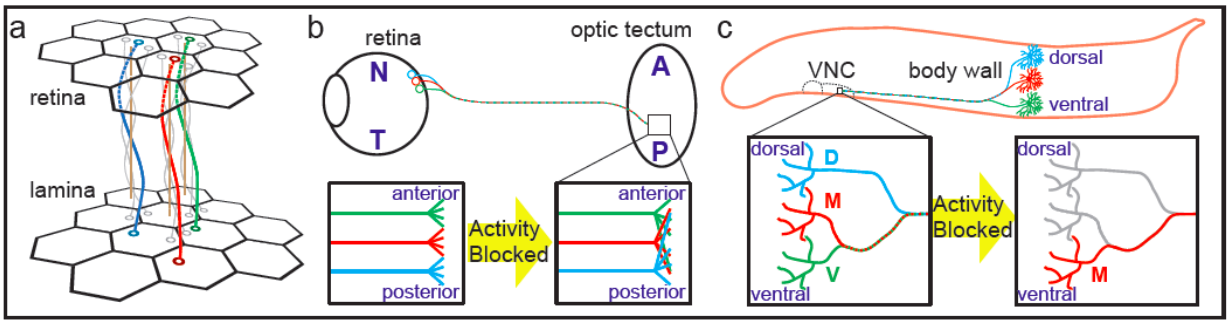


Figure 1.3. Fine-scale topography in *Drosophila* and vertebrates

(a) In the *Drosophila* visual system, R1-R6 photoreceptors form a retinotopic map. R4 photoreceptors from neighboring three ommatidia (labeled in blue, red, or green) terminate at adjacent cartridges in the lamina. Gray: R2 and R5 photoreceptors. Brown: R7 and R8 photoreceptors. Modified from Clandinin and Zipursky 2000.

(b) In the vertebrate visual system, the fine-scale topographic projections of RGCs require neuronal activity. Upon the inhibition of neuronal activity, RGCs exhibit enlarged presynaptic terminals which extensively overlap with adjacent RGC terminals. The square in the tectum is magnified at the bottom. N: nasal. T: temporal. A: anterior. P: posterior.

(c) In the nociceptive system in *Drosophila* larva, the topographic projections of M (in red) and V (in green) neurons depend on the relative levels of neuronal activity. The activity inhibition of M neurons shifts their axon terminals ventrally. VNC: ventral nerve cord.

CHAPTER 2

Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in *Drosophila*

This chapter consists of material from a research article in Neuron, Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in *Drosophila*, written by **Takuya Kaneko**, Ann Marie Macara, Ruonan Li, Yujia Hu, Kenichi Iwasaki, Zane Dunnings, Ethan Firestone, Shawn Horvatic, Ananya Guntur, Orie T. Shafer, Chung-Hui Yang, Jie Zhou and Bing Ye in 2017.

Author contributions are as follows:

T.K., A.M.M., and B.Y. conceived the project and designed the experiments. T.K., A.M.M., R.L., Y.H., K.I., and Z.D. performed behavior analyses. A.M.M. set up the live-imaging system for the larval nervous system. A.M.M., T.K., and R.L. performed the calcium and cAMP imaging. O.T.S. provided reagents and expertise in stimulating neurons with P2X2 and cAMP imaging. Y.H., K.I., and T.K. carried out the studies on neuronal morphology and connectivity. E.F. and Z.D. set up the optogenetic system for studying larval nociceptive behavior. S.H. and J.Z. developed the method and software

for quantifying body angles in larval nociceptive curling. R.L. and A.M.M. developed the assay for AITC-induced nociceptive behavior. A.G. and C.-H.Y. provided initial data suggesting that serotonergic neurons are downstream of the nociceptors. B.Y. supervised the project. T.K., A.M.M., and B.Y. wrote the paper.

I thank all the coauthors of this paper. Special thanks to the co-first author, Ann Marie Macara, who is the main collaborator for this project.

Summary

How experiences during development cause long-lasting changes in sensory circuits and affect behavior in mature animals are poorly understood. Here we establish a novel system for mechanistic analysis of the plasticity of developing neural circuits by showing that sensory experience during development alters nociceptive behavior and circuit physiology in *Drosophila* larvae. Despite the convergence of nociceptive and mechanosensory inputs on common second-order neurons (SONs), developmental noxious input modifies transmission from nociceptors to their SONs, but not from mechanosensors to the same SONs, which suggests striking sensory pathway specificity. These SONs activate serotonergic neurons to inhibit nociceptor-to-SON transmission; stimulation of nociceptors during development sensitizes nociceptor presynapses to this feedback inhibition. Our results demonstrate that, unlike associative learning, which involves inputs from two sensory pathways, sensory pathway-specific plasticity in the *Drosophila* nociceptive circuit is in part established

through feedback modulation. This study elucidates a novel mechanism that enables pathway-specific plasticity in sensory systems.

Noxious Experience during Development Suppresses Larval Nociceptive Behavior

Stimulation of the nociceptors (i.e. C4da neurons) of *Drosophila* larvae elicits a series of behavioral responses, which begin as an abrupt curling of the body and are often followed by rolling the body along the rostrocaudal axis (Hwang et al., 2007) (Figure 2.1A). 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. Among these compounds, allyl-isothiocyanate (AITC), which is found in cruciferous plants and used as a food flavoring, preservative, and, in high concentrations, insecticide (Wu et al., 2009), acts through TrpA1 channels to excite C4da nociceptors in *Drosophila* larvae (Iwasaki et al., 2008). Consistently, we found that AITC activated C4da neurons (Figure 2.1B). To test the consequences of exposure to AITC during development, we reared larvae in an environment containing AITC at a concentration comparable to that found 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; Hwang et al., 2007). Larvae raised on AITC exhibited suppression of nociceptive rolling behavior (Figures 2.1C and

2.2B), suggesting that noxious stimulation during development suppresses nociceptive behavior.

To determine whether developmental activation of nociceptors is sufficient to suppress nociceptive behavior, we specifically activated C4da neurons during development using an optogenetic approach. ChR2 was specifically expressed in C4da neurons and activated in developing larvae with brief pulses of blue light (5 s of illumination followed by a 5-min break). Red light, which does not activate ChR2, was used as a negative control. After larval development was complete 5 days after egg laying (AEL), 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 during development dramatically suppressed nociceptive responses, including rolling, curling, and overall response (as demonstrated by changes in body angle) (Figures 2.1D, 2.2C, and 2.2D).

The extent of the sensory input-induced suppression of nociceptive behavior depended on the intensity of developmental stimulation (Figure 2.1E). A low intensity of optogenetic stimulation during development led to a behavioral suppression that was comparable to AITC-induced suppression (Figure 2.1C). Regardless of the intensity, developmental stimulation suppressed the maximal responses (Figure 2.1E), which effectively reduced the gain of the nociceptive circuit.

ChR2-mediated suppression of nociceptive behavior is not due to the bleaching of all-trans-retinal (ATR), a key component in ChR2 function (Figures 2.2E and 2.2F). Consistent with a previous report that intense blue light activates nociceptors (Xiang et al., 2010), illumination with blue light alone (without ATR) during development led to a mild decrease in nociceptive rolling (Figure 2.2G). Moreover, developmental activation

of nociceptors did not change the size or targeting of their presynaptic terminals (Figure 2.3).

This behavioral suppression was not due to acute changes, because it was absent in larvae reared under constant darkness for 5 days before being illuminated with pulsed red or blue light for 1 hr (Figure 2.1F). Furthermore, stimulating nociceptors on days 3 and 4 (late second and early third instar larval stages) suppressed the nociceptive response in mature larvae that were tested on day 5 (Figure 2.1G), suggesting that the functional development of larval nociceptive behavior is regulated by nociceptor activity. This result also demonstrates that the plasticity is long lasting (>24 hr).

Taken together, these results show that noxious experience during development leads to long-lasting suppression of nociceptive behavior in the *Drosophila* larva (Figure 2.1H).

A08n Neurons Are Specific Postsynaptic Targets of Nociceptors

To identify the mechanism that underlies the sensory input induced 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, 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 2.4A). Using an improved GFP reconstitution across synaptic partners (GRASP) technique, termed

synaptobrevin-GRASP (syb-GRASP) (Macpherson et al., 2015), we found that A08n dendrites are synaptic partners of C4da axon terminals, but not those of mechanosensory neurons (Figures 2.4B and 2.4C), raising the possibility that C4da-to-A08n synaptic transmission is dedicated to the nociceptive circuit. To test this, 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 2.4D). These AITC-elicited responses were nociceptor dependent, because no response was observed when C4da neurons were genetically ablated or the peripheral nervous system (PNS) was disconnected from the CNS. As shown later in Figure 2.6, the nociceptor-elicited responses in A08n neurons were further confirmed by chemogenetic stimulation of nociceptors. In contrast, activation of mechanosensors did not elicit any response in A08n neurons (Figure 2.4E), suggesting that C4dato-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 activation of both nociceptors and mechanosensors (Figures 2.4F and 2.4G).

The role of A08n neurons in larval behavior has not been identified, although optogenetic stimulation of GMR82E12-GAL4-expressing neurons, which include 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 elicited abrupt body curling (Klapoetke et al., 2014), indicating that these neurons play a major role in the initial step of nociceptive behavior (Figure 2.4H).

In addition to the A08n neurons in the ventral nerve chord (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 2.4I and 2.5). 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 responses at the same level as larvae that expressed CsChrimson in all GMR82E12-GAL4 neurons; larvae expressing CsChrimson in one of the two A08n neurons responded at about half the level of those that expressed in both neurons. Therefore, activating A08n neurons is sufficient to elicit nociceptive behavior. We then 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 we recorded behavioral responses. Larvae with inhibited A08n neurons showed a reduction in nociceptive behavior in response to C4da activation (Figure 2.4J). 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.

Nociceptor Input-Induced Plasticity Is Sensory Pathway Specific

Results presented so far show that increased input through nociceptors during development leads to long-lasting suppression of nociceptive behavior. Moreover, while Basin-4 neurons receive synaptic inputs from both nociceptors and mechanosensors, A08n neurons receive synaptic inputs from nociceptors, but not mechanosensors. Next, we set out to identify where the plasticity occurs. We first examined the activity of the nociceptive pathway-specific target neurons A08n by calcium imaging. Nociceptors were stimulated during development by either ChR2-mediated optogenetics followed by acute AITC stimulation in mature larvae for calcium imaging (Figures 2.6A and 2.7) or by AITC during development followed by acute chemogenetic activation of nociceptors for calcium imaging (Yao et al., 2012) (Figures 2.6B–D). In the chemogenetic approach, the vertebrate P2X2 receptor, an ATP-activated cation channel absent in *Drosophila* (Hu et al., 2010; Lima and Miesenbo¨ ck, 2005), was specifically expressed in nociceptors. Perfusion with solutions containing ATP activates P2X2 (Yao et al., 2012; Zemelman et al., 2002) and, consequently, stimulates nociceptors.

A08n neurons' response to nociceptor stimulation was significantly reduced after developmental stimulation (Figures 2.6A and B). A similar reduction in A08n response was caused by C4da activation on days 3 and 4 of development (Figure 2.7), which is consistent with the behavioral output (Figure 2.1G).

We next took advantage of the multimodal inputs to Basin-4 to study the specificity of sensory input-induced plasticity of the larval nociceptive circuit. After developmental treatment of AITC, Basin-4's responses to nociceptor stimulation were significantly reduced (Figure 2.6C). In contrast, Basin-4 response to mechanosensor

stimulation was not affected by enhanced noxious inputs during development (Figure 2.6D).

Suppression of C4da-to-SON 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 2.6E and F).

These results suggest that nociceptive inputs during development specifically suppress synaptic transmission from nociceptors to SONs, but not from mechanosensors—even to the same SONs (Figure 2.6G).

Serotonergic Neurons Are Required to Establish Sensory Input-Induced Plasticity of the Larval Nociceptive Circuit

Next, we set out to determine what mediates developmental experience-dependent suppression of nociceptive behavior. 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 express tryptophan hydroxylase (TRH) (Huser et al., 2012), are near the C4da axon terminals (Figure 2.8A). This raises the possibility that serotonin (5-HT) modulates synaptic transmission from C4da neurons to their targets and contributes to the synaptic and behavioral plasticity of the larval nociceptive circuit.

To test this, we silenced serotonergic neurons and examined the consequences for nociceptive behavior. Silencing serotonergic neurons partially rescued nociceptive

behavioral responses in larvae whose nociceptors had been stimulated during development (Figures 2.8B and C). Silencing serotonergic neurons did not affect nociceptive behavioral responses when nociceptors were not stimulated during development (Figure 2.9). These results suggest that serotonergic neurons are required to establish nociceptive input-induced plasticity in larvae.

We next investigated whether enhancing serotonergic signaling during development leads to suppression of nociceptive behavior. Feeding larvae with the precursor to serotonin, 5-hydroxytryptophan (5-HTP), which increases serotonin levels in the body (Yuan et al., 2006), led to subdued nociceptive behavior (Figure 2.8D). Feeding 5-HTP, which has a half-life of about 4 hr (Westenberg et al., 1982), to larvae during the first 2 days of development also suppressed nociceptive behavior (Figure 2.8E), suggesting that the effects on behavior are due to developmental changes in the nociceptive circuit.

Consistent with the results from behavioral studies, silencing serotonergic neurons rescued A08n responses to nociceptors in mature larvae that had experienced chronic noxious inputs (Figure 2.8F), while feeding larvae with 5-HTP led to a suppression of C4da-to-A08n transmission (Figure 2.8G). Furthermore, optogenetic activation of serotonergic neurons during development reduced C4da-to-A08n transmission (Figure 2.8H). Since serotonergic neurons were not activated during calcium imaging in these experiments, this result again demonstrates that developmental activation of serotonergic neurons suppresses C4da-to- SON synaptic transmission.

Taken together, these results suggest that serotonergic neurons play an essential role in establishing nociceptive inputinduced plasticity in the developing larval nociceptive circuit.

Stimulation of Nociceptors Activates Serotonergic Neurons via SONs in the Nociceptive Pathway

To understand how serotonergic neurons contribute to nociceptor input-induced plasticity, we investigated whether nociceptors regulate the activity of serotonergic neurons. Stimulation of nociceptors activated serotonergic neurons in larval VNC (Figure 2.10A). Silencing A08n, Basin-4, or all four Basin (Basin-1–4) neurons partially reduced nociceptor-induced activation of serotonergic neurons (Figure 2.10B), suggesting that nociceptors activate serotonergic neurons through the SONs. To directly test this, we stimulated A08n or Basin-4 neurons with ATP/P2X2, and we found that both SONs activated serotonergic neurons (Figure 2.10C). These results suggest that stimulation of nociceptors activates serotonergic neurons through the SONs.

Serotonergic Signaling Inhibits Nociceptor-to-SON Transmission

We then investigated how serotonergic signaling modulates nociceptor-to-SON synaptic transmission. Our results suggest that serotonergic signaling modulates this synapse through presynaptic inhibition (Figure 2.10D). First, 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 2.10D and E). In *Drosophila*, ipsapirone may act as an agonist to both 5-HT1a and 5-HT1b, since these two receptors are similarly homologous to mammalian 5-HT1a receptors (Saudou et al.,

1992). Second, ipsapirone reduced nociceptor-evoked increases in cyclic AMP (cAMP) levels in C4da presynaptic terminals (Figure 2.10F). Because 5-HT1 receptors are G protein-coupled receptors (GPCRs) that downregulate cAMP levels in cells (Raymond et al., 1999), this result suggests serotonergic modulation of C4da presynaptic terminals. Last, RNAi-mediated knockdown of 5-HT1b in C4da neurons, but not that of 5-HT1a or an unrelated protein (mCherry), reduced inhibition of C4da-to-A08n transmission by ipsapirone (Figure 2.10G). In contrast, knockdown of 5-HT1b in A08n neurons had no effect (Figure 2.10H). These results suggest that 5-HT1b in C4da axon terminals mediates inhibition of nociceptor-to-A08n transmission by serotonin.

Consistent with physiological results, we found that the terminals of serotonergic neurons intimately intertwined with C4da presynaptic terminals (Figure 2.11A). However, we did not detect significant syb-GRASP signal from serotonergic terminals to C4da presynaptic terminals (Figure 2.11B). This suggests that the distance between serotonergic terminals and C4da presynaptic sites is larger than that of conventional synapses (<100 nm). This finding may not be surprising because serotonergic terminals are known to modulate synaptic transmission through volume transmission or extra-synaptic transmission. In fact, 5-HT1b receptors are found predominantly at extra-synaptic and nonsynaptic sites in rat brains (Riad et al., 2000).

Taken together, these results suggest that SONs activate serotonergic neurons to inhibit nociceptor-to-SON synaptic transmission by acting on nociceptor presynaptic terminals, forming an inhibitory feedback loop (Figure 2.10I).

Developmental Stimulation of Nociceptors Sensitizes Nociceptor Presynaptic Terminals to Serotonergic Inhibition

Our finding of feedback serotonergic modulation led us to hypothesize that developmental stimulation of nociceptors alters the level of this modulation to establish plasticity in C4da-to-SON transmission. Developmental stimulation of C4da neurons did not affect C4da-elicited responses of serotonergic neurons (Figure 2.12A). Thus, we tested the possibility that developmental stimulation sensitizes nociceptor presynaptic terminals to serotonergic inhibition of C4da-to-SON transmission. Results from two experiments support this possibility. First, 1 mM ipsapirone significantly inhibited C4da-to-A08n transmission after developmental stimulation of nociceptors, but not when nociceptors were not stimulated during development (Figure 2.12B). Similarly, while 10 mM ipsapirone only partially inhibited C4da-to-A08n transmission when nociceptors were not stimulated during development, it completely inhibited this synaptic transmission after developmental stimulation of nociceptors. These results indicate enhanced sensitivity to ipsapirone of C4da-to-A08n synaptic transmission. Second, developmental stimulation of nociceptors significantly reduced the sensory input-induced increase in cAMP levels in C4da axon terminals (Figures 2.12C and D), without affecting basal cAMP levels (Figure 2.12E).

Consistent with a change in presynaptic modulation of nociceptor-to-target transmission, developmental stimulation of nociceptors did not reduce the responsiveness of SONs. Larval sensory neurons are known to use acetylcholine (ACh) as the transmitter (Salvaterra and Kitamoto, 2001). We tested A08n responses to different concentrations of nicotine, which specifically activates ionotropic ACh

receptors (AChRs), and we recorded A08n response by calcium imaging. Developmental activation of nociceptors did not reduce A08n's response to nicotine (Figure 2.12F).

Taken together, these results suggest that nociceptor activity during development enhances 5-HT1R-mediated inhibition of nociceptor-to-SON transmission by regulating presynaptic terminals. Such sensory afferent-specific regulation of presynaptic terminals likely maintains the function of other sensory pathways that share central neurons with the nociceptive circuit.

We define here a novel mechanistic model that explains pathway specific, experience-dependent plasticity during development. Nociceptors activate SONs in the nociceptive circuit, which in turn activate modulatory serotonergic neurons; the latter suppress transmission from nociceptors to SONs, thereby forming a feedback circuit motif. During development, stimulation of nociceptors activates serotonergic neurons to sensitize nociceptor presynaptic terminals to serotonergic inhibition, reducing nociceptive behavioral responses in mature larvae (Figure 2.12G).

Materials and Methods

Drosophila melanogaster strains

The following fly stocks are used in this study. Both male and female wandering 3rd-instar larvae are used unless otherwise noted. All experiments were conducted on age- and size-matched larvae.

GAL4/LexA stocks: GMR82E12-GAL4 (Bloomington *Drosophila* Stock Center, stock number B-40153) and 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), GMR57F07-lexA (B-54899), and GMR72F11-Gal4 (B-39786) (Ohyama et al., 2015); TRH-GAL4 (B-38389); TRH-LexA (B-52248).

UAS/LexAop stocks: UAS-CD4-GFP (BL-35836); UAS-GCaMP6f (Chen et al., 2013); LexAop-GCaMP6f (Chen et al., 2013) (B-44277); UAS-Epac1-camps (Shafer et al., 2008); UAS-ChR2::YFP (Honjo et al., 2012); UAS-CsChrimson::Venus (B-55139) (Klapoetke et al., 2014); LexAop-P2X2 (Yao et al., 2012); UAS-DenMark (B-33062) (Nicolaı¨ 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::spGFP1-10 and LexAop-CD4::spGFP11 (Macpherson et al., 2015); UAS-RNAi-5-HT1b (B-33418, designated as #1); UAS-RNAi-5-HT1b (B-27635, designated as #2); UAS-RNAi-5-HT1a (B-33885); UAS-RNAi-5-HT7 (B-32471); UAS-FRT-rCD2-stop-FRT-CD8::GFP, UAS-FRT-rCD2-stop-FRT-CD4::tdTomato (Yang et al., 2014).

Other stocks: hs-flp122 (B-1929), tubP-FRT-Gal80-FRT (B-38880), and ppk-ChR2::YFP. 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 between the attR1 and attR2 sites, which are downstream of the 1-Kb promoter of the ppk gene. The resulting plasmid, pDEST-APPHIH-ChR2, was then used for transgenesis with the fC31 system.

FLP-out mosaic labeling

Mosaic A08n labeling (Figure 2.4B) and stimulation (Figures 2.4I and 2.5) were achieved by the combination of a FRT-flanked Gal80 transgene that is under the control of a tubulin promoter (tubP-FRT-Gal80-FRT), and a transgene expressing heat-inducible flippase (hs-FLP) (Gordon and Scott, 2009). Upon flippase-mediated removal of Gal80, A08n neurons express CD4-GFP, DenMark, and Syt-HA (Figure 2.4B), or CsChrimson::Venus (Figures 2.4I and 2.5). UAS-FRT-rCD2-stop-FRT-CD8::GFP and UAS-FRT-rCD2-stop-FRTCD4::tdTomato (Yang et al., 2014) were used in combination with hs-FLP and TRH-GAL4 to label single serotonergic neurons with fluorescent proteins in Figure 2.8A. Genetic mosaic clones were generated using the method described previously (Yang et al., 2014).

Behavioral tests

Embryos were collected for 6-12 hr at 25C on basic fly food. The fly food was mixed with 4 mM ATR for optogenetic stimulation, 2.5 to 5 mM allyl isothiocyanate (AITC, Sigma) for AITC-mediated developmental stimulation, or 5 mM 5-hydroxytryptophan (5-HTP, Sigma) for 5-HTP feeding. On day 5, mature (wandering 3rd instar) larvae were transferred to room temperature, removed from food, rinsed, and left in the dark for 1 hr before being tested for behavioral responses. Control and experimental groups were paired in all behavioral tests.

Optogenetic behavioral tests were done on 35 mm grape-agar plates covered with 1 mL water in a dark room. For optogenetic stimulations, a light intensity of 26 mW/mm2 was used unless otherwise stated. 470nmBlue LED (CREE XP-E

Blue3WLED, RapidLED) was used for ChR2 activation. ChR2-mediated nociceptor stimulation was performed by ppk-GAL4/UAS-ChR2::YFP or ppk-ChR2::YFP, as indicated in the figures. Neurons in the CNS—A08n and TRH—were stimulated by expressing CsChrimson::Venus using 617 nm red LED (CREE XP-E Red LED, RapidLED). LED stimulation was for 5 s.

AITC behavioral tests were performed on 12-well grape-agar plates. Each well was covered with 300 mL of 25 mM AITC before transferring one larva to the well. Only single larvae were placed on each well so that individual larvae could be tracked for two min.

Sample sizes were estimated based on previous publications in the field (Jovanic et al., 2016; Ohyama et al., 2015). To reduce possible variation in each batch of fly cultures, samples from more than 3 separate trials, each of which included similar numbers of samples, were analyzed. The behavioral tests with AITC as the stimulant were more subject to variations of experimental conditions. To eliminate experimenter bias, these experiments were done in double-blind fashion. The video recorded by the primary experimenter was coded and randomized by another experimenter. After the primary experimenter quantified the data, the recordings were decoded for statistical analysis. All behavioral data were included in the statistical analyses.

Developmental optogenetic stimulation

Automatic optogenetic stimulation (26 mW/mm2) of nociceptors during development was achieved by a custom-made array of LEDs. Illumination duration and frequency were controlled by a BASIC Stamp microcontroller, and intensity by aPWMDimmer.

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 passed through an 80_CREE XP-E/XP-G lens. Developmental optogenetic stimulation was performed in a 25_C incubator. LEDs were programmed to turn on for 5 s every 5 min throughout the appropriate experimental duration.

Calcium and cAMP imaging

Live imaging of calcium and cAMP levels was conducted on a Leica SP5 confocal microscope equipped with a resonant scanner, an acousto-optical beam splitter, and an HC Fluotar L 25x/0.95WVISIR immersion objective (Leica). Calcium levels were measured by imaging with the GCaMP6f indicator (Chen et al., 2013). Larvae were dissected as previously described (Matsunaga et al., 2013). Dissection and imaging were performed in a modified hemolymph-like 3 (HL3) saline (70mM NaCl, 5 mM KCl, 0.5 mMCaCl2, 20mM MgCl2, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.2) (Stewart et al., 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., 2002, 2005). A low concentration of calcium was also used to reduce muscle contraction and nerve damage during dissection (Caldwell et al., 2013). Calcium responses were recorded in xyzt mode such that an image stack was taken every 1 s or 2 s over the course of the experiment.

ATP/P2X2 technique was used to stimulate neurons for calcium imaging with the expression of rat ATP-sensitive cation channel P2X2 (Yao et al., 2012). Because there

are no ATP-sensitive channels in *Drosophila*, ATP application excites neurons that express P2X2 (Yao et al., 2012). This approach is particularly instrumental when GCaMP-implemented calcium imaging is used to record neuronal activity due to an overlap in the excitation wavelengths of ChR2, CsChrimson (activated by both red and blue light), and GCaMP. Unless otherwise stated, 1mMATP was used to activate neurons expressing P2X2. ATP (Sigma) was first dissolved in water to 100mMand then diluted to the final concentrations with HL-3. We used the ValveBank 4 Pinch Valve Perfusion System (Automate Scientific) to control perfusion of ATP.

In addition to the ATP/P2X2 technique, AITC was used as a natural stimulant to activate nociceptive neurons, and nicotine to activate A08n neurons. AITC, ipsapirone (Tocris), and (-)-nicotine tartrate (Fisher Scientific) were bath-applied to the specimen; concentrations are indicated in the figure legends. AITC (Sigma) was first dissolved in DMSO to 1 M, and then diluted to the final concentrations with HL-3. In experiments that test the effects of ipsapirone while stimulating nociceptors, ipsapirone was mixed with AITC before being applied to the samples.

cAMP levels were measured by imaging with the Epac1-camps indicator (Shafer et al., 2008; Yao et al., 2012). The procedure for cAMP imaging was the same as that for Ca2+ imaging described above except for the following. The Epac1-camps indicator was specifically expressed in nociceptors, whose presynaptic terminals were imaged for FRET. Hence the observed cAMP levels were only those in C4da presynaptic terminals. 2 mM AITC was used to activate nociceptive neurons. In Figure 6C, ipsapirone was mixed with AITC before being applied to the specimen. A Z stack was taken every 3 s over the course of the experiment.

Sample sizes were estimated based on previous publications in the field (Jovanic et al., 2016; Ohyama et al., 2015; Yao et al., 2012). To reduce possible variation in each batch of fly cultures, samples from more than 3 separate trials, each of which included similar numbers of samples, were analyzed. Several key findings, including the effects of developmental stimulation on behavior and synaptic transmission and the pathway-specificity of the plasticity were repeated by multiple experimenters. All data were included in the analyses with the exception that neurons with low levels of AITC-evoked responses (< 50% MaxDF/F0) before ipsapirone treatment were not included in Figures 5G and 5H. This is to minimize the masking of ipsapirone suppression caused by low levels of initial response.

Immunostaining

Third instar larvae were dissected and stained as described previously (Kim et al., 2013; Wang et al., 2013). Primary antibodies used were: mouse anti-GFP (1:100, Sigma, RRID: AB_259941); chicken anti-GFP (1:2500, Aves Laboratories, RRID: AB_2307313); rabbit anti-RFP (1:5000, Rockland, RRID: AB_2209751); and rat anti-HA (1:250, Roche, RRID: AB_390915). Secondary antibodies used were (1:500, Jackson ImmunoResearch): donkey anti-mouse Alexa Fluor 488 (RRID: AB_2340846), anti-chicken Alexa Fluor 488 (RRID: AB_2340375), anti-chicken Alexa Fluor 647 (RRID: AB_2340380), anti-rabbit Rhodamine RX (RRID: AB_2340613), and anti-rat Alexa Fluor 647 (RRID: AB_2340694).

Synaptobrevin-GRASP

Syb-GRASP was performed as described previously (Macpherson et al., 2015). Reconstituted GFP (i.e., GRASP) signals were detected by anti-mouse monoclonal GFP antibody (1:100, Sigma, RRID: AB_259941, referred to as anti-GRASP). Syb::spGFP1-10 was preferentially detected by anti-chicken polyclonal antibody (1:2500, Aves Laboratories, RRID: AB_2307313, referred to as anti-spGFP1-10).

Quantification of behaviors

Rolling was counted only when a larva exhibited at least one complete (360_) rotation of the body along a rostrocaudal axis (Hwang et al., 2007). 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 larvae that rolled and curled were manually counted in slow motion (0.5 - 0.25 x speed) over the 5 s stimulation interval for optogenetic assays or over the 2 min period of AITC stimulation. Percent rolling and curling were the number of larvae that rolled or curled divided by the total number of larvae counted (number of larvae that rolled or curled x 100/Total larvae).

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 and its middle and end points, a custom ImageJ plugin was developed to first detect and track the larvae at each frame. Contours of the detected larvae were then smoothed using a 3x3 mean filter. A 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 above.

Quantification of calcium and cAMP Responses

The ImageJ software (NIH) with the Time Series Analyzer plugin was used to analyze the xyzt projection movie. The average intensity of a selected region of interest (ROI) was measured over the course of the experiment.

To quantify calcium responses, the change in GCaMP6f fluorescence was calculated by DF/F0 = (Ft - F0)/F0, where Ft is the fluorescent mean value of an ROI in a given frame. F0 is the baseline 30 s interval before stimulation. Max DF is the maximum value during the stimulation period of the recording. To analyze the activity levels of A08n neurons, Max DF/F0 for individual A08n neurons were plotted on the graph and used for statistical analyses (i.e., each dot indicates one A08n). Because the responses of Basin-4 neurons—unlike A08n neurons—are highly variable within each VNC (data not shown) (Jovanic et al., 2016), the average of Max DF/F0 of 10 Basin-4 neurons from abdominal segments 3-7 in each VNC were calculated to represent the Basin-4 activity in each VNC.

The cAMP response level was quantified as changes in the inverse FRET ratio, which is CFP intensity divided by YFP intensity (DCFP/YFP) scanned with a 458-nm confocal laser. The cAMP basal level was quantified as YFP intensity scanned with a

458-nm divided by that scanned with a 515-nm laser (YFP458/YFP515). The n represents the number of larvae tested.

Statistical analysis

Statistical analysis was performed using Prism software. Fractions of rolling and curling behavior under stimulation were analyzed using the chi-square test. Calcium and cAMP imaging, and axon terminal length of single C4da neurons were analyzed using the nonparametric Mann-Whitney U-test. For multi-group comparisons, Kruskal-Wallis tests were conducted. 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 errors of the mean.

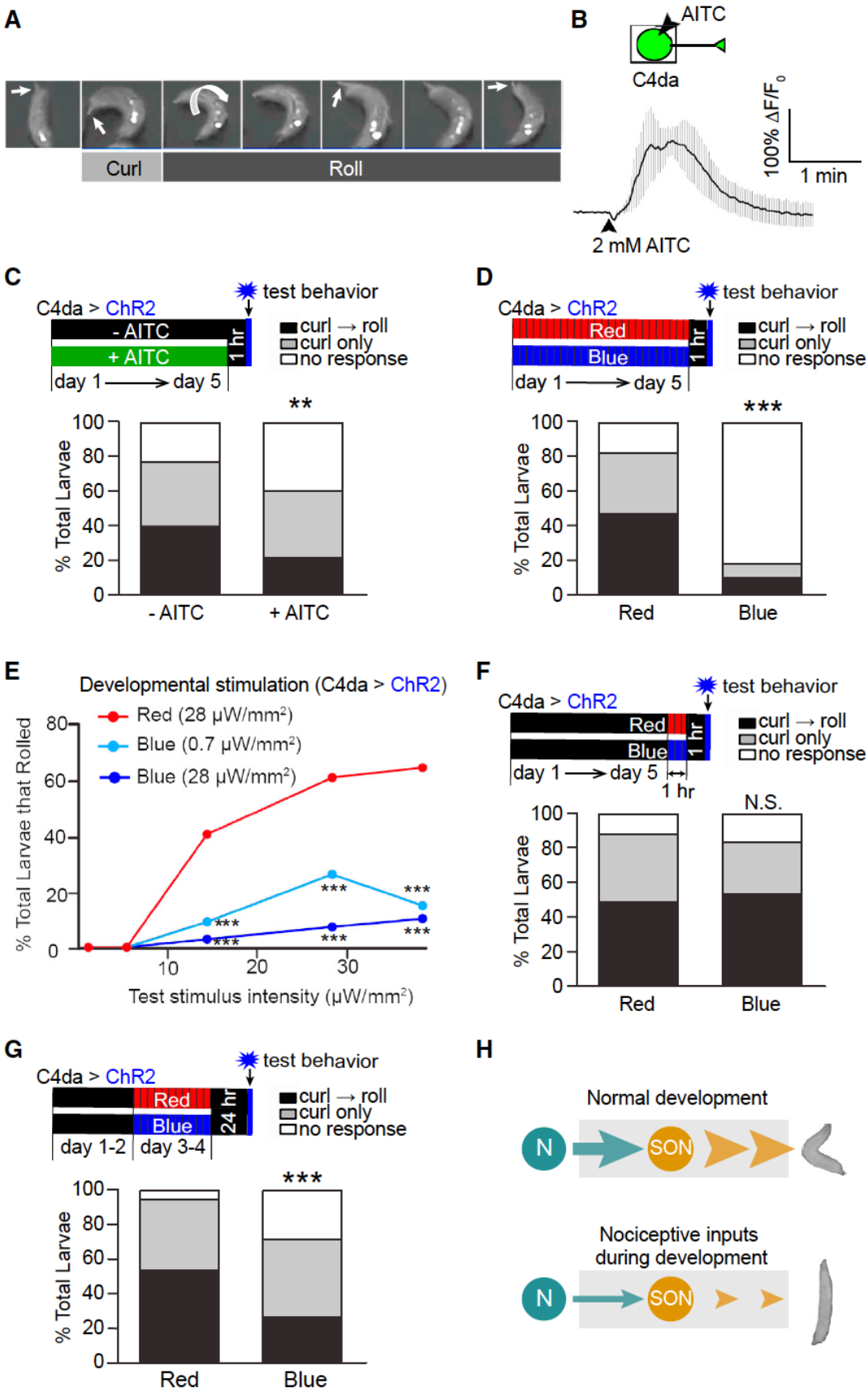

A
Curl
Roll
B
AITC
C4da
100% ΔF/F0
1 min
2 mM AITC
C
C4da > ChR2
- AITC
+ AITC
1 hr
day 1
day 5
test behavior
curl → roll
curl only
no response
**
% Total Larvae
- AITC
+ AITC
D
C4da > ChR2
Red
Blue
***
Red
Blue
E
Developmental stimulation (C4da > ChR2)
Red (28 μW/mm²)
Blue (0.7 μW/mm²)
Blue (28 μW/mm²)
% Total Larvae that Rolled
Test stimulus intensity (μW/mm²)
F
C4da > ChR2
1 hr
N.S.
G
C4da > ChR2
24 hr
day 1-2
day 3-4
H
Normal development
N
SON
Nociceptive inputs
during development

Figure 2.1. Activation of Larval Nociceptors during Development Suppresses Nociceptive Behavior in Mature Larvae

(A) A montage showing nociceptive behavior responses, including curling and rolling, in a third instar larva. ChR2 was expressed specifically in C4da nociceptors and activated by illumination with blue light. Small arrows point to the openings of dorsal trachea to indicate the larva's body position, and the large arrow indicates the direction of rolling.

(B) AITC activates C4da neurons. The boxed area in the schematic indicates the part of the cell imaged for calcium signals. The trace shows the average of responses (n = 3).

(C) Larvae raised on AITC exhibit reduced nociceptive behavior. Top panel: a diagram shows 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 the time when the larvae are at the late third instar stage. Control larvae were reared on regular food throughout development (black bar), whereas experimental larvae were reared on food containing 2.5 mM AITC (green bar). The vertical blue bar indicates the blue light used in optogenetic stimulation for behavioral tests on day 5, and the vertical black bar indicates the 1 hr of darkness prior to the tests. Throughout the paper, the following color coding is used: green, AITC; black, darkness or no AITC; red, red light (617 nm); blue, blue light (470 nm); and gray, ATP. Moreover, the expression of proteins for stimulating nociceptors (i.e., ChR2, CsChrimson, and P2X2) is indicated by ''C4da > proteins'' above the timeline bars. Bars represent the percentage of total larvae that performed rolling following curling (curl /roll), curling only, and no response (n = 75 larvae per group). Note that every roll starts with curling.

(D) Optogenetic stimulation of nociceptors during larval development leads to a dramatic suppression of nociceptive behavior (n = 45 larvae per group).

(E) Developmental stimulation suppresses the maximal response ofnociceptivebehavior inmature larvae. Larvae that expressed ChR2 in C4da neurons were reared under pulses of red (28 mW/mm2) or blue light (0.7 or 28 mW/mm2) during development. Rolling responses of late third instar larvae were tested with five different intensities of blue light (0.7, 5, 14, 28, and 38 mW/mm2) (n = 115, 88, and 69 larvae for red, 0.7 mW/mm2 blue, and 28 mW/mm2 blue, respectively).

(F) Larvae that received acute stimulation (1 hr) of nociceptors do not exhibit suppression of nociceptive behavior (n = 60 larvae per group).

(G) Stimulating nociceptors on days 3 and 4 AEL suppressed nociceptive behavior in mature larvae tested on day 5 (i.e., 24 hr after pulse stimulation) (n = 100 larvae for each group).

(H) A set of diagrams that summarize the results in this figure. Stimulation of nociceptors during development leads to suppression of nociceptive behavior in mature larvae.

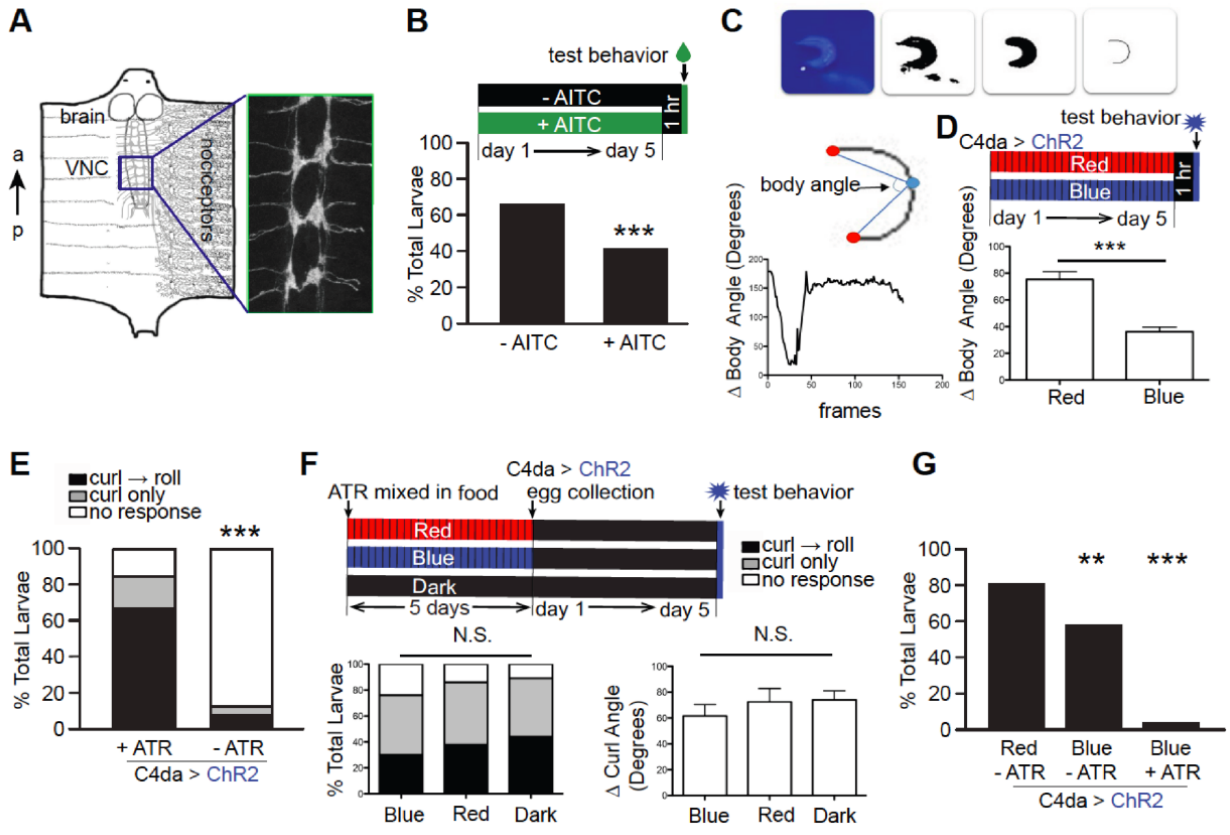


Figure 2.2. Developmental stimulation of nociceptors suppresses nociceptive behavior.

(**A**) Schematic of a larval fillet used for live-imaging studies. "a" and "p" indicate anterior and posterior of the larval fillet, respectively. The axon terminals of C4da neurons in several segments are magnified.

(**B**) Raising larvae on AITC (5 mM) leads to suppression of nociceptive behavior. The behavioral tests were performed with AITC on 3rd instar larvae. The graph indicates the percentage of larvae showing a complete (i.e. 360°) rolling within 2 min in response to 25 mM AITC. *n* = 440 and 124 larvae for – AITC and + AITC, respectively.

(**C**) Diagram illustrating the automatic measurement of larval body angle to quantify 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, and 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/sec.

(**D**) Optogenetic activation of C4da neurons during development results in a significant reduction in curling (*n* = 70 larvae for each group).

(**E**) All-trans-retinal (ATR) is required for ChR2 activation of C4da neurons (*n* = 115 larvae for each group). Larvae were developed in constant darkness for 5 days in

food with or without ATR. Larvae developed without ATR (right) do not exhibit robust responses to blue light compared to larvae with ATR (left).

(**F**) ChR2-mediated suppression of nociceptive behavior is not due to ATR bleaching. 4 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 for 5 days before being tested for behavior. No change in nociceptive behavior was observed between the tested conditions, as analyzed using Kruskal-Wallis tests. *n* = 30, 30 and 20 larvae for blue, red and dark, respectively, for curling angle analysis; *n* = 60, 60 and 45 larvae for blue, red and dark, respectively, for percent of total larvae that exhibited nociceptive responses.

(**G**) Illumination with blue light alone (without ATR) during development led to a mild decrease in nociceptive rolling. The behavior test was performed with 100 mM AITC. *n* = 58, 59, and 83 larvae for Red – ATR, Blue – ATR, and Blue + ATR, respectively.

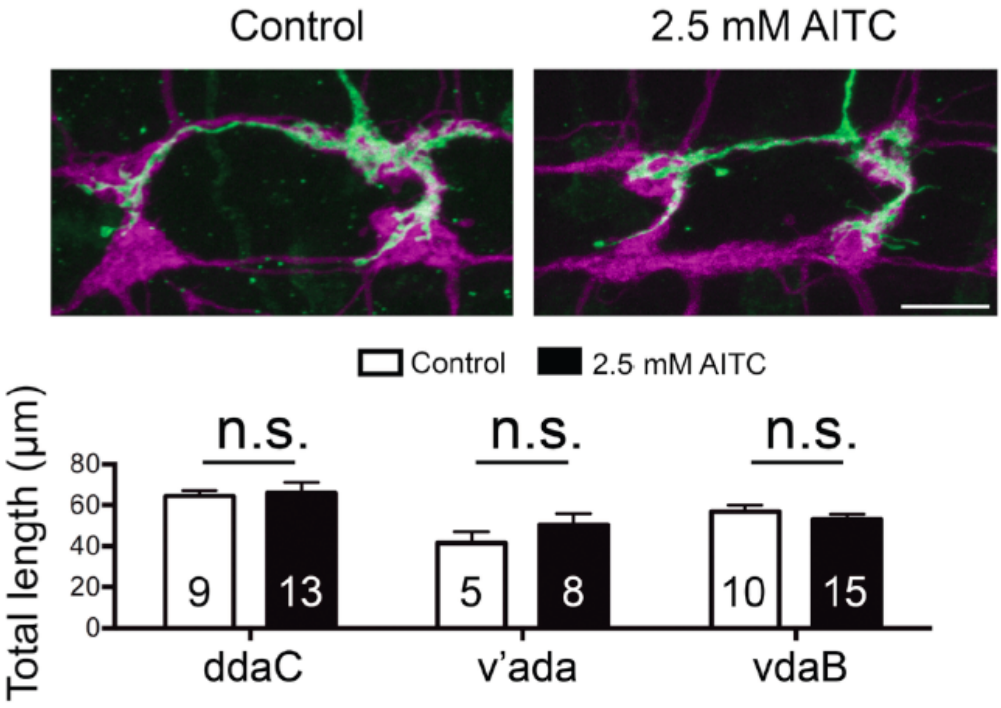


Figure 2.3. Developmental activation of C4da nociceptors with AITC does not change the size or targeting of their presynaptic terminals.
Micrographs show the presynaptic terminal of a representative ddaC neuron (green) from each group. Presynaptic terminals of the other C4da neurons in the same body segment were labeled using a different fluorescent protein (magenta). Quantification of each subtype of C4da neurons (ddaC, v'ada, and vdaB) is shown in the bar chart. Scale bar: 10 μm.

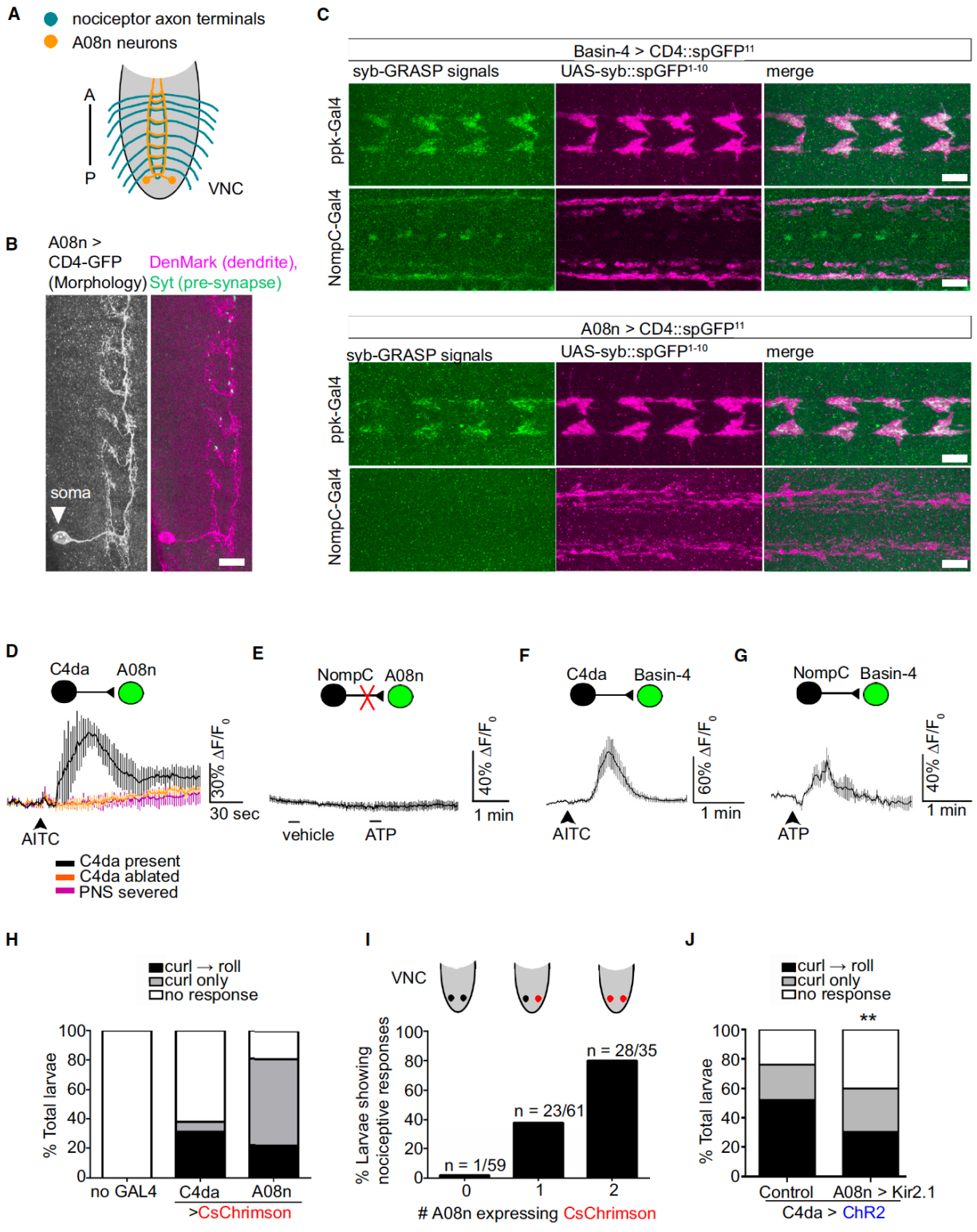


Figure 2.4. A08n Neurons Are Specific Postsynaptic Targets of Nociceptors

(A) A schematic of the axon projections of nociceptors (blue) and the neurites of A08n within the VNC (orange). A, anterior; P, posterior.

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

(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. SpGFP11 expressed in Basin-4 neurons produces syb-GRASP signals (green) with spGFP1–10 (magenta) expressed in either nociceptors (ppk-GAL4) or mechanosensors (NompC-GAL4). In contrast, spGFP11 expressed in A08n neurons only produces syb-GRASP signals with spGFP1–10 in nociceptors. The discrete GFP signals along the VNC midline are artifacts that show up in the absence of spGFP11 and can be observed with various antibodies. Scale bar, 10 mm.

(D) AITC activates A08n neurons specifically through C4da nociceptors, as shown by calcium imaging. A schematic is included to show pre- and postsynaptic neurons. The neuron that was recorded by GCaMP calcium imaging is shown in green. Traces show the averages of responses. 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, n = 4, and n = 4 in C4da present, C4da ablated, and PNS severed, respectively).

(E) Stimulation of NompC-expressing mechanosensors does not activate A08n neurons. Mechanosensors expressing P2X2 were stimulated by 1 mM ATP for Ca2+ imaging (n = 10 neurons [five larvae]).

(F) Stimulation of C4da nociceptors activates Basin-4 neurons (n = 9 neurons [five larvae]). Note that AITC does not activate NompC-expressing mechanosensory neurons (data not shown).

(G) Stimulating NompC-expressing mechanosensors activates Basin-4 neurons (n = 8 neurons [four larvae]).

(H) Activation of C4da or A08n neurons by CsChrimson elicits nociceptive response (n = 90 larvae for each group). Note that activation of A08n neurons mostly results in abrupt body curling.

(I) Larvae expressing CsChrimson in one or two A08n neurons exhibit an increase in nociceptive behavior. Results combine curling and rolling. Most of the responders showed curling only. Sample numbers are indicated in the graph.

(J) Larvae with silenced A08n neurons show a significant reduction in nociceptive response. Nociceptors were activated by ChR2. A08n neurons were silenced by Kir2.1 expression with the GMR82E12-Gal4 driver. The control group lacked the driver (n = 100 larvae for each group).

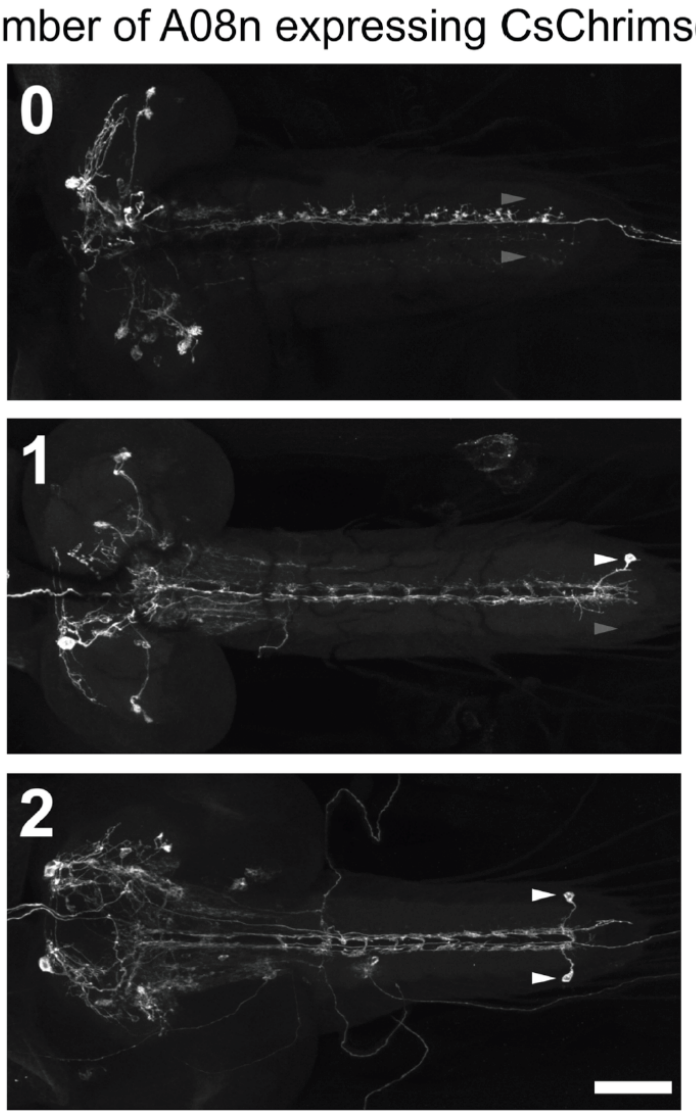


Figure 2.5. Mosaic activation of A08n neurons.
CsChrimson::Venus was expressed in none (“0”), one (“1”), or both (“2”) A08n neurons, in addition to several neurons in the brain. The FLP-out mosaic technique with GMR82E12-Gal4 driver was used for the mosaic expression of CsChrimson. Arrowheads point to the cell bodies of A08n. Scale bar: 50 μm.

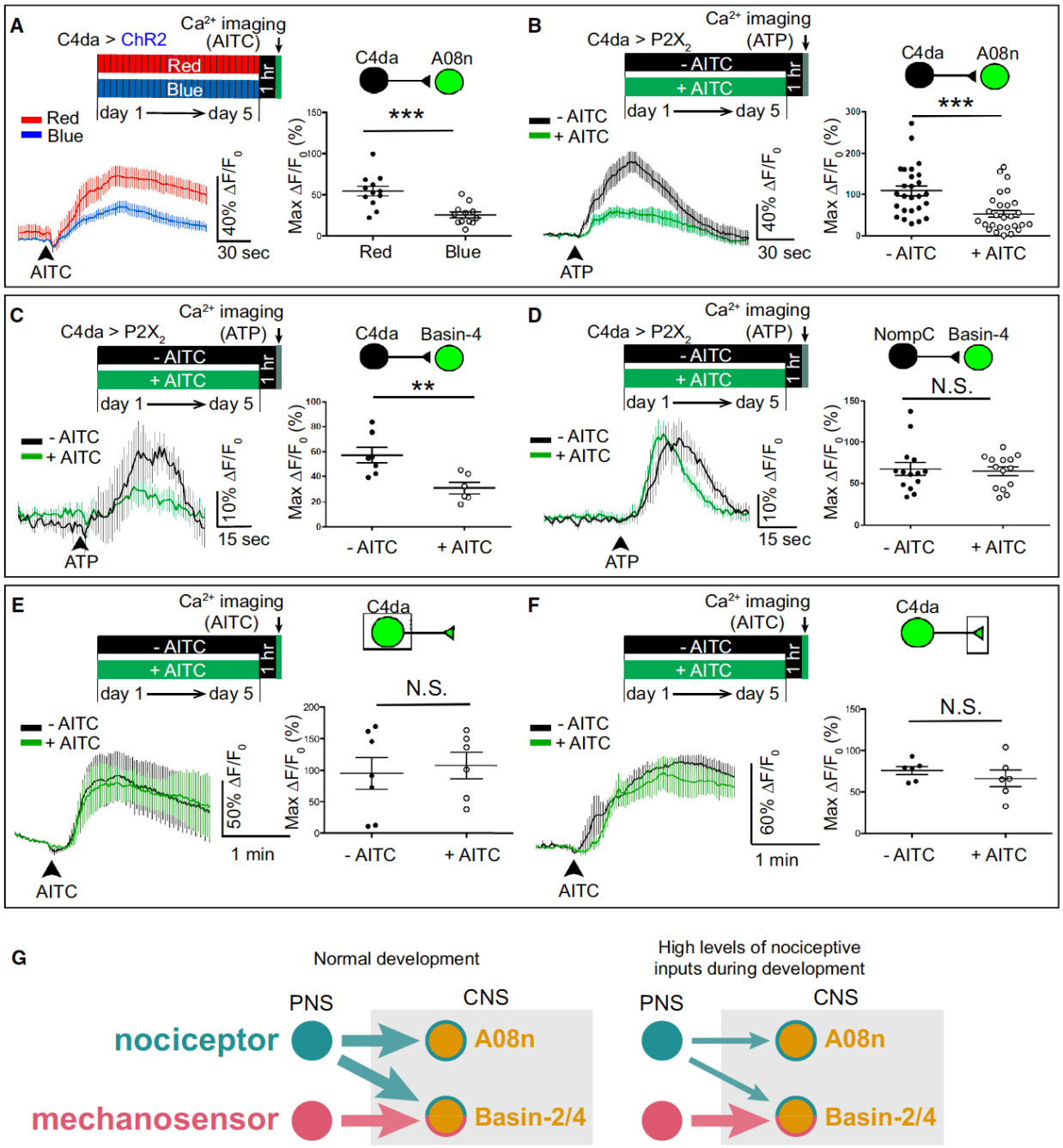


Figure 2.6. The Sensory Input-Induced Plasticity of Larval Nociceptive Circuit Is Pathway Specific

(A) Optogenetic activation of C4da neurons during development inhibits C4da-to-A08n transmission. C4da neurons in mature larvae were stimulated by AITC for Ca2+ imaging. Maximum DF/F0 for individual A08n neurons is plotted on the graph and used for statistical analyses (i.e., each dot indicates one A08n) (n = 12 neurons [from six larvae] per group).

(B) Stimulation of C4da neurons by AITC (5 mM) during development inhibits C4da-to-A08n transmission. C4da neurons in mature larvae were stimulated by P2X2 for Ca2+ imaging (n = 26 [from 13 larvae] and 28 neurons [from 14 larvae] in _AITC and +AITC, respectively).

(C) Stimulation of C4da neurons by AITC (2.5 mM) during development inhibits C4da-to-Basin-4 transmission. Because the responses of Basin-4 neurons are highly variable within each VNC (data not shown) (Jovanic et al., 2016), the average of maximum DF/F0 of ten Basin-4 neurons from abdominal segments 3–7 in each VNC was calculated to represent Basin-4 activity in each VNC (i.e., each dot indicates one larva) (n = 7 and 6 larvae in _AITC and +AITC, respectively).

(D) Treating larvae with AITC (2.5 mM) during development does not alter the mechanosensor-to-Basin-4 transmission (n = 14 larvae per group).

(E and F) Larvae raised in environments with and without AITC (2.5 mM) have similar levels of noxious stimulation-induced calcium responses in C4da somata (E) and axon terminals (F) (n = 6 and 7 neurons [four larvae per group] in _AITC and +AITC, respectively, E; n = 6 neurons from three larvae per group, F).

(G) Schematics showing the pathway specificity of nociceptor input-induced plasticity in synaptic connections between larval nociceptors and SONs in the circuit. Left: circuit diagram shows connections under normal developmental conditions. Right: high levels of nociceptive input specifically suppress synaptic transmission from nociceptors to SONs.

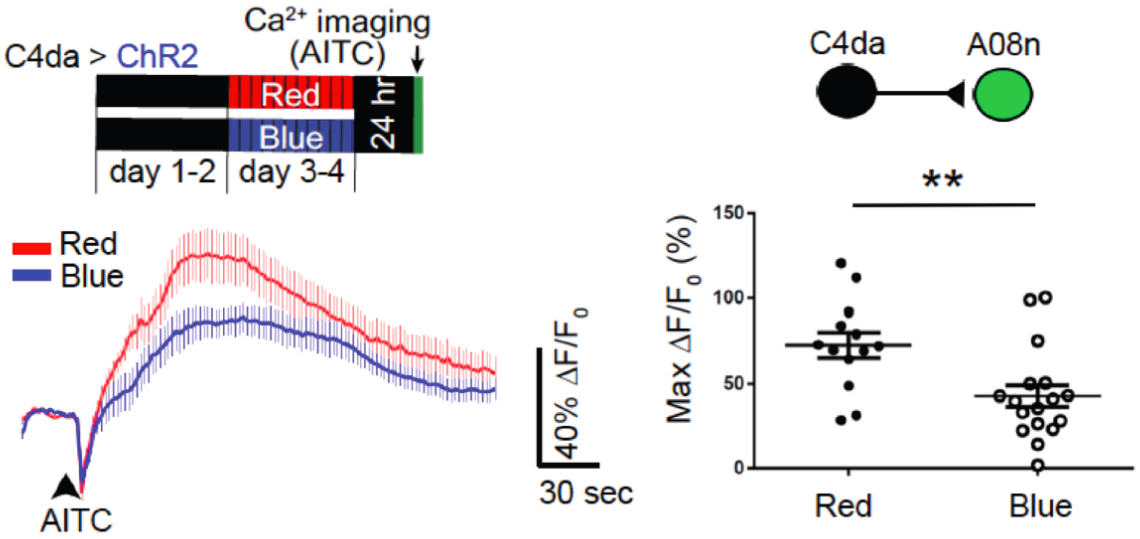


Figure 2.7. High levels of nociceptive inputs during development suppress C4da-to-A08n synaptic transmission.
Nociceptor activation during day 3-4 AEL of larval development resulted in reduced A08n responses. *n* = 13 neurons (7 larvae) and 17 neurons (10 larvae) in red and blue groups, respectively.

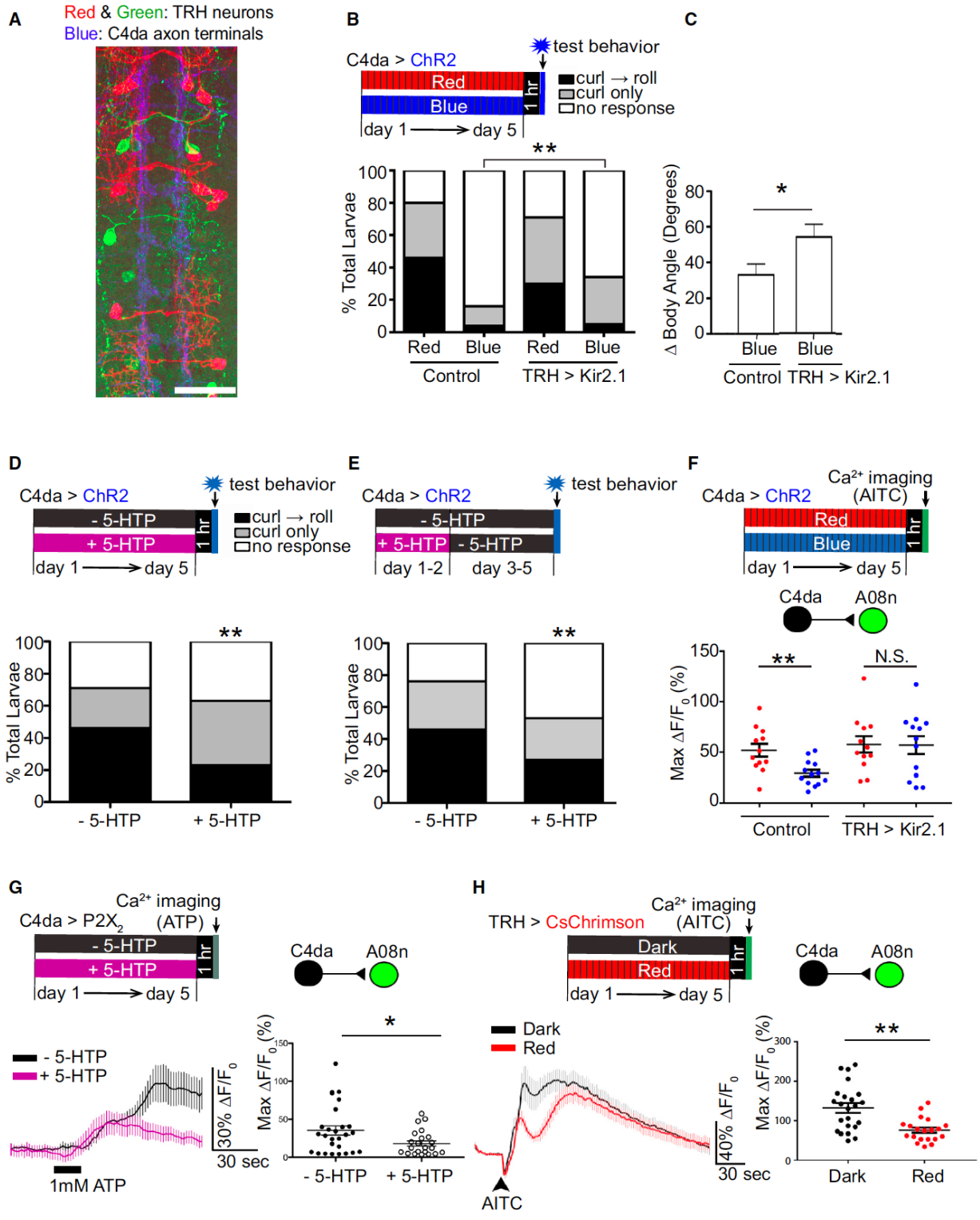


Figure 2.8. Serotonergic Neurons Are Required to Establish Sensory Input-Induced Plasticity of Larval Nociceptive Behavior

(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 mm.

(B and C) Silencing TRH neurons partially rescued nociceptive behavioral responses in larvae whose nociceptors were optogenetically stimulated during development. TRH neurons were silenced by Kir2.1 expression with TRH-Gal4. The control lacked TRH-Gal4. In (B), n = 120 (control + red), 120 (control + blue), 135 (TRH > Kir2.1 + red), and 120 (TRH > Kir2.1 + blue) larvae. In (C), n = 42 (TRH) and 40 (TRH > Kir.21) larvae.

(D) Larvae fed 5-HTP throughout development show a 2-fold reduction in rolling (n = 135 [_5-HTP] and 105 [+5-HTP] larvae).

(E) Larvae fed 5-HTP during days 1 and 2 of larval development show a 2-fold reduction in nociceptive rolling compared to control (n = 50 [_5-HTP] and 60 [+5-HTP] larvae).

(F) Silencing TRH neurons rescues A08n activity in larvae that experience high levels of nociceptive inputs during development. Control, larvae that have normal TRH activity due to the lack of TRH-Gal4 driver for expressing UAS-Kir2.1 (n = 12 and 13, respectively, for red and blue illuminations); TRH > Kir2.1, larvae whose TRH neurons were silenced by the expression of Kir2.1 (n = 12 and 13, respectively, for red and blue illuminations).

(G) Larvae fed 5-HTP throughout development show a reduction in C4da-to-A08n synaptic transmission (n = 27 [_5-HTP] and 23 [+5-HTP] neurons).

(H) Optogenetic stimulation of serotonergic neurons during development, without the stimulation during calcium imaging in mature larvae, reduced AITC-elicited A08n responses. Pulses of red light were used to chronically stimulate CsChrimson-expressing serotonergic neurons. Larvae in the control group were reared in the dark (n = 23 neurons [from 13 larvae] and 20 neurons [from 11 larvae] in dark and red, respectively).

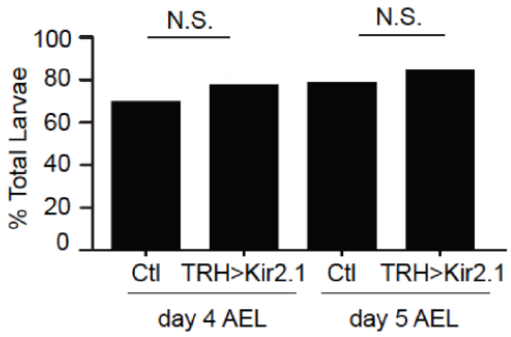


Figure 2.9. Silencing serotonergic neurons does not affect the nociceptive behavioral responses if nociceptors are not stimulated during development. Kir2.1 was expressed in serotonergic neurons to silence these neurons (TRH>Kir2.1). The negative control (Ctl) was UAS-Kir2.1 alone without the TRH-GAL4 driver. 25 mM AITC was used to assess larval rolling behavior on day 4 AEL (*n* = 106 larvae for Ctl and 72 for TRH>Kir2.1) and day 5 AEL (*n* = 57 for Ctl and 59 for TRH>Kir2.1). The percentage of larvae showing a complete rolling within 2 min was quantified. No significant difference was observed between the two groups. Larvae younger than day 4 AEL were not tested because they rarely exhibit nociceptive rolling (data not shown).

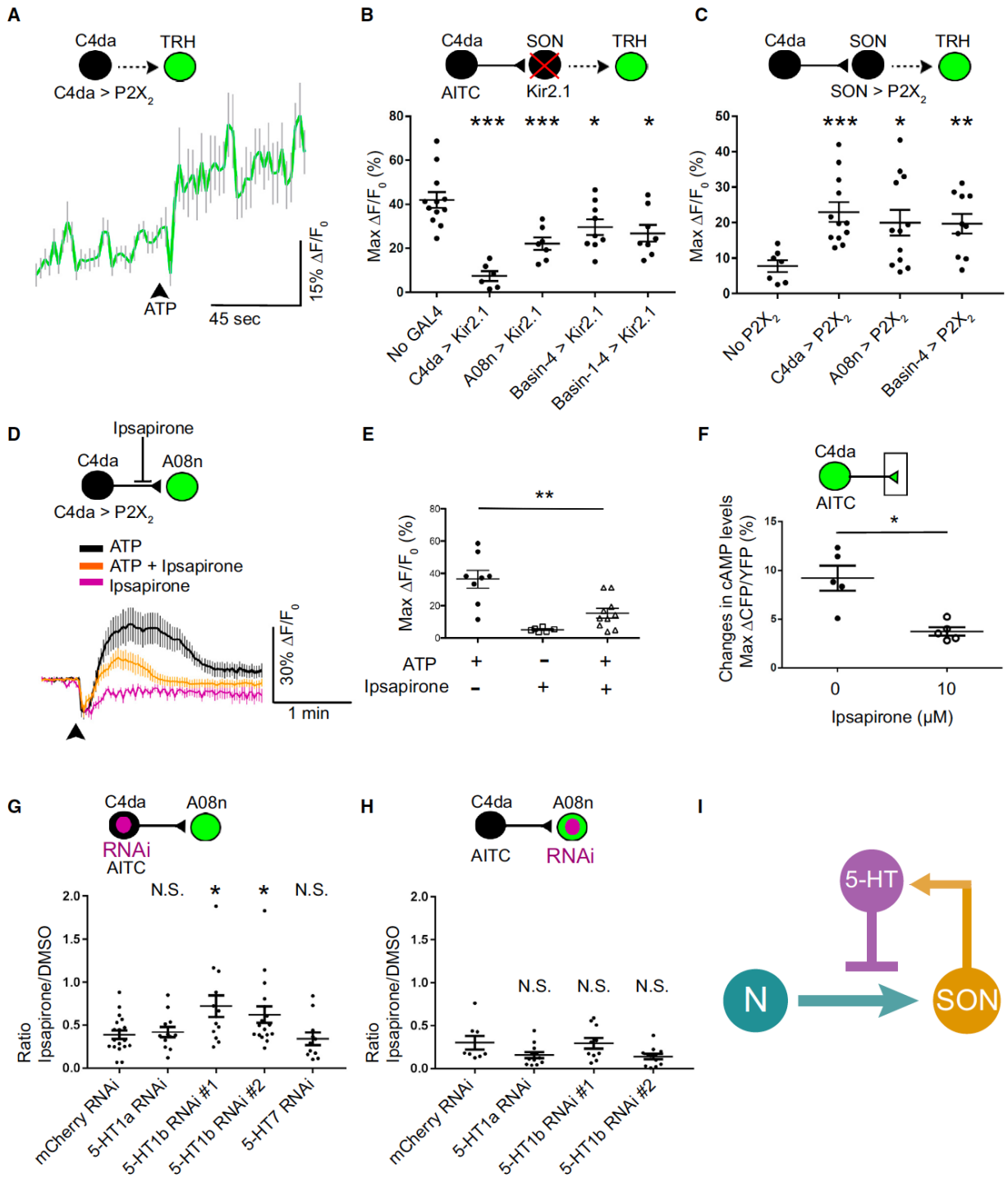


Figure 2.10. Feedback Inhibition of Nociceptor-to-SON Transmission through Serotonergic Neurons

(A) Stimulating C4da nociceptors activates serotonergic neurons. The trace shows the averages of GCaMP6f intensities in TRH neurons caused by stimulating

C4da neurons via ATP/P2X2. The black arrowhead indicates the start of ATP/P2X2-mediated stimulation (n = 33).

(B) Silencing SONs suppresses nociceptor-induced activation of serotonergic neurons. C4da, A08n, Basin-4, and Basin-1–4 were inhibited by expressing Kir2.1. AITC was used to stimulate C4da neurons (n = 12, 6, 7, 9, and 8 larvae in control, C4da, A08n, Basin-4, and Basin-1–4 groups, respectively).

(C) Stimulation of SONs activates serotonergic neurons. ATP was used to activate neurons expressing P2X2. Larvae in ‘‘no P2X2’’ group had no P2X2 expression due to the lack of a GAL4 driver and a P2X2 transgene (n = 7, 12, 12, and 10 larvae in control, C4da, A08n, and Basin-4 groups, respectively).

(D and E) Ipsapirone (100 mM) inhibits C4da-to-A08n synaptic transmission. ATP/P2X2 was used to activate C4da neurons. (D) Traces show the averages of responses. (E) Quantification and statistical analysis are shown (n = 8, 6, and 10 neurons, five larvae per group).

(F) Ipsapirone (10 mM) diminishes AITC-elicited increase in cAMP levels in C4da axon terminals. Imaging-based cAMP sensor Epac1-camps was expressed specifically in nociceptors, and their presynaptic terminals were imaged for Fo¨ rster resonance energy transfer (FRET). AITC was used to activate nociceptors. Levels of cAMP were quantified as changes in the inverse FRET ratio, which is CFP intensity divided by YFP intensity (DCFP/YFP) scanned with a 458-nm confocal laser (n = 5 larvae per group).

(G and H) Knockdown of 5-HT1b in C4da neurons, but not that in A08n, significantly blocks the effect of ipsapirone on nociceptor-to-SON transmission. The graphs show the ratio of AITC-elicited maximum DF/F0 in the presence of 10 mM ipsapirone over that in the presence of DMSO (vehicle). This ratio indicates the extent of serotonergic inhibition of AITC-elicited responses in A08n. In the schematic, neurons expressing GCaMP6f and RNAi are shown in green and purple, respectively. An RNAi line against mCherry was used as a negative control. 5-HT1b #1 (B-33418) and 5-HT1b #2 (B-27635) were used to knock down 5-HT1b (n = 18 neurons [nine larvae], n = 12 neurons [six larvae], n = 13 neurons [seven larvae], n = 17 neurons [nine larvae], and n = 12 neurons [seven larvae] for mCherry, 5-HT1a, 5-HT1b #1, 5-HT1b #2, and 5-HT7, respectively, G; n = 8 [five larvae], n = 12 [six larvae], n = 10 neurons [five larvae], and n = 12 neurons [six larvae] for mCherry, 5-HT1a, 5-HT1b #1, and 5-HT1b #2, respectively, H).

(I) Schematic model showing that serotonergic neurons modulate nociceptor-to-SON transmission by providing feedback inhibition.

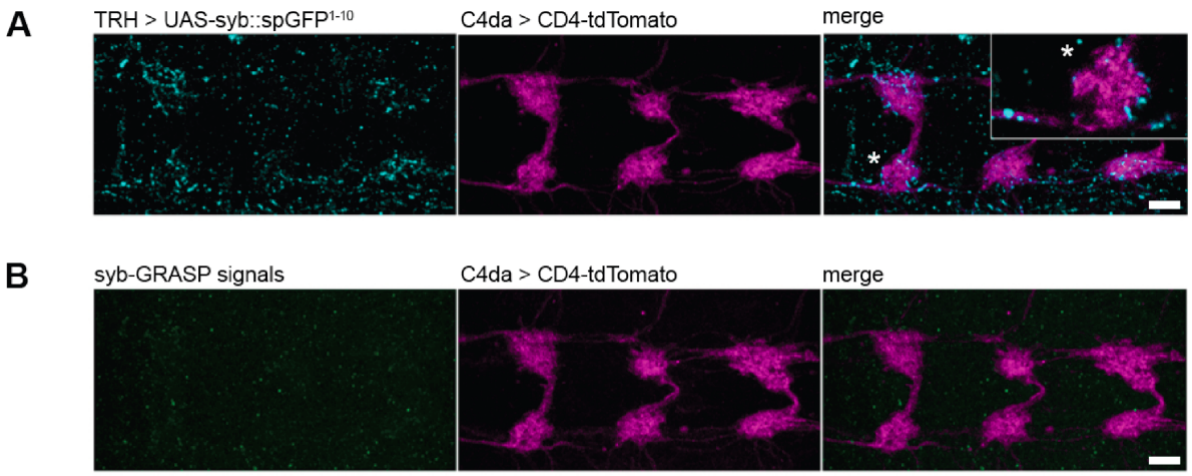


Figure 2.11. The terminals of serotonergic neurons intimately intertwine with C4da presynaptic terminals.

(A) Terminals of TRH neurons expressing the presynaptic marker, syb-spGFP1-10 (cyan), near C4da neuropils (magenta). The inset in "merge" shows one C4da neuropil (marked with "*") in a single focal plane. Scale bar: 5 μm.

(B) No syb-GRASP signal was observed from TRH terminals to C4da presynaptic terminals. Syb-spGFP1-10 was expressed in TRH neurons; CD4-tdTomato and CD4-spGFP11 were expressed in C4da neurons.

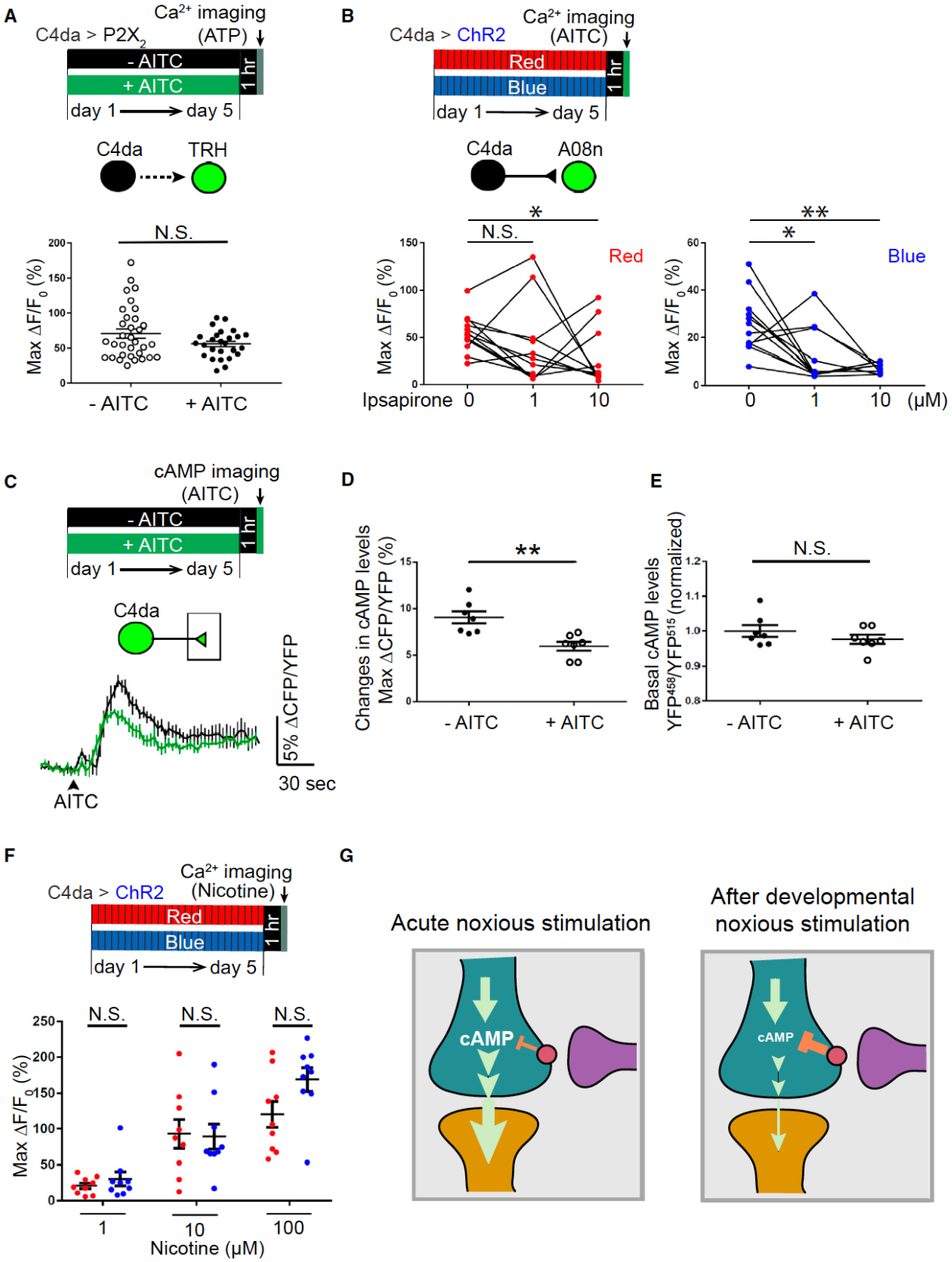

A
Ca2+ imaging
C4da > P2X2
(ATP)
- AITC
+ AITC
1 hr
day 1
day 5
C4da
TRH
N.S.
Max ΔF/F0 (%)
- AITC
+ AITC
B
Ca2+ imaging
C4da > ChR2
(AITC)
Red
Blue
C4da
A08n
*
N.S.
Red
**
*
Blue
Ipsapirone 0 1 10
0 1 10 (μM)
C
cAMP imaging
(AITC)
C4da
5% ΔCFP/YFP
30 sec
AITC
D
Changes in cAMP levels
Max ΔCFP/YFP (%)
**
E
Basal cAMP levels
YFP458/YFP515 (normalized)
N.S.
F
Ca2+ imaging
C4da > ChR2
(Nicotine)
N.S.
Nicotine (μM)
G
Acute noxious stimulation
After developmental
noxious stimulation
cAMP

Figure 2.12. Developmental Stimulation of Nociceptors Enhances 5-HT Receptor-Mediated Inhibition of Nociceptor-to-SON Transmission

(A) Noxious stimulation during development (2.5mMAITC) does not affect nociceptor-induced responses in serotonergic neurons (n = 33 and 25 larvae for _AITC and +AITC, respectively).

(B) Developmental stimulation of C4da neurons sensitizes C4da-to-A08n transmission to ipsapirone. AITC was used to stimulate C4da neurons for calcium imaging. 0, 1, or 10 mMipsapirone was mixed with AITC and applied sequentially from low to high concentrations (n = 12 neurons [from six larvae] for each group).

(C–E) Noxious stimulation during development (2.5 mM AITC) significantly diminishes AITC-elicited increase in cAMP levels in C4da axon terminals without affecting basal cAMP levels. Average traces of cAMP responses (C), quantification and statistical analysis of cAMP levels (n = 7 for each group, D), and quantification and statistical analysis of basal cAMP levels (E) are shown. The value YFP458/YFP515 was normalized by dividing the average of the _AITC group (n = 7 for each group).

(F) Developmental activation of nociceptors does not reduce A08n responses to nicotine. Three different concentrations of nicotine were applied to the brains of mature larvae, and changes in calcium levels in A08n somata were analyzed. Since A08n is specific to nociceptive inputs, A08n responses to nicotine reflect its responses to nociceptive inputs (n = 9 in each group).

(G) A model that explains the experience-dependent sensory input-induced plasticity in nociceptor-to-SON transmission. Left panel: acute noxious stimulation in mature larvae increases cAMP levels in the presynaptic terminals of nociceptors (shown in teal), and it leads to robust synaptic transmission to SONs (shown in orange). Serotonergic neurons (shown in purple) suppress cAMP levels in nociceptor presynaptic terminals. Right panel: developmental noxious stimulation enhances the responsiveness of nociceptor presynaptic terminals to 5-HT modulation in mature larvae, leading to further suppression of cAMP production and reduced synaptic transmission to target neurons.

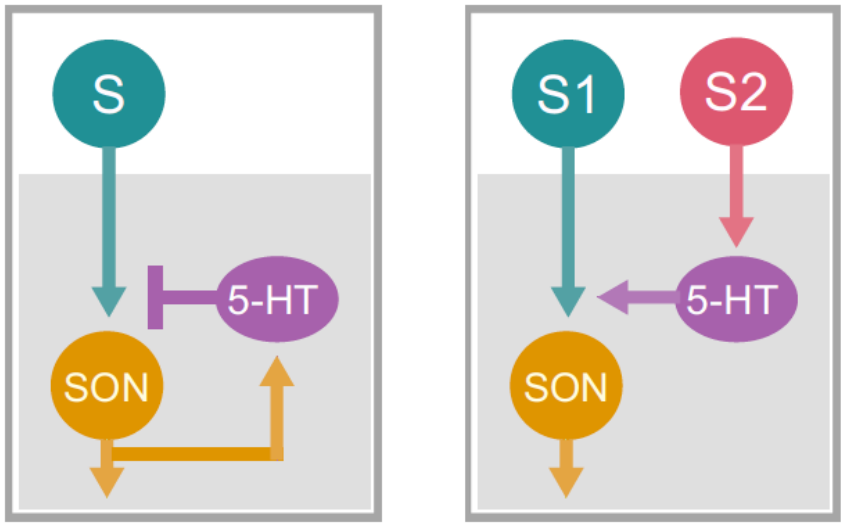


Figure 2.13. Feedback Modulation Enables Pathway-Specific Plasticity in Developing Sensory Circuits in *Drosophila*

The pathway-specific plasticity of the developing nociceptive circuit in *Drosophila* is different from the serotonergic facilitation that occurs during sensitization of the defensive gill-withdrawal reflex in *Aplysia*. In the *Drosophila* nociceptive circuit, activity in nociceptors (S) leads to activation of both SONs in the circuit and serotonergic interneurons (5-HT), while in *Aplysia* gill-withdrawal reflex circuit, serotonergic interneurons are activated by another sensory pathway (S2).

CHAPTER 3

Neural Activity Instructs Fine-Scale Topography in a Developing Sensory Circuit in *Drosophila*

A part of this chapter includes excerpts from a review article in Journal of Comprehensive Physiology A, Fine-scale topography in sensory systems: insights from *Drosophila* and vertebrates, written by **Takuya Kaneko** and Bing Ye in 2016.

In addition, Materials and Methods section incorporates material from a research article in Current Biology, Trim9 regulates activity-dependent fine-scale topography in *Drosophila,* written by Limin Yang, Ruonan Li, **Takuya Kaneko**, Kendra Takle, Rei K. Morikawa, Laura Essex, Xin Wang, Jie Zhou, Kazuo Emoto, Yang Xiang, and Bing Ye in 2014.

I acknowledge Limin Yang, a collaborator for this project.

Summary

To encode the positions of sensory stimuli, sensory circuits form topographic maps in the central nervous system (CNS) through specific point-to-point connections between pre- and postsynaptic neurons. In vertebrate visual systems, the establishment of topographic maps involves the formation of a coarse topography followed by that of fine-scale topography that distinguishes the axon terminals of neighboring neurons. Intrinsic differences in the form of broad gradients of guidance molecules instruct coarse topography while neuronal activity is required for fine-scale topography. On the other hand, studies in the *Drosophila* visual system have shown that intrinsic differences in cell adhesion among the axon terminals of neighboring neurons instruct the fine-scale topography, independent of neural activity. Here, we identify the first activity-dependent topography in *Drosophila*. The central projections of nociceptive sensory neurons in *Drosophila* exhibit fine-scale topography, which depends on the activity levels of nociceptive neurons. This study reveals a role of neuronal activity in creating molecular differences among nociceptive neurons. Our study further demonstrates that topography of nociceptors requires their postsynaptic targets, similar to activity-dependent topography in vertebrates, implicating a conserved principle.

The topographic map in the nociceptive system in *Drosophila* larva

Do C4da nociceptive neurons exhibit topography in C4da synaptic areas ("C4da neuropils")? In order to address this question, we took advantage of the C4da-specific promoter from the *pickpocket* gene (Grueber et al. 2003) to generate a reference of the C4da neuropils by marking these neurons with a membrane marker, e.g. the red

fluorescence protein tdTomato tagged with the membrane protein CD4. We then labeled single C4da neurons in the background of this reference. Automated image analysis was used to deduce the relative position of the synaptic terminal of the GFP-labeled mutant in the C4da neuropil. An advantage of the C4da system is that randomly labeled single neurons can be reliably identified based on the position of the cell bodies on the body wall. As a consequence, the differences among the three C4da neurons can be systematically studied.

This analysis identified that the synaptic terminals of the three C4da neurons in each hemi-segment form a dorsal-to-ventral topographic arrangement in the VNC, which corresponds to the dorsal-to-ventral distribution of their dendritic fields on the body wall. This topographic system is fine-scale because it distinguishes the locations of neighboring neurons. Unlike the dendritic tiling on the body wall, which is large in size, the fine-scale topography formed by the C4da synaptic terminals in the VNC is confined along a distance of roughly 6 microns. Moreover, the synaptic terminals are intimately adjacent to each other, leaving little space in between. These features allow for direct assessment of fine-scale topography, but also call for new techniques for efficient analysis.

Although it consists of only three neurons, the C4da topography is composed of two aspects, which are likely to be established through different mechanisms. The three C4da neurons in each hemi-segment are termed the dorsal (D), middle (M), and ventral (V) neurons based on the locations of their cell bodies and dendritic fields in the body wall. After the axons of the three neurons reach the VNC, the D axon immediately defasciculates from the M and V axons. The D axon projects dorsally while the M and V

axons project ventrally. Both then converge on the C4da neuropil situated in the most medioventral portion of the general synaptic area in the VNC. Once inside the C4da neuropil, the terminal of the D axon stays dorsally to those of the M and V axons. The M and V axons, which reach the C4da neuropil as a single nerve, separate from each other gradually during development so that the M terminal locates dorsally to the V terminal. Consequently, the presynaptic terminals of the three C4da neurons occupy distinct areas in the C4da neuropil, establishing fine-scale topography (Figure 1.3c).

Neural activity-dependent regulation of fine-scale topography in the larval nociceptive circuit

The mechanism separating the D terminal from the M/V terminals is different from that separating the M and V terminals; neural activity specifically regulates the projections of M and V neurons. When the activity of M neurons is blocked by the expression of Kir2.1, an inward rectifier potassium channel that blocks both spontaneous and evoked activity, M terminals end in the ventral portion of C4da neuropil where V neurons normally terminate (Figure 1.3c). The effect of Kir2.1 is specific to M neurons, and its expression in D or V neurons has no effect on the topographic locations of their terminals. Conversely, activation of V neurons through dTrpA1 (at 30°C) shifts their terminals to the middle portion of the C4da neuropil. Expression of dTrpA1 in either D or M neurons does not affect the topography. These results suggest that the levels of neuronal activity in M and V neurons direct the separation of their terminals. The separation of the D terminals from the M/V terminals is independent of neuronal activity, though the underlying mechanism remains unknown.

In the C4da system, it is likely that neural activity instructs fine-scale topography by setting up different levels of intracellular signaling activities in M and V neurons. In other words, neuronal activity creates an intrinsic signaling gradient among neighboring afferents. First, although C4da neurons are almost genetically identical, they exhibit different levels of the *Drosophila* ortholog of the tripartite motif protein Trim9 (dTrim9), a putative E3 ubiquitin ligase of the TRIM family (Reymond et al. 2001). The protein level of dTrim9 is higher in V neurons than M neurons (Morikawa et al. 2011). Second, the dTrim9 protein level is regulated by neuronal activity. When the activity of M neurons is blocked by Kir2.1, dTrim9 levels in these neurons increase to levels comparable to V neurons. Conversely, activating V neurons by dTrpA1 decreases dTrim9 level and consequently eliminates the difference between M and V neurons. Third, the gradient in dTrim9 protein level is essential for the topographic projections. When dTrim9 is overexpressed specifically in M neurons to abolish the M-V difference, the M axons terminate in the ventral portion of the C4da neuropil, which is a similar phenotype to Kir2.1 expression. Conversely, the removal of dTrim9 gene from V neurons recapitulates the phenotype of those with activation by dTripA1. Finally, restoring the dTrim9 gradient rescues the topographic defects caused by activity manipulation. Therefore, genetic manipulation to establish the gradient of dTrim9 is sufficient to specify the projections of M and V neurons regardless of their activity levels.

Altogether, these results suggest that neuronal activity regulates the levels of dTrim9, a cell-intrinsic molecule that is differentially expressed in individual neurons, to separate the M and V terminals. How might neural activity establish such a molecular difference among neighboring afferents? One possibility is that the activity levels of M

and V neurons are different. Although this could be caused by genetic programs, it is equally possible that M and V neurons in developing larvae, which are behaving animals, might be activated at different levels. Another possibility is that the responses to neuronal activity differ between M and V neurons so that the same level of neuronal activity leads to different levels of dTrim9 proteins. This would be caused by a genetically programmed difference, and neuronal activity would just be a factor linking an intrinsic difference to differential expressions of signaling molecules.

C4da neurons require postsynaptic neurons for fine-scale topography

The identification of activity-dependent topography in *Drosophila* has opened up the door for mechanistic analysis of the role of neural activity, which might be in part conserved in vertebrates. In the vertebrate visual system, neural activity in retinal ganglion cells (RGCs) instructs fine-scale topography by regulating interactions of the RGC axons with postsynaptic neurons. In order to address whether this role of neural activity is conserved in the *Drosophila* nociceptive system, we examined the necessity of postsynaptic targets of C4da neurons for fine-scale topography. For this purpose, we genetically ablated one type of second-order neuron of this circuit, A08n neurons, through the expression of reaper (rpr) and head involution defective (hid) (Hsu et al., 2002). The genetic deletion of A08n postsynaptic neurons significantly disrupted the C4da projections with incomplete separation of M and V presynaptic terminals (Figure 3.1A). This result suggests that the C4da topographic projections require interactions of the C4da axons with their postsynaptic targets, and the axon-target interactions may be the site of neural activity-mediated regulation as in the vertebrate visual system.

Despite this similarity across species, the pathway by which neural activity impacts axon-target interactions seems different. In the vertebrate visual system, neural activity in RGCs instructs topography through activation of NMDA receptors of postsynaptic neurons, whereas, in the *Drosophila* nociceptive system, neural activity is mediated by dTrim9 in presynaptic C4da neurons as mentioned above. This difference may reflect that C4da neurons are presumably cholinergic and, as a result, their axon-target interactions are independent of the NMDA glutamate receptor. Consistent with this difference, our result suggests that neural activity is capable of instructing the C4da projections even without synaptic transmission to activate postsynaptic neurons. In this experiment, we expressed tetanus toxin light chain (TNT) in single C4da neurons, which blocks synaptic transmission from these neurons (Robertson et al., 2013; Sweeney et al., 1995). We found that C4da neurons with TNT expression exhibits normal topographic projections (Figure 3.1B), indicating that activation of postsynaptic neurons is a dispensable role of neural activity in C4da neurons for establishing fine-scale topography.

C4da fine-scale topography requires BMP signaling

How do postsynaptic neurons contribute to activity-dependent C4da topography? Since our result indicates synaptic transmission from C4da neurons is unnecessary for topography, we hypothesize that postsynaptic neurons regulate the C4da projections through retrograde signaling (i.e signaling from post to presynaptic terminals). The most characterized retrograde signaling in *Drosophila* is BMP signaling at the larval neuromuscular junction (NMJ), where BMP signaling is active in motor neurons in

response to BMP ligands secreted from muscle cells (Marques and Zhang, 2006). Taking a clue from this work, we first asked whether BMP signaling is similarly active in C4da sensory neurons. When BMP signaling is active, the BMP effector, Mad, becomes phosphorylated and trafficked to the nucleus to initiate transcription of target genes; therefore, the levels of BMP signaling are reflected by the expression levels of nuclear phosphor Mad (pMad) (Tanimoto et al., 2000). Through immunostaining, we identified all of the three C4da subtypes express pMad in the nucleus, demonstrating that BMP is active in C4da neurons as we hypothesize (Figure 3.1C).

Next, we examined whether postsynaptic A08n neurons express BMP ligands. For this purpose, we used a Gal4 reporter line for the BMP ligand, Dpp, in combination with UAS-nuclear GFP. We identified that A08n expresses high levels of GFP expression although only a small population of neurons are positive for the reporter expression in the VNC (Figure 3.1D). This result is consistent with our hypothesis that A08n neurons communicate with C4da neurons through retrograde BMP signaling.

Is BMP signaling in C4da neurons required for their topographic projections? To address this question, we first deleted the type II BMP receptor, Wit, in single C4da neurons through MARCM technique. We found that Wit deletion from D or V neurons has no effect on their topographic projections while deletion from M neurons disrupts their projections by shifting the presynaptic terminals ventrally (Figure 3.2A). A similar phenotype was observed when we mutated the BMP effector, Mad, in single C4da neurons. M neurons that lack Mad expression project their presynaptic terminals to the ventral side of the C4da neuropil (Figure 3.2B). Thus, BMP signaling is required specifically in M neurons, where neural activity is required, for fine-scale topography.

One possible model is that M neurons exhibit higher levels of BMP signaling than V neurons and this difference causes the separation of the M/V presynaptic terminals. However, our results show this is unlikely the case. First, our immunostaining for nuclear pMad showed no obvious difference in the levels of BMP signaling between M and V neurons (Figure 3.2B). Second, elevation of BMP signaling specifically in V neurons does not shift their presynaptic terminals dorsally. We elevated the levels of BMP signaling through the expression of a constitutively active form of the BMP type I receptor, Tkv, (Tkv[CA]). Tkv[CA] expression in single C4da neurons resulted in overgrowth of their axon terminals along the A-P axis, but has no effect on their orientation along the D-V axis (Figures 3.2B-C). Consistently, overexpression of the Wit receptor to increase BMP signaling does not disrupt the topographic projections. (Figure 3.2B). Taken together, despite the requirement of BMP signaling, the C4da projections are not determined by the levels of BMP signaling.

Then, what is the function of BMP signaling? Given that blocking BMP signaling phenocopies the effect of inhibiting neural activity, we suspect that BMP signaling mediates the role of neural activity in instructing topography. This model is consistent with the function of retrograde BMP signaling at the NMJ, where BMP signaling is required for activity-dependent stabilization of synaptic connections (Berke et al., 2013). BMP signaling in C4da neurons may similarly mediate the formation of synapses to stabilize only topographically correct connections (i.e. synapses of M neurons at the meddle layer of the C4da neuropil). Besides this favored model, it is equally possible that BMP signaling controls topography by instructing the levels of neural activity. In fact, a recent study demonstrates that BMP signaling impacts the levels of calcium

increases at the presynaptic terminals of C4da neurons (Tracey and Honjo, 2018). A future study will distinguish these two models by determining whether neural activity requires BMP signaling. The first model would be supported if the presynaptic terminals of V neurons that lack Mad function are not shifted dorsally by enhancing neural activity. Future research on this system is expected to elucidate detailed molecular mechanisms by which neural activity instructs fine-scale synaptic connections.

Materials and Methods

MARCM for Analyzing Topographic Mapping

MARCM experiments were done as previously described (Ye et al., 2011). We dissected only size-matched 3-day-old third-instar larvae to ensure consistency of the developmental stages of the analyzed animals. Three hours after egg laying, the eggs were heat shocked at 37.5°C for ~15–20 min. For using MARCM to overexpress dTrpA1 in single C4da neurons, the eggs were kept at 30°C after heat shock and dissected 2.5 days later. The same procedure was followed for the control experiments.

Immunostaining, Imaging, Image Preprocessing, and Image Analysis

Third-instar larvae were immunostained as described (Ye et al., 2004), with minor modifications. All images were collected as 3D stacks using an SP5 confocal system (Leica Microsystems) equipped with a 63× oil lens (Plan-Apochromat, numerical aperture [NA] = 1.4, Leica Microsystems). The axial sampling step (z-step) was 0.3 μm. Images were collected with minimum signal saturation. Three steps were necessary to preprocess images for analyzing the topographic index, volume, and boundary location

of presynaptic terminals. First, confocal image stacks were deconvolved with Huygens software (Scientific Volume Imaging). Next, the VNC in each stack was aligned to uniform orientation with the 3D image analysis software Amira (FEI Visualization Sciences Group). Third, the 3D image stacks were cropped to contain only the C4da neuropil with the single MARCM or FLP-out clones in them. After preprocessing, the image stack was automatically analyzed by custom-designed software for topographic index, volume ratio, and boundary location.

Algorithm for Analyzing the Topographic Index and Volume of Presynaptic Terminals

The neuropil channel for TI and volume ratio calculation was obtained by combining the raw neuropil signal with the clone channel signal. Because it was typically dim, the neuropil channel was enhanced by iterative histogram normalization: The maximum-intensity parameter during normalization was iteratively adjusted so that the mean foreground intensity was increased to 80. The extraction of signals of C4 da neuropil and clone was extracted from background by using robust adaptive threshold selection (RATS) (Wilkinson, 1998).

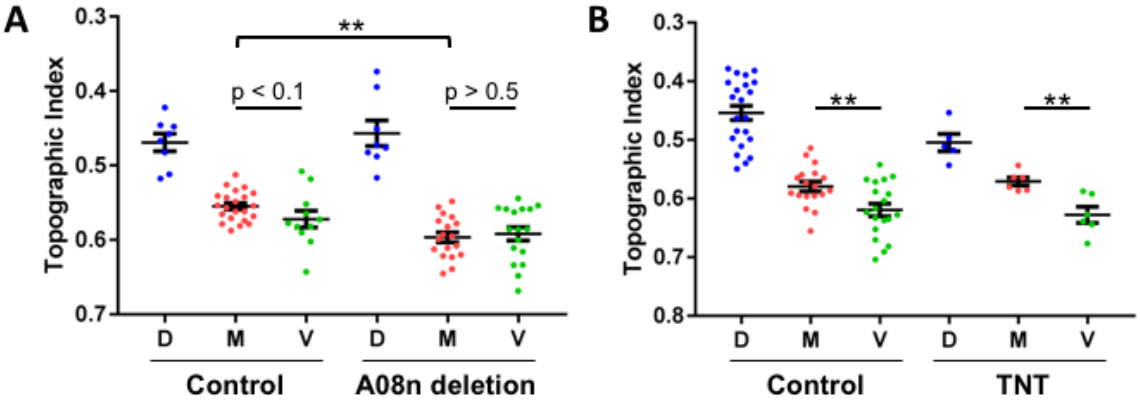
RATS is a segmentation method for extracting the foreground object out of a gray level image based on robust and adaptive thresholding. It selects the thresholds by recursively dividing the image using quadtree structure and then automatically calculating the thresholds using intensity values as well as their gradients in the local region. The thresholds for all local regions are then bilinearly interpolated across the entire image. The advantage of the RATS approach is its local adaptability, which suits well to microscopic images with contrast variation among different local regions. RATS

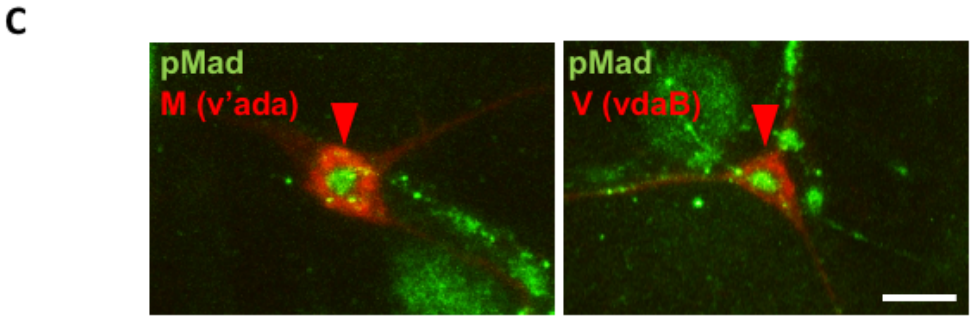
was applied to each slice of the 3D image stack. The minimum region size (also called leaf size) of the quadrant was set to 5 pixels per side.

The TI of each clone voxel was calculated by measuring its relative position between dorsal and ventral neuropil boundaries: $TI_i = d_i/(d_i + v_i)$, where d_i was the distance of the voxel *i* to the dorsal boundary and v_i was its distance to the ventral boundary. As such, the voxels at the dorsal side of the neuropil had TIs closer to 0; the voxels at the ventral side of the neuropil had TIs closer to 1. The overall TI of a clone was the averaged sum calculated by $TI = \Sigma TI_i/n$, where n was the total number of clone voxels in the 3D image stack. Note that an overall $TI \in (0, 1)$ can never be 0 or 1. The volume ratio was the ratio between the volumes of clone and neuropil, with the volume being measured by the number of foreground voxels in the 3D volume. The volume of a clone or neuropil was represented by the total number of voxels in the 3D images of the clone or neuropil. Thus, the result of this analysis is not affected by the "holes" in the 3D image of a presynaptic terminal caused by the convoluted morphology of the terminal.

The average dorsal boundary position of each clone was calculated by taking the mean of the normalized positions of the dorsal-most voxels of the clone. To obtain the normalized position, we measured the distance (d_i) between the dorsal-most voxel of the clone and that of the neuropil at the same x position, and the distance between the dorsal-most and ventral-most voxels of the neuropil at that x position (D_i). The normalized boundary position (B_i) at a particular x position is calculated as the ratio between the two distances: $B_i = d_i/D_i$. The average boundary position of a clone was the averaged sum of the B_i for all x positions.

The software for quantifying TI, volume ratio, and boundaries was developed as an ImageJ (NIH) plugin.


A
Topographic Index
0.3
0.4
0.5
0.6
0.7
**
p < 0.1
p > 0.5
D
M
V
D
M
V
Control
A08n deletion
B
Topographic Index
0.3
0.4
0.5
0.6
0.7
0.8
**
**
D
M
V
D
M
V
Control
TNT


C
pMad
M (v'ada)
pMad
V (vdaB)

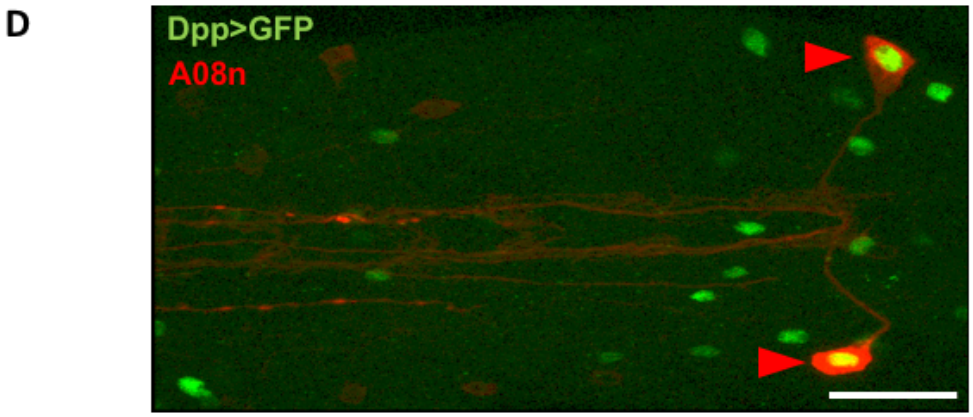

D
Dpp>GFP
A08n

Figure 3.1. Postsynaptic neurons support C4da topography possibly through retrograde BMP signaling

(A) Deleting A08n neurons disrupts C4da topography. Topographic Index (TI) indicates the relative positions of individual presynaptic terminals; a low TI means a dorsal position while a high TI means a ventral position (please see Materials and Methods for details). In the control group, M neurons exhibit lower TI than V neurons, suggesting the presynaptic terminals of M neurons are located more dorsally than those of V neurons. The deletion of A08n neurons through the expression of rpr and hid abolishes the topographic separation between the M and V terminals.

(B) Inhibition of synaptic transmission has no effect on C4da topography. The release of neurotransmitter is blocked by the expression of TNT.

(C) BMP signaling is active in C4da neurons. All of the three C4da subtypes express nuclear pMad, an indicator of BMP levels. The M and V neurons (labeled in red by ppk-tdTomato) exhibit seemingly comparable levels of nuclear pMad signals (green). Arrows indicate soma of C4da neurons. Scale bar: 7.5 μm.

(D) A08n neurons express the BMP ligand, Dpp. The image shows the larval VNC. Dpp-Gal4 reporter drives the expression of UAS-nuclear GFP (in green) in A08n neurons labeled by cytosolic mcherry (in red). Arrows indicate soma of A08n neurons. Scale bar: 25 μm.

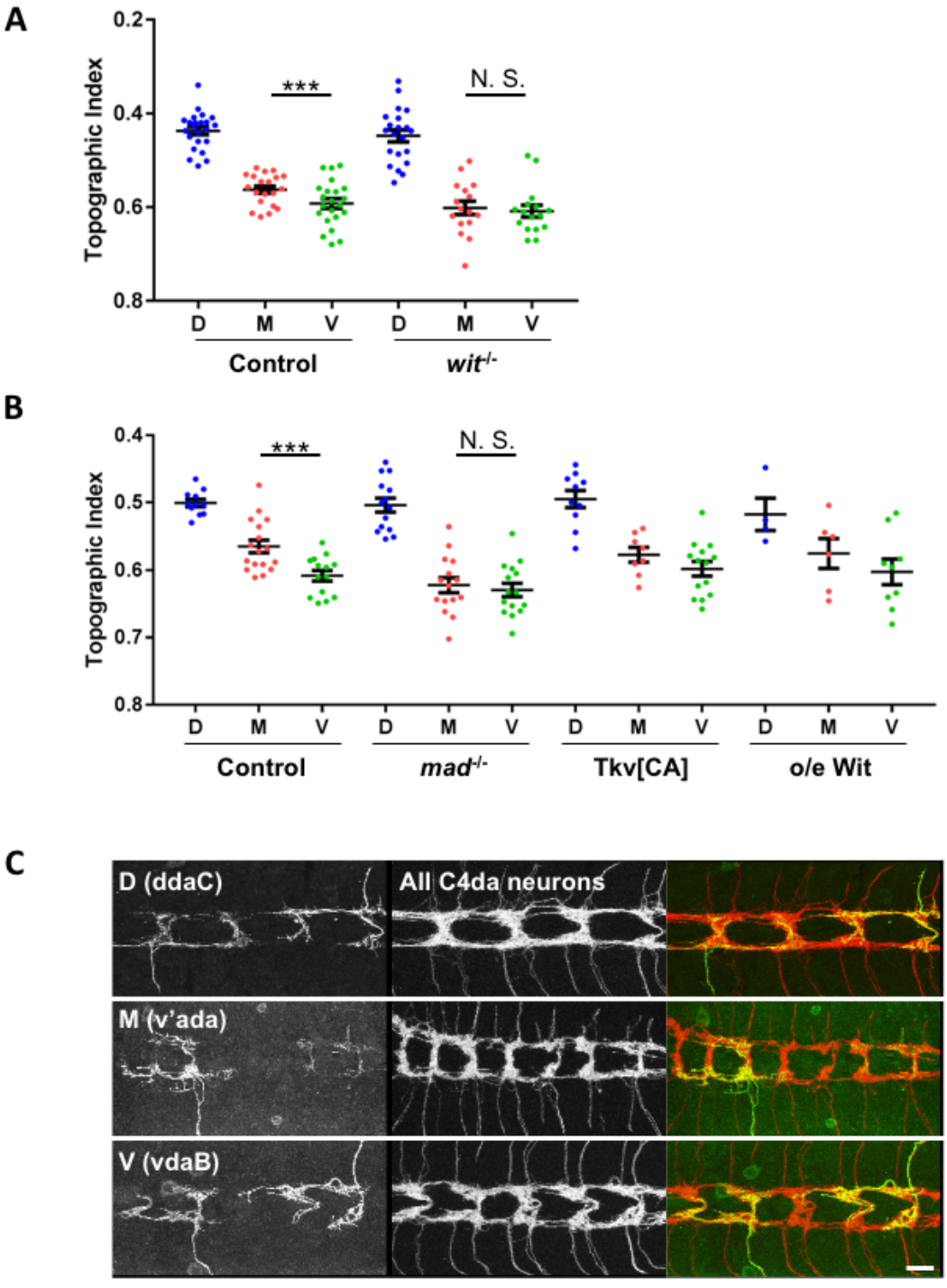

A
Topographic Index
0.2
0.4
0.6
0.8
***
N. S.
D
M
V
D
M
V
Control
wit-/-
B
Topographic Index
0.4
0.5
0.6
0.7
0.8
***
N. S.
D
M
V
D
M
V
D
M
V
D
M
V
Control
mad-/-
Tkv[CA]
o/e Wit
C
D (ddaC)
All C4da neurons
M (v'ada)
V (vdaB)

Figure 3.2. C4da fine-scale topography requires BMP signaling

(A) Deleting the BMP receptor, Wit, disrupts C4da topography. MARCM technique was used to induce *wit* mutation in single C4da neurons.

(B) Deleting the BMP effector, Mad, abolishes the M/V separation, whereas elevating BMP signaling through Tkv[CA] expression or Wit overexpression (o/e) has little effect.

(C) The expression of Tkv[CA] results in overgrowth of the C4da axon terminals. The presynaptic terminals of all C4da neurons are labeled by ppk-tdTomato in red, and single C4da neurons (MARCM clones) are labeled by GFP in green. Scale bar: 10 μm.

CHAPTER 4

Discussion and Future Directions

A part of this chapter includes excerpts from a review article in Journal of Comprehensive Physiology A, Fine-scale topography in sensory systems: insights from *Drosophila* and vertebrates, written by **Takuya Kaneko** and Bing Ye in 2016, and a research article in Neuron, Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in *Drosophila*, written by **Takuya Kaneko**, Ann Marie Macara, Ruonan Li, Yujia Hu, Kenichi Iwasaki, Zane Dunnings, Ethan Firestone, Shawn Horvatic, Ananya Guntur, Orie T. Shafer, Chung-Hui Yang, Jie Zhou and Bing Ye in 2017.

Sensory-pathway-specific plasticity: insights from *Drosophila*

In this study, we demonstrate that a feedback circuit motif provides the basis for sensory-input-induced plasticity in the developing *Drosophil*a nociceptive circuit. We first show that functional development of the nociceptive circuit in *Drosophila* is regulated by noxious sensory inputs. In investigating the underlying mechanism, we identified a group of second-order neurons (SONs) that receive inputs from nociceptors, but not mechanosensors, which reveals that the larval nociceptive circuit contains components for both multimodality integration and modality-specific processing. Taking

advantage of this feature of the circuit, we demonstrate that sensory input-induced plasticity of the nociceptive circuit exhibits a striking degree of pathway-specific adaptation to noxious inputs. We further show that this pathway specificity is, at least in part, achieved through feedback inhibition of the nociceptor presynaptic terminals by serotonergic interneurons. This unique mechanism enables the nervous system to establish long-lasting functional changes in a sensory pathway-specific manner, without disrupting other modalities.

Sensory Gating through Presynaptic Inhibition at the First Synapse of the Nociceptive Circuit

To prevent an overload of irrelevant or low-priority information, the nervous system filters out sensory afferents elicited by redundant or unnecessary stimuli. Although this process, referred to as sensory gating, occurs at multiple levels in the nervous system (Freedman et al., 1996; McCormick and Bal, 1994), presynaptic inhibition at the first synapse of sensory pathways appears to be a recurrent theme in sensory gating across species. For example, in the *Drosophila* olfactory system, GABAergic inhibition of the terminals of olfactory receptor neurons (ORNs) controls the information flow in the olfactory circuit (Olsen and Wilson, 2008; Root et al., 2008); the levels of presynaptic inhibition, which are mediated by GABABR2 receptors, are different in distinct types of ORNs to achieve appropriate responses to various olfactory cues (Root et al., 2008).

Previous studies in other organisms have shown that presynaptic inhibition of nociceptor terminals controls the sensory afferent in the nociceptive pathway. Both external stimuli and internal activities can inhibit nociceptor-to-SON synaptic

transmission and, consequently, alter the animal's response to noxious stimuli (Fields, 2004; Kuner, 2010). This allows the animal to perform other behavior(s) that may be more important than nociceptive behavior even in the presence of noxious inputs. Whether or not the presynaptic inhibition of nociceptor terminals exists in *Drosophila* was unknown.

In the present study, we discovered presynaptic inhibition at the first synapse of nociceptive circuit in the *Drosophila* larva. Moreover, we found that serotonergic signaling mediates this presynaptic inhibition. Serotoninergic systems are known to modulate sensory gating in several animal species. For example, in the medicinal leech, feeding inhibits the synaptic transmission from tactile mechanosensory neurons to all their SONs (Gaudry and Kristan, 2009), which can be mimicked by serotonin and blocked by an antagonist of serotonin receptors. Such a modulation of sensory gating establishes the priority of feeding over tactile behaviors. Moreover, in *Drosophila*, serotonin modulates olfactory processing by enhancing olfactory responses of the SONs, the projection neurons, in an odorant-specific fashion (Dacks et al., 2009). Therefore, serotonergic inhibition of the first synapse of sensory circuits is a mechanism that underlies sensory gating in multiple sensory systems. Our study shows that serotonergic neurons are part of a feedback loop that inhibits nociceptor-to-SON synaptic transmission in the *Drosophila* larva, which reveals a circuit motif that underlies the presynaptic inhibition of the first synapse of nociceptive circuit (Figure 2.13).

A Novel Mechanism that Underlies Sensory Pathway Specificity in Sensory Input-Induced Plasticity

Our results demonstrate pathway specificity in the plasticity of the *Drosophila* nociceptive circuit. Although mechanosensory and nociceptive pathways converge on shared SONs (e.g., Basin-4) to integrate inputs from two different sensory modalities (Ohyama et al., 2015), developmental noxious input only modifies nociceptor-to-SON 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 receive inputs from SONs (Figure 2.13). Several aspects of this circuit motif contribute to sensory pathway specificity of the plasticity. First, it is different from the serotonergic facilitation that occurs during sensitization of the defensive gill-withdrawal reflex in *Aplysia*, in which serotonergic neurons are activated by another sensory pathway (Kandel, 2001). This makes sense, because the pathway-specific plasticity described here is not a form of associative learning. Co-activation of SONs in the sensory circuit and modulatory interneurons, which provide a feedback control, forms a circuit motif that allows the establishment of long-lasting changes in the nociceptive circuit while maintaining the normal functions of other sensory modalities. Second, the nociceptive circuit establishes plasticity by sensitizing nociceptor presynaptic terminals to serotonin, rather than by enhancing the activity of serotonergic neurons; this allows the nervous system to maintain other serotonin-dependent functions. It is interesting to note that the plasticity achieved through this feedback motif implies a homeostatic mechanism that maintains the activity levels of serotonergic neurons when those of SONs are reduced. Last, by modulating the presynaptic terminals of nociceptors, but not the postsynaptic

neurons, nociceptor input-induced plasticity allows the postsynaptic neurons to maintain their normal responses in other modalities (e.g., mechanosensation).

The Role of Serotonergic System in the Plasticity of Sensory Gating

We found that the strength of serotonergic modulation is plastic, such that stimulation of nociceptors during development sensitizes nociceptor presynaptic terminals to serotonergic inhibition. This plasticity is probably established through use-dependent strengthening of the serotonergic modulation; noxious input during development likely leads to chronic activation of serotonin receptors in nociceptor presynaptic terminals, which in turn sensitizes these presynaptic terminals to serotonergic modulation. The enhanced sensitivity may be achieved through (1) an increase in the expression level of serotonin receptors in the presynaptic terminals, for example, through CREB-dependent gene expression (Bartsch et al., 1998; Dash et al., 1990); (2) modification of a signaling transduction pathway that leads to reduced presynaptic neurotransmitter release, which would be a novel form of synaptic scaling; or (3) modification of the function of the serotonin transporter in presynaptic terminals (Fabre et al., 2000). Our study lays the foundation for future studies of the molecular pathways that underlie nociceptor input-induced plasticity.

Ethological Significance of Sensory Input-Induced Plasticity in the Nociceptive Circuit

Developmental plasticity of the nervous system allows animals to adapt their behaviors to survive in a unique, yet stable, environment. Larvae have fewer degrees of freedomin choosing their environments than do adult flies and vertebrates. As

nociceptive curling and rolling are disruptive to baseline functions of the larval nervous system, it is conceivable that developing in an environment in which there is excessive noxious sensory input requires that the animal suppresses nociceptive circuit function for survival. Identification of nociceptive circuit-specific 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 environments (Singh et al., 1982). Moreover, some noxious chemicals have antimicrobial properties (Billing and Sherman, 1998; Zhang, 2010) and could potentially protect *Drosophila* from bacteria or deleterious fungi in rotten fruits. Finally, our data show that exposure to low levels of the insecticide AITC results in modulation of insect behavior, which indicates potential unintentional ecological consequences of insecticide off-target effects.

Sensory-pathway-specific plasticity: future directions

Although we provided a novel insight into the mechanism that enables sensory-pathway-specificity, our study has opened up more questions to be addressed. Most importantly, it needs to be determined whether the mechanosensory pathway is capable of expressing plasticity in response to enhanced mechanosensory inputs during development. If mechanosensory synapses of Basin-4 neurons are incapable of expressing synaptic plasticity, it is no wonder that noxious inputs modify nociceptive synapses specifically without changing mechanosensory synapses. The effect of mechanosensory experience can be examined by providing developing larvae with high

levels of vibration, because Basin-4 neurons receive inputs from mechanosensors that detect vibration. As observed in *C. elegans*, enhanced mechanosensory experience may increase the sensitivity to mechanosensory inputs, which could help animals to prepare for future mechanosensory stimuli.

Another question would arise if mechanosensory synapses of Basin-4 exhibit synaptic plasticity. How do serotonergic neurons specifically modify one type of sensory neurons? Considering the broad distribution of serotonergic neurites and the wide diffusion range of serotonin (Bunin and Wightman, 1999), it is conceivable that serotonin reaches both the presynaptic terminals of mechanosensors and those of nociceptors, which are immediately adjacent to each other (Grueber et al., 2007). One possibility is that sensory-input-induced plasticity largely depends on the timing of neural activities in serotonergic neurons and sensory neurons. The feedback motif in the nociceptive circuit causes the activation of serotonergic neurons immediately after the activation of nociceptive neurons. This is consistent with the timing that enables serotonin to establish associative learning in *Aplysia*, where serotonergic neurons need to be activated slightly after the activation of sensory neurons (Kandel, 2013). Similarly, this model 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 the nociceptive circuit, inhibition of the nociceptor-to-target transmission may be attributable to Hebbian plasticity in the connection between serotonergic neurons and nociceptor presynaptic terminals. To test this model, future study can address whether silencing serotonergic neurons throughout development

prevents noxious inputs from sensitizing the nociceptor presynaptic terminals to serotonin.

Activity-dependent topographic projections: insights from *Drosophila*

Topographic maps are unique in that, although neurons in the map are of the same type, the axon terminals of neighboring neurons are continuously aligned to form point-to-point connection specificity. In the fly visual system, genetic programs sufficiently establish fine-scale topographic maps, whereas both vertebrate visual system and fly nociceptive system require the activity of afferent neurons for their fine-scale topographic alignments. Despite these findings, many important questions remain to be answered in order to understand the mechanisms underlying activity-dependent fine-scale topography.

Genetic code versus neural activity in specifying synaptic connections

Why is the genetic code insufficient for establishing fine-scale topography in the vertebrate visual system and fly nociceptive system but sufficient for the fly visual system? The six photoreceptor axons from each ommatidium are bundled together in a fashion that preserves the spatial arrangement of the cell bodies (Clandinin and Zipursky 2002), which may require Eph gradients in the targets (Dearborn et al. 2002). Intrinsic differences in cell adhesion then precisely instruct the projections of the six axons to form specific connections with their respective targets. These two steps prevent adjacent presynaptic terminals from overlapping with each other at any stage of

the development, resulting in a continuous map composed of a large number of neurons of the same type.

The vertebrate visual systems take a different strategy. Morphogens such as BMP construct broad gradients of Ephs and ephrins over large populations of pre- and post-synaptic neurons (Peters 2002). These broad gradients are insufficient for endowing distinct identities to neighboring axon terminals, producing overlapping synaptic terminals among the neighbors. In order to refine the coarse topography, neuronal activity is used as an additional cue to distinguish adjacent axons for their fine-scale separation. The sorting of the larger number of axons in vertebrates might be beyond the capacity of genetic programs, and the difference in the strategies might be attributed to differences in the brain size across species.

Our finding of activity-dependent topography in the fly nociceptive system demonstrates cross-species conservation of the activity-dependent fine-scale sorting of sensory afferents. Among the three nociceptive neurons forming topography in one hemi-segment, two project their axons as a single nerve (Yang et al. 2014). The axons of the two neurons are not patterned topographically in the nerve, and their presynaptic terminals overlap initially. Despite the small number of afferents, the sorting of the adjacent terminals relies on the relative levels of neuronal activity. Elucidating how neuronal activity generates molecular differences among neighboring neurons will shed light on how the nervous system uses neuronal activity for its development.

The link between neural activity and synaptic specificity in fine-scale topography

Despite remarkable progresses in understanding activity-dependent fine-scale topography, how neural activity leads to specific synaptic connections in fine-scale topography remains undetermined. Studies of the fly visual system revealed that fine-scale topography requires a network of cell-cell adhesion, which is based on differential regulation of adhesion properties between neighboring neurons (Figure 4.1). In this system, interactions among axons with different levels of cadherin activity direct the axons to their correct targets.

Axon-axon interactions appear to be a conserved mechanism underlying the formation of neural maps. In the vertebrate olfactory system, receptor neurons detecting different odors project their axons to anatomically separated glomeruli. The formation of such a discrete map relies on axon-axon interactions of olfactory receptor neurons, which are partly regulated by stimulus-induced activity of these neurons (Sakano 2010; Nishizumi and Sakano 2015). Odor-driven neuronal activity produces a difference in the expression profile of cell surface molecules among neurons with different receptors (Serizawa et al. 2006). The activity-dependent membrane molecules include adhesive molecules, such as kirrel2/3, and repulsive molecules, such as ephrinA/EphA. Interactions between afferents with different levels of these proteins contribute to the receptor-type specific axonal segregation in the olfactory system.

In the vertebrate visual system, it is possible that the retinal waves dynamically control adhesive characteristics of individual RGC axons (Figure 4.1). For example, the waves might produce transient gradients of adhesion activity among neighboring axons along the axis of its propagation. Such local gradients can instruct individual synaptic

connections through a network of cell-cell interactions. In this regard, the activity-dependent regulation of Trim9 levels might act as a mediator between neural activity and cell adhesion. It is interesting to note that Trim9 protein, which is specifically expressed in the nervous system in mammals (Berti et al. 2002; Tanji et al. 2010), exhibits a gradient along the dorsal-to-ventral axis in mouse spinal cord (Berti et al. 2002). This raises the possibility that Trim9 gradient instructs fine-scale topography in vertebrates.

The role of axon-target interactions and BMP signaling in establishing topography

Besides axon-axon interactions mentioned above, topographic maps of sensory afferents are commonly established through interactions of the afferents with their postsynaptic targets, i.e. axon-target interactions. In the fly visual system, topographic projections of photoreceptors require Ncad-mediated attractive interactions of the photoreceptors with their postsynaptic neurons in the lamina. In the case of the vertebrate visual system, a necessary step for establishing RGC topography is activation (i.e. depolarization) of postsynaptic neurons by neuronal activity of presynaptic RGCs. Consequent synchronization of neural activities in pre- and postsynaptic terminals leads to activation of NMDA receptors on the postsynaptic terminals, which in turn stabilizes their synaptic connections and establishes fine-scale topography.

Our study further identified that topographic projections of fly larval nociceptors require A08n neurons, one group of postsynaptic targets of nociceptors. Although similar to the vertebrate visual system, fine-scale topography of nociceptors is instructed

by neural activity of these nociceptors, it is unlikely that nociceptor activity does so through activation of postsynaptic A08n neurons. Indeed, our result demonstrated that neural activity of nociceptors can instruct topography, even in the absence of neurotransmitter release from the nociceptors, ruling out the possibility that synchronized neural activities between pre- and postsynaptic terminals instruct topography even in this fly system. It is easy to speculate that a different strategy has been evolved to accommodate particular needs of the fly nervous system, where the majority of neurons (likely including nociceptors) are cholinergic and communicate with postsynaptic targets through acetylcholine receptors rather than through NMDA glutamate receptors necessary for co-activity detection.

Rather than by directly activating postsynaptic targets, neural activity in nociceptors possibly impacts axon-target interactions indirectly by regulating intrinsic signaling molecules. In fact, at the fly NMJ, neuronal activity in motor neurons indirectly affects axon-target interactions by regulating signaling molecules inside the motor neurons. In this system, individual motor neurons connect with specific muscle cells with high fidelity during the embryonic stage (Ruiz-Canada and Budnik, 2006). This target selection is partially regulated by motor neuron activity, whose role exhibits at least three similarities to that of nociceptor activity (Carrillo et al., 2010). First, inhibition of neural activity of motor neurons results in their synaptic connections with incorrect muscle cells. Second, neural activity regulates motor neuron projections, even in the absence of depolarization of postsynaptic muscle cells. Third, the function of neural activity in motor neurons is likely mediated by the intrinsic signaling molecule, CaMKII, whose overexpression in motor neurons suppresses the defects caused by the

inhibition of motor neuron activity. Importantly, in addition to these three similarities, activity-dependent CaMKII signaling seems to instruct target selection by sensitizing motor neurons to Semaphorin-2a signaling, a retrograde repellent signaling derived from muscle cells. The potential targets of this activity-dependent regulation are signaling pathways downstream of plexin B, a receptor for Semaphorin-2a. Based on this important research, we favor a model of nociceptor topography in which neural activity in nociceptors similarly control nociceptors' responses to retrograde signaling from postsynaptic A08n neurons through Trim9-mediated signaling pathways.

We identified Dpp as a potential retrograde signaling molecule secreted from A08n neurons to nociceptors, which may act as a guidance molecule. The role of Dpp as an attractive guidance cue was demonstrated in two tissues in the fruit fly. First, in the developing wing disc, Dpp-expressing epithelial cells physically contact distant tracheal primordial cells by recruiting long protrusions of the tracheal cells that contain the Dpp receptor, Tkv (Roy et al., 2014; Roy and Kornberg, 2011). Second, in the testis of adult flies, Dpp-expressing somatic "niche" cells recruit Tkv-containing cellular protrusion of male germline stem cells in order to provide niche signals to the stem cells (Inaba et al., 2015). In both of these two tissues, Dpp is required and sufficient to attract Tkv-expressing cellular processes, and ectopic Dpp expressions cause incorrect targeting of these processes, suggesting that Dpp-mediated BMP signaling directs cell-cell connections.

Given the possible role of Dpp as an attractive guidance cue, we propose that neural activity in nociceptors instructs fine-scale topography by sensitizing nociceptors' responses to retrograde Dpp signaling, which may involve translocation of Tkv to the

presynaptic terminals. We further suspect that this activity-dependent regulation sensitizes the presynaptic terminals of M neurons, more than those of V neurons, to Dpp-mediated recruitment by postsynaptic A08n neurons. Consistent with this model, a recent EM reconstruction demonstrated that Dpp-expressing A08n neurons form more synapses with M neurons than V neurons (Gerhard et al., 2017). This EM study further determined that the primary presynaptic partners of A08n neurons are D neurons, suggesting that postsynaptic terminals of A08n neurons are dorsally located within the synaptic areas of nociceptors (i.e. C4da neuropils). The recruitment of the M axons to this dorsally-located A08n postsynaptic terminals may be the direct cause of the termination of the M axons dorsally to the V axons.

BMP signaling may regulate nociceptor topography through more indirect pathways, such as the induction of other guidance molecules. In the vertebrate visual system, RGC topography is indirectly regulated by the mammalian homologs of Dpp, BMP2 and BMP4, which are expressed specifically in the dorsal side of the retina. This dorsally restricted BMP expression drives the formation of D-V gradients in signaling molecules that directly instruct RGC topography as guidance cues (e.g. EphB2/EphrinB2) (Peters, 2002; Plas et al., 2008). Similarly, in the fly visual system, Dpp-mediated BMP signaling indirectly regulates topographic projections of photoreceptors by specifying the cell fate of photoreceptor targets in the lamina (Yoshida et al., 2005). Besides these functions of BMP signaling, a recent study of fly nociceptors revealed an additional one; BMP signaling enhances calcium responses in the presynaptic terminals of nociceptors (Tracey and Honjo, 2018). This interesting finding raises the possibility that this BMP-induced calcium enhancement mediates the

role of neural activity in establishing topography. To examine this possibility, future research needs to address whether the levels of calcium responses in the nociceptor presynaptic terminals determine their topographic projections, and whether the calcium responses are controlled by axon-target interactions (e.g. A08n-derived Dpp signaling).

The role of topography of the nociceptive system in fly larvae

Topographic maps generally encode the positional information of sensory stimuli, and allow animals to respond differently to sensory inputs from different body locations. Indeed, fly larvae respond differently to noxious inputs based on the location of the stimuli along the larval body axis (Robertson et al., 2013). For example, noxious stimuli on the posterior region usually elicit rapid forward locomotion, whereas the same type of stimuli on the middle part of the larval body often elicits rolling behavior. Additionally, the initial direction of rolling behavior tends to be towards the side of the body that is stimulated by the noxious inputs; larvae preferentially roll to the right in response to noxious inputs on the right side of the body (Hwang et al., 2007). Each behavior seems to allow larvae to efficiently escape from individual noxious stimuli.

However, although we identified the topographic alignment of nociceptors along the D-V axis, no study has demonstrated any difference in larval behavior in response to sensory inputs from the dorsal side vs. ventral side. If the positional information of stimuli along the D-V axis is unnecessary for larval behavior, the topographic alignment of three neurons along the D-V axis may merely be a strategy to allow the synaptic areas to be equally occupied by the presynaptic terminals of the three neurons. Whether larvae exhibit distinct behavior in responses to different noxious inputs along

the D-V axis of the larval body needs careful examination to better understand the role of topographic maps and to study the behavioral consequences of topography defects.

Activity-dependent topographic projections: future directions

The formation of a topographic map of sensory axons is a general strategy used by the nervous system to detect the locations of sensory stimuli. It is demonstrated that stimulating sensory neurons in different locations elicits different responses in the downstream circuit. However, do spatially separated sensory neurons of the same type connect to the same types of target neurons? This is a fundamental open question in developmental neuroscience and sensory neuroscience. Moreover, whether sensory experience during development affects the types of postsynaptic neurons that nociceptors connect to is another important open question. Topography in the *Drosophila* nociceptive system, which we identified, offers an opportunity to address these issues.

Our finding that the nociceptor topographic projections require neuronal activity raised the possibility that the activity of nociceptors instructs the choice of postsynaptic targets during the establishment of nociceptor-to-target connections. Moreover, the other project of my dissertation research revealed that the nociceptor-to-target transmission is suppressed by sensory inputs during development. This suppression may be caused by changes in the postsynaptic neuron types that nociceptors select. Based on these findings, I suspect that the levels of neuronal activities in the nociceptors direct their selection of postsynaptic partners.

In the past few years, we and others have identified many different types of postsynaptic targets of nociceptors in *Drosophila* larva (Burgos et al., 2018; Gerhard et al., 2017; Kaneko et al., 2017; Ohyama et al., 2015; Takagi et al., 2017; Vogelstein et al., 2014; Yoshino et al., 2017). I expect that individual nociceptors structurally connect to different groups of target neurons and elicit responses differently in the downstream circuit. This idea is consistent with a recent EM reconstruction demonstrating that postsynaptic target neurons form different numbers of synapses with individual nociceptors, although this study needs further verification with more samples (Gerhard et al., 2017). Future studies will analyze synaptic connections of individual nociceptors in detail and address whether the connections change upon manipulation of neural activity. This study would further improve our understanding of how sensory inputs and neuronal activities contribute to the formation of functional neural networks.

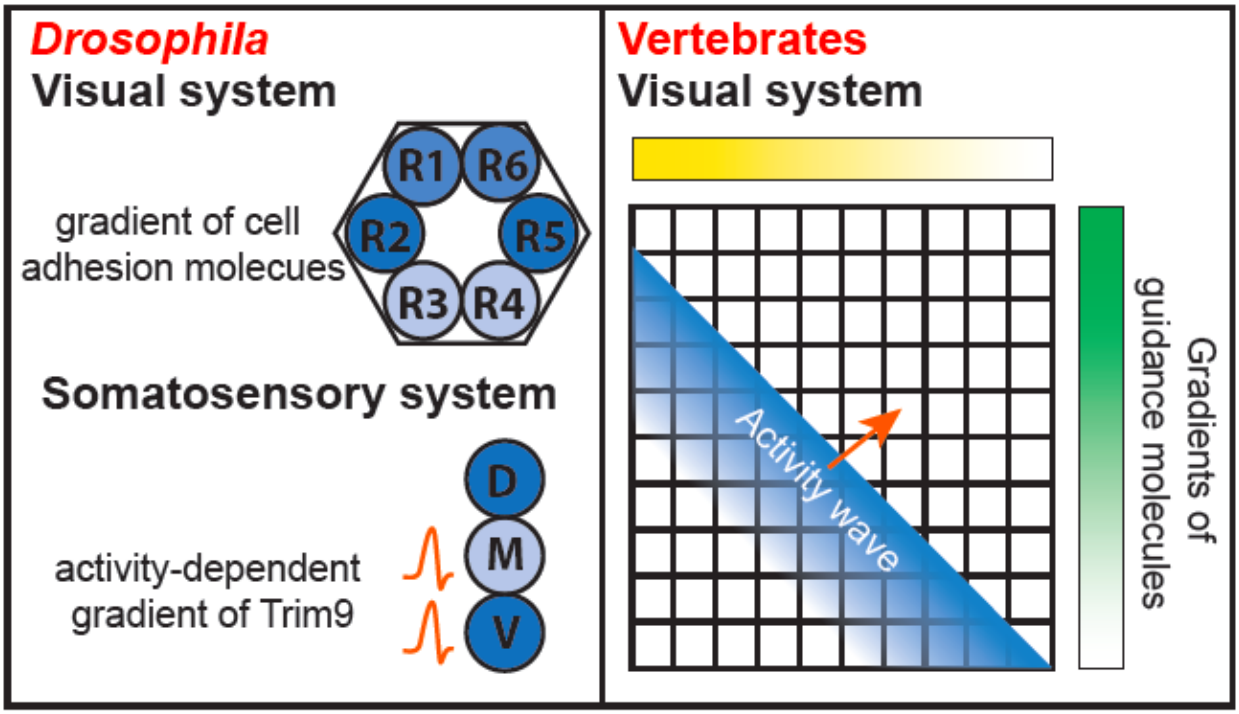


Figure 4.1. Mechanisms underlying fine-scale topography
In *Drosophila*, local gradients of intrinsic molecules (e.g. cell adhesion molecules in the visual system and Trim9 in the somatosensory system) instruct fine-scale topography. In the vertebrate visual system, broad gradients of guidance molecules are not sufficient to distinguish neighboring afferents. The retinal wave of neuronal activity might transiently provide a local gradient of intrinsic molecules, which in turn specifies individual connections.

BIBLIOGRAPHY

References from “Fine-scale topography in sensory systems: insights from Drosophila and vertebrates” in Journal of Comprehensive Physiology A (2016):

Ackman JB, Burbridge TJ, Crair MC (2012) Retinal waves coordinate patterned activity throughout the developing visual system Nature 490:219-225 doi:10.1038/nature11529

Ackman JB, Crair MC (2014) Role of emergent neural activity in visual map development Current opinion in neurobiology 24:166-175 doi:10.1016/j.conb.2013.11.011

Berti C, Messali S, Ballabio A, Reymond A, Meroni G (2002) TRIM9 is specifically expressed in the embryonic and adult nervous system Mechanisms of development 113:159-162 doi:Pii S0925-4773(02)00013-8

Brigidi GS et al. (2014) Palmitoylation of delta-catenin by DHHC5 mediates activity-induced synapse plasticity Nature Neuroscience 17:522-U571 doi:Doi 10.1038/Nn.3657

Butts DA, Kanold PO, Shatz CJ (2007) A burst-based "Hebbian" learning rule at retinogeniculate synapses links retinal waves to activity-dependent refinement PLoS Biol 5:e61 doi:10.1371/journal.pbio.0050061

Cang J, Feldheim DA (2013) Developmental mechanisms of topographic map formation and alignment Annual review of neuroscience 36:51-77 doi:10.1146/annurev-neuro-062012-170341

Chalupa LM (2009) Retinal waves are unlikely to instruct the formation of eye-specific retinogeniculate projections Neural development 4:25 doi:10.1186/1749-8104-4-25

Chandrasekaran AR, Plas DT, Gonzalez E, Crair MC (2005) Evidence for an instructive role of retinal activity in retinotopic map refinement in the superior colliculus of the mouse J Neurosci 25:6929-6938

Chen PL, Clandinin TR (2008) The cadherin Flamingo mediates level-dependent interactions that guide photoreceptor target choice in *Drosophila* Neuron 58:26-33 doi:10.1016/j.neuron.2008.01.007

Clandinin TR, Zipursky SL (2000) Afferent growth cone interactions control synaptic specificity in the *Drosophila* visual system Neuron 28:427-436

Clandinin TR, Zipursky SL (2002) Making connections in the fly visual system Neuron 35:827-841

Cline HT (1991) Activity-dependent plasticity in the visual systems of frogs and fish Trends in neurosciences 14:104-111

Cline HT, Constantine-Paton M (1989) NMDA receptor antagonists disrupt the retinotectal topographic map Neuron 3:413-426

Constantine-Paton M, Cline HT, Debski E (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways Annual review of neuroscience 13:129-154 doi:10.1146/annurev.ne.13.030190.001021

Debski EA, Cline HT (2002) Activity-dependent mapping in the retinotectal projection Current opinion in neurobiology 12:93-99

Feller MB (2009) Retinal waves are likely to instruct the formation of eye-specific retinogeniculate projections Neural development 4:24 doi:10.1186/1749-8104-4-24

Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ (1996) Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves Science (New York, NY 272:1182-1187

Flanagan JG (2006) Neural map specification by gradients Current opinion in neurobiology 16:59-66 doi:10.1016/j.conb.2006.01.010

Flanagan JG, Vanderhaeghen P (1998) The ephrins and Eph receptors in neural development Annual review of neuroscience 21:309-345 doi:DOI 10.1146/annurev.neuro.21.1.309

Furman M, Xu HP, Crair MC (2013) Competition driven by retinal waves promotes morphological and functional synaptic development of neurons in the superior colliculus Journal of neurophysiology 110:1441-1454 doi:10.1152/jn.01066.2012

Grubb MS, Rossi FM, Changeux JP, Thompson ID (2003) Abnormal functional organization in the dorsal lateral geniculate nucleus of mice lacking the beta 2 subunit of the nicotinic acetylcholine receptor Neuron 40:1161-1172

Grueber WB, Jan LY, Jan YN (2002) Tiling of the *Drosophila* epidermis by multidendritic sensory neurons Development 129:2867-2878

Grueber WB, Ye B, Moore AW, Jan LY, Jan YN (2003) Dendrites of distinct classes of *Drosophila* sensory neurons show different capacities for homotypic repulsion Curr Biol 13:618-626

Hadjieconomou D, Timofeev K, Salecker I (2011) A step-by-step guide to visual circuit assembly in *Drosophila* Current opinion in neurobiology 21:76-84 doi:10.1016/j.conb.2010.07.012

Harris WA, Stark WS, Walker JA (1976) Genetic dissection of the photoreceptor system in the compound eye of *Drosophila* melanogaster The Journal of physiology 256:415-439

Hebb DO (1949) The organization of behavior; a neuropsychological theory. Wiley book in clinical psychology. Wiley, New York,

Hiesinger PR et al. (2006) Activity-independent prespecification of synaptic partners in the visual map of *Drosophila* Current biology : CB 16:1835-1843 doi:10.1016/j.cub.2006.07.047

Huang L, Pallas SL (2001) NMDA antagonists in the superior colliculus prevent developmental plasticity but not visual transmission or map compression Journal of neurophysiology 86:1179-1194

Huberman AD, Feller MB, Chapman B (2008) Mechanisms Underlying Development of Visual Maps and Receptive Fields Annual review of neuroscience 31:479-509 doi:doi:10.1146/annurev.neuro.31.060407.125533

Itoh K, Ozaki M, Stevens B, Fields RD (1997) Activity-dependent regulation of N-cadherin in DRG neurons: Differential regulation of N-cadherin, NCAM, and L1 by distinct patterns of action potentials J Neurobiol 33:735-748 doi:Doi 10.1002/(Sici)1097-4695(19971120)33:6<735::Aid-Neu3>3.0.Co;2-A

Kobayashi T, Nakamura H, Yasuda M (1990) Disturbance of refinement of retinotectal projection in chick embryos by tetrodotoxin and grayanotoxin Brain research Developmental brain research 57:29-35

Lee CH, Herman T, Clandinin TR, Lee R, Zipursky SL (2001) N-cadherin regulates target specificity in the *Drosophila* visual system Neuron 30:437-450

Lee RC, Clandinin TR, Lee CH, Chen PL, Meinertzhagen IA, Zipursky SL (2003) The protocadherin Flamingo is required for axon target selection in the *Drosophila* visual system Nat Neurosci 6:557-563

Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis Neuron 22:451-461

Luo L, Flanagan JG (2007) Development of continuous and discrete neural maps Neuron 56:284-300

McLaughlin T, O'Leary DD (2005) Molecular gradients and development of retinotopic maps Annual review of neuroscience 28:327-355

McLaughlin T, Torborg CL, Feller MB, O'Leary DD (2003) Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development Neuron 40:1147-1160

Meister M, Wong RO, Baylor DA, Shatz CJ (1991) Synchronous bursts of action potentials in ganglion cells of the developing mammalian retina Science (New York, NY 252:939-943

Menon KP, Carrillo RA, Zinn K (2013) Development and plasticity of the *Drosophila* larval neuromuscular junction Wiley interdisciplinary reviews Developmental biology 2:647-670 doi:10.1002/wdev.108

Morikawa RK, Kanamori T, Yasunaga KI, Emoto K (2011) Different levels of the Tripartite motif protein, Anomalies in sensory axon patterning (Asap), regulate distinct axonal projections of *Drosophila* sensory neurons Proceedings of the National Academy of Sciences of the United States of America 108:19389-19394

Mrsic-Flogel TD, Hofer SB, Creutzfeldt C, Cloez-Tayarani I, Changeux JP, Bonhoeffer T, Hubener M (2005) Altered map of visual space in the superior colliculus of mice lacking early retinal waves J Neurosci 25:6921-6928 doi:10.1523/JNEUROSCI.1555-05.2005

Nicol X, Voyatzis S, Muzerelle A, Narboux-Neme N, Sudhof TC, Miles R, Gaspar P (2007) cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map Nat Neurosci 10:340-347 doi:10.1038/nn1842

Peters MA (2002) Patterning the neural retina Current opinion in neurobiology 12:43-48

Pfeiffenberger C, Yamada J, Feldheim DA (2006) Ephrin-As and patterned retinal activity act together in the development of topographic maps in the primary visual system J Neurosci 26:12873-12884 doi:10.1523/JNEUROSCI.3595-06.2006

Prakash S, Caldwell JC, Eberl DF, Clandinin TR (2005) *Drosophila* N-cadherin mediates an attractive interaction between photoreceptor axons and their targets Nat Neurosci 8:443-450 doi:10.1038/nn1415

Reymond A et al. (2001) The tripartite motif family identifies cell compartments The EMBO journal 20:2140-2151 doi:10.1093/emboj/20.9.2140

Ruthazer ES, Cline HT (2004) Insights into activity-dependent map formation from the retinotectal system: a middle-of-the-brain perspective J Neurobiol 59:134-146 doi:10.1002/neu.10344

Schwabe T, Neuert H, Clandinin TR (2013) A network of cadherin-mediated interactions polarizes growth cones to determine targeting specificity Cell 154:351-364 doi:10.1016/j.cell.2013.06.011

Shah RD, Crair MC (2008) Retinocollicular synapse maturation and plasticity are regulated by correlated retinal waves J Neurosci 28:292-303 doi:10.1523/JNEUROSCI.4276-07.2008

Simon DK, Prusky GT, O'Leary DD, Constantine-Paton M (1992) N-methyl-D-aspartate receptor antagonists disrupt the formation of a mammalian neural map Proceedings of the National Academy of Sciences of the United States of America 89:10593-10597

Sperry RW (1943) Visuomotor coordination in the newt (Triturus viridescens) after regeneration of the optic nerve Journal of Comparative Neurology 79:33-55 doi:DOI 10.1002/cne.900790104

Sperry RW (1947) Nature of functional recovery following regeneration of the oculomotor nerve in amphibians The Anatomical record 97:293-316

Sperry RW (1963) Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections Proceedings of the National Academy of Sciences of the United States of America 50:703-710

Stafford BK, Sher A, Litke AM, Feldheim DA (2009) Spatial-temporal patterns of retinal waves underlying activity-dependent refinement of retinofugal projections Neuron 64:200-212 doi:10.1016/j.neuron.2009.09.021

Sun C, Warland DK, Ballesteros JM, van der List D, Chalupa LM (2008) Retinal waves in mice lacking the beta2 subunit of the nicotinic acetylcholine receptor Proceedings of the National Academy of Sciences of the United States of America 105:13638-13643 doi:10.1073/pnas.0807178105

Tanaka H et al. (2000) Molecular modification of N-cadherin in response to synaptic activity Neuron 25:93-107 doi:Doi 10.1016/S0896-6273(00)80874-0

Tanji K, Kamitani T, Mori F, Kakita A, Takahashi H, Wakabayashi K (2010) TRIM9, a novel brain-specific E3 ubiquitin ligase, is repressed in the brain of Parkinson's disease and dementia with Lewy bodies Neurobiology of disease 38:210-218 doi:DOI 10.1016/j.nbd.2010.01.007

Willshaw DJ, Sterratt DC, Teriakidis A (2014) Analysis of local and global topographic order in mouse retinocollicular maps J Neurosci 34:1791-1805 doi:10.1523/JNEUROSCI.5602-12.2014

Wong RO, Meister M, Shatz CJ (1993) Transient period of correlated bursting activity during development of the mammalian retina Neuron 11:923-938

Xu HP et al. (2011) An instructive role for patterned spontaneous retinal activity in mouse visual map development Neuron 70:1115-1127 doi:10.1016/j.neuron.2011.04.028

Yang L et al. (2014) Trim9 regulates activity-dependent fine-scale topography in *Drosophila* Curr Biol 24:1024-1030 doi:10.1016/j.cub.2014.03.041

Zhang J, Ackman JB, Xu HP, Crair MC (2012) Visual map development depends on the temporal pattern of binocular activity in mice Nat Neurosci 15:298-307 doi:10.1038/nn.3007

References from “Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in *Drosophila*” in Neuron (2017):

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J Neurosci *21*, 1523-1531.

Bartsch, D., Casadio, A., Karl, K.A., Serodio, P., and Kandel, E.R. (1998). CREB1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation. Cell *95*, 211-223.

Caldwell, L., Harries, P., Sydlik, S., and Schwiening, C.J. (2013). Presynaptic pH and vesicle fusion in *Drosophila* larvae neurones. Synapse *67*, 729-740.

Chen, T.W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., Schreiter, E. R., Kerr, R. A., Orger, M. B., Jayaraman, V., Looger, L. L., Svoboda, K., Kim, D. S. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature *499*.

Corty, M.M., Matthews, B.J., and Grueber, W.B. (2009). Molecules and mechanisms of dendrite development in *Drosophila*. Development *136*, 1049-1061.

Dacks, A.M., Green, D.S., Root, C.M., Nighorn, A.J., and Wang, J.W. (2009). Serotonin modulates olfactory processing in the antennal lobe of *Drosophila*. Journal of neurogenetics *23*, 366-377.

Dash, P.K., Hochner, B., and Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. Nature *345*, 718-721.

Deeg, K.E., and Aizenman, C.D. (2011). Sensory modality-specific homeostatic plasticity in the developing optic tectum. Nat Neurosci *14*, 548-550.

Fabre, V., Boutrel, B., Hanoun, N., Lanfumey, L., Fattaccini, C.M., Demeneix, B., Adrien, J., Hamon, M., and Martres, M.P. (2000). Homeostatic regulation of serotonergic function by the serotonin transporter as revealed by nonviral gene transfer. J Neurosci *20*, 5065-5075.

Fields, H. (2004). State-dependent opioid control of pain. Nat Rev Neurosci *5*, 565-575.

Freedman, R., Adler, L.E., Myles-Worsley, M., Nagamoto, H.T., Miller, C., Kisley, M., McRae, K., Cawthra, E., and Waldo, M. (1996). Inhibitory gating of an evoked response to repeated auditory stimuli in schizophrenic and normal subjects. Human recordings, computer simulation, and an animal model. Arch Gen Psychiatry *53*, 1114-1121.

Gaudry, Q., and Kristan, W.B., Jr. (2009). Behavioral choice by presynaptic inhibition of tactile sensory terminals. Nat Neurosci *12*, 1450-1457.

Glaser, T., and Traber, J. (1985). Binding of the putative anxiolytic TVX Q 7821 to hippocampal 5-hydroxytryptamine (5-HT) recognition sites. Naunyn Schmiedebergs Arch Pharmacol *329*, 211-215.

Gordon, M.D., and Scott, K. (2009). Motor control in a *Drosophila* taste circuit. Neuron *61*, 373-384.

Gou, B., Liu, Y., Guntur, A.R., Stern, U., and Yang, C.H. (2014). Mechanosensitive neurons on the internal reproductive tract contribute to egg-laying-induced acetic acid attraction in *Drosophila*. Cell Rep *9*, 522-530.

Griffith, L.C., and Budnik, V. (2006). Plasticity and second messengers during synapse development. Int Rev Neurobiol *75*, 237-265.

Grueber, W.B., Ye, B., Moore, A.W., Jan, L.Y., and Jan, Y.N. (2003). Dendrites of distinct classes of *Drosophila* sensory neurons show different capacities for homotypic repulsion. Curr Biol *13*, 618-626.

Grueber, W.B., Ye, B., Yang, C.H., Younger, S., Borden, K., Jan, L.Y., and Jan, Y.N. (2007). Projections of *Drosophila* multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. Development *134*, 55-64.

Guo, J.D., and Rainnie, D.G. (2010). Presynaptic 5-HT(1B) receptor-mediated serotonergic inhibition of glutamate transmission in the bed nucleus of the stria terminalis. Neuroscience *165*, 1390-1401.

Hodge, J.J. (2009). Ion channels to inactivate neurons in *Drosophila*. Frontiers in molecular neuroscience *2*, 13.

Honjo, K., Hwang, R.Y., and Tracey, W.D., Jr. (2012). Optogenetic manipulation of neural circuits and behavior in *Drosophila* larvae. Nat Protoc *7*, 1470-1478.

Hu, A., Zhang, W., and Wang, Z. (2010). Functional feedback from mushroom bodies to antennal lobes in the *Drosophila* olfactory pathway. Proceedings of the National Academy of Sciences of the United States of America *107*, 10262-10267.

Hubel, D.H., and Wiesel, T.N. (1970). The period of susceptibility to the physiological effects of unilateral eye closure in kittens. The Journal of physiology *206*, 419-436.

Huser, A., Rohwedder, A., Apostolopoulou, A.A., Widmann, A., Pfitzenmaier, J.E., Maiolo, E.M., Selcho, M., Pauls, D., von Essen, A., Gupta, T.*, et al.* (2012). The serotonergic central nervous system of the *Drosophila* larva: anatomy and behavioral function. PLoS One *7*, e47518.

Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., and Tracey, W.D. (2007). Nociceptive neurons protect *Drosophila* larvae from parasitoid wasps. Curr Biol *17*, 2105-2116.

Iwasaki, Y., Tanabe, M., Kobata, K., and Watanabe, T. (2008). TRPA1 agonists--allyl isothiocyanate and cinnamaldehyde--induce adrenaline secretion. Bioscience, biotechnology, and biochemistry *72*, 2608-2614.

Johns, D.C., Marx, R., Mains, R.E., O'Rourke, B., and Marban, E. (1999). Inducible genetic suppression of neuronal excitability. J Neurosci *19*, 1691-1697.

Jovanic, T., Schneider-Mizell, C.M., Shao, M., Masson, J.B., Denisov, G., Fetter, R.D., Mensh, B.D., Truman, J.W., Cardona, A., and Zlatic, M. (2016). Competitive Disinhibition Mediates Behavioral Choice and Sequences in *Drosophila*. Cell *167*, 858-870 e819.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science (New York, NY *294*, 1030-1038.

Kim, J.H., Wang, X., Coolon, R., and Ye, B. (2013). Dscam expression levels determine presynaptic arbor sizes in *Drosophila* sensory neurons. Neuron *78*, 827-838.

Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z*., et al.* (2014). Independent optical excitation of distinct neural populations. Nature methods *11*, 338-346.

Kuner, R. (2010). Central mechanisms of pathological pain. Nature medicine *16*, 1258-1266.

Lesch, K.P., and Waider, J. (2012). Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron *76*, 175-191.

Lima, S.Q., and Miesenbock, G. (2005). Remote control of behavior through genetically targeted photostimulation of neurons. Cell *121*, 141-152.

Macleod, G.T., Marin, L., Charlton, M.P., and Atwood, H.L. (2004). Synaptic vesicles: test for a role in presynaptic calcium regulation. J Neurosci *24*, 2496-2505.

Macpherson, L.J., Zaharieva, E.E., Kearney, P.J., Alpert, M.H., Lin, T.Y., Turan, Z., Lee, C.H., and Gallio, M. (2015). Dynamic labelling of neural connections in multiple colours by trans-synaptic fluorescence complementation. Nature communications *6*, 10024.

Maj, J., Chojnacka-Wojcik, E., Tatarczynska, E., and Klodzinska, A. (1987). Central action of ipsapirone, a new anxiolytic drug, on serotoninergic, noradrenergic and dopaminergic functions. J Neural Transm *70*, 1-17.

Matsunaga, T., Fushiki, A., Nose, A., and Kohsaka, H. (2013). Optogenetic perturbation of neural activity with laser illumination in semi-intact *drosophila* larvae in motion. J Vis Exp, e50513.

McCormick, D.A., and Bal, T. (1994). Sensory gating mechanisms of the thalamus. Current opinion in neurobiology *4*, 550-556.

Menon, K.P., Carrillo, R.A., and Zinn, K. (2013). Development and plasticity of the *Drosophila* larval neuromuscular junction. Wiley Interdiscip Rev Dev Biol *2*, 647-670.

Nicolai, L.J., Ramaekers, A., Raemaekers, T., Drozdzecki, A., Mauss, A.S., Yan, J., Landgraf, M., Annaert, W., and Hassan, B.A. (2010). Genetically encoded dendritic marker sheds light on neuronal connectivity in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America *107*, 20553-20558.

Nitabach, M.N., Blau, J., and Holmes, T.C. (2002). Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell *109*, 485-495.

Ohyama, T., Schneider-Mizell, C.M., Fetter, R.D., Aleman, J.V., Franconville, R., Rivera-Alba, M., Mensh, B.D., Branson, K.M., Simpson, J.H., Truman, J.W.*, et al.* (2015). A multilevel multimodal circuit enhances action selection in *Drosophila*. Nature *520*, 633-639.

Olsen, S.R., and Wilson, R.I. (2008). Lateral presynaptic inhibition mediates gain control in an olfactory circuit. Nature *452*, 956-960.

Prieto-Godino, L.L., Diegelmann, S., and Bate, M. (2012). Embryonic origin of olfactory circuitry in *Drosophila*: contact and activity-mediated interactions pattern connectivity in the antennal lobe. PLoS Biol *10*, e1001400.

Raymond, J.R., Mukhin, Y.V., Gettys, T.W., and Garnovskaya, M.N. (1999). The recombinant 5-HT1A receptor: G protein coupling and signalling pathways. Br J Pharmacol *127*, 1751-1764.

Reiff, D.F., Ihring, A., Guerrero, G., Isacoff, E.Y., Joesch, M., Nakai, J., and Borst, A. (2005). In vivo performance of genetically encoded indicators of neural activity in flies. J Neurosci *25*, 4766-4778.

Reiff, D.F., Thiel, P.R., and Schuster, C.M. (2002). Differential regulation of active zone density during long-term strengthening of *Drosophila* neuromuscular junctions. J Neurosci *22*, 9399-9409.

Riad, M., Garcia, S., Watkins, K.C., Jodoin, N., Doucet, E., Langlois, X., el Mestikawy, S., Hamon, M., and Descarries, L. (2000). Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. The Journal of comparative neurology *417*, 181-194.

Robinson, I.M., Ranjan, R., and Schwarz, T.L. (2002). Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain. Nature *418*, 336-340.

Root, C.M., Masuyama, K., Green, D.S., Enell, L.E., Nassel, D.R., Lee, C.H., and Wang, J.W. (2008). A presynaptic gain control mechanism fine-tunes olfactory behavior. Neuron *59*, 311-321.

Ruiz-Canada, C., and Budnik, V. (2006). Synaptic cytoskeleton at the neuromuscular junction. Int Rev Neurobiol *75*, 217-236.

Salvaterra, P.M., and Kitamoto, T. (2001). *Drosophila* cholinergic neurons and processes visualized with Gal4/UAS-GFP. Brain Res Gene Expr Patterns *1*, 73-82.

Shafer, O.T., Kim, D.J., Dunbar-Yaffe, R., Nikolaev, V.O., Lohse, M.J., and Taghert, P.H. (2008). Widespread Receptivity to Neuropeptide PDF throughout the Neuronal Circadian Clock Network of *Drosophila* Revealed by Real-Time Cyclic AMP Imaging. Neuron *58*, 223-237.

Singh, R.S., Hickey, D.A., and David, J. (1982). Genetic Differentiation between Geographically Distant Populations of *DROSOPHILA MELANOGASTER*. Genetics *101*, 235-256.

Stewart, B.A., Atwood, H. L., Renger, J. J., Wang, J., Wu, C. F. (1994). Improved stability of *Drosophila* larval neuromuscular preparations in hemolymph-like physiological solutions. Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology *175*.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. Cell *72*, 527-540.

Sultana, T., Savage, G.P., McNeil, D.L., Porter, N.G., and Clark, B. (2003). Comparison of flavour compounds in wasabi and horseradish. J Food Agric Environ *1*, 117-121.

Traber, J., Davies, M.A., Dompert, W.U., Glaser, T., Schuurman, T., and Seidel, P.R. (1984). Brain serotonin receptors as a target for the putative anxiolytic TVX Q 7821. Brain Res Bull *12*, 741-744.

Vogelstein, J.T., Park, Y., Ohyama, T., Kerr, R.A., Truman, J.W., Priebe, C.E., and Zlatic, M. (2014). Discovery of brainwide neural-behavioral maps via multiscale unsupervised structure learning. Science (New York, NY *344*, 386-392.

Wang, X., Kim, J.H., Bazzi, M., Robinson, S., Collins, C.A., and Ye, B. (2013). Bimodal control of dendritic and axonal growth by the dual leucine zipper kinase pathway. PLoS Biol *11*, e1001572.

Westenberg, H.G., Gerritsen, T.W., Meijer, B.A., and van Praag, H.M. (1982). Kinetics of l-5-hydroxytryptophan in healthy subjects. Psychiatry Res *7*, 373-385.

Xiang, Y., Yuan, Q., Vogt, N., Looger, L.L., Jan, L.Y., and Jan, Y.N. (2010). Light-avoidance-mediating photoreceptors tile the *Drosophila* larval body wall. Nature *468*, 921-926.

Yang, L., Li, R., Kaneko, T., Takle, K., Morikawa, R.K., Essex, L., Wang, X., Zhou, J., Emoto, K., Xiang, Y.*, et al.* (2014). Trim9 regulates activity-dependent fine-scale topography in *Drosophila*. Curr Biol *24*, 1024-1030.

Yao, Z.P., Macara, A. M., Lelito, K. R., Minosyan, T. Y., Shafer, O. T. (2012). Analysis of functional neuronal connectivity in the *Drosophila* brain. Journal of neurophysiology *108*.

Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. Curr Biol *16*, 1051-1062.

Yuan, Q., Xiang, Y., Yan, Z., Han, C., Jan, L.Y., and Jan, Y.N. (2011). Light-induced structural and functional plasticity in *Drosophila* larval visual system. Science (New York, NY *333*, 1458-1462.

Zemelman, B.V., Lee, G.A., Ng, M., and Miesenbock, G. (2002). Selective photostimulation of genetically chARGed neurons. Neuron *33*, 15-22.

Zhang, Y. (2010). Allyl isothiocyanate as a cancer chemopreventive phytochemical. Mol Nutr Food Res *54*, 127-135.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. Nature *438*, 179-184.

Zhong, L., Bellemer, A., Yan, H., Ken, H., Jessica, R., Hwang, R.Y., Pitt, G.S., and Tracey, W.D. (2012). Thermosensory and nonthermosensory isoforms of *Drosophila* melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. Cell Rep. 1, 43–55.

Other references:

Allen, C.B., Celikel, T., and Feldman, D.E. (2003). Long-term depression induced by sensory deprivation during cortical map plasticity in vivo. Nat Neurosci *6*, 291-299.

Bailey, C.H., and Chen, M. (1989). Structural Plasticity at Identified Synapses during Long-Term-Memory in Aplysia. J Neurobiol *20*, 356-372.

Berke, B., Wittnam, J., McNeill, E., Van Vactor, D.L., and Keshishian, H. (2013). Retrograde BMP signaling at the synapse: a permissive signal for synapse maturation and activity-dependent plasticity. J Neurosci *33*, 17937-17950.

Bliss, T.V.P., and Collingridge, G.L. (1993). A Synaptic Model of Memory - Long-Term Potentiation in the Hippocampus. Nature *361*, 31-39.

Bunin, M.A., and Wightman, R.M. (1999). Paracrine neurotransmission in the CNS: involvement of 5-HT. Trends in neurosciences *22*, 377-382.

Burgos, A., Honjo, K., Ohyama, T., Qian, C.S., Shin, G.J., Gohl, D.M., Silies, M., Tracey, W.D., Zlatic, M., Cardona, A.*, et al.* (2018). Nociceptive interneurons control modular motor pathways to promote escape behavior in Drosophila. Elife *7*.

Carew, T.J., Walters, E.T., and Kandel, E.R. (1981). Classical conditioning in a simple withdrawal reflex in Aplysia californica. J Neurosci *1*, 1426-1437.

Carrillo, R.A., Olsen, D.P., Yoon, K.S., and Keshishian, H. (2010). Presynaptic Activity and CaMKII Modulate Retrograde Semaphorin Signaling and Synaptic Refinement. Neuron *68*, 32-44.

Castellucci, V., Pinsker, H., Kupfermann, I., and Kandel, E.R. (1970). Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in Aplysia. Science (New York, NY *167*, 1745-1748.

Deeg, K.E., and Aizenman, C.D. (2011). Sensory modality-specific homeostatic plasticity in the developing optic tectum. Nature Neuroscience *14*, 548-550.

Emoto, K., He, Y., Ye, B., Grueber, W.B., Adler, P.N., Jan, L.Y., and Jan, Y.N. (2004). Control of dendritic branching and tiling by the tricornered-kinase/furry signaling pathway in Drosophila sensory neurons. Cell *119*, 245-256.

Engert, F., and Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature *399*, 66-70.

Foeller, E., and Feldman, D.E. (2004). Synaptic basis for developmental plasticity in somatosensory cortex. Current opinion in neurobiology *14*, 89-95.

Frey, U., and Morris, R.G.M. (1997). Synaptic tagging and long-term potentiation. Nature *385*, 533-536.

Gerhard, S., Andrade, I., Fetter, R.D., Cardona, A., and Schneider-Mizell, C.M. (2017). Conserved neural circuit structure across Drosophila larval development revealed by comparative connectomics. Elife *6*.

Grueber, W.B., Ye, B., Moore, A.W., Jan, L.Y., and Jan, Y.N. (2003). Dendrites of distinct classes of Drosophila sensory neurons show different capacities for homotypic repulsion. Curr Biol *13*, 618-626.

Grueber, W.B., Ye, B., Yang, C.H., Younger, S., Borden, K., Jan, L.Y., and Jan, Y.N. (2007). Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. Development *134*, 55-64.

Hebb, D.O. (1949). The Organization of Behavior: A Neuropsychological Theory (New York: Wiley & Sons).

Hsu, C.D., Adams, S.M., and O'Tousa, J.E. (2002). Rpr- and hid-driven cell death in Drosophila photoreceptors. Vision Res *42*, 507-516.

Hubel, D.H., and Wiesel, T.N. (1970). The period of susceptibility to the physiological effects of unilateral eye closure in kittens. The Journal of physiology *206*, 419-436.

Huganir, R.L., and Nicoll, R.A. (2013). AMPARs and Synaptic Plasticity: The Last 25 Years. Neuron *80*, 704-717.

Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., and Tracey, W.D. (2007). Nociceptive Neurons Protect Drosophila Larvae from Parasitoid Wasps. Current Biology *17*, 2105-2116.

Inaba, M., Buszczak, M., and Yamashita, Y.M. (2015). Nanotubes mediate niche-stem-cell signalling in the Drosophila testis. Nature *523*, 329-332.

Kandel, E.R. (2001). The molecular biology of memory storage: A dialog between genes and synapses. Bioscience Rep *21*, 565-611.

Kandel, E.R. (2013). Principles of neural science, 5th edn (New York: McGraw-Hill).

Kandel, E.R., Frazier, W.T., Waziri, R., and Coggeshall, R.E. (1967). Direct and Common Connections among Identified Neurons in Aplysia. Journal of neurophysiology *30*, 1352-+.

Kaneko, T., Macara, A.M., Li, R., Hu, Y., Iwasaki, K., Dunnings, Z., Firestone, E., Horvatic, S., Guntur, A., Shafer, O.T.*, et al.* (2017). Serotonergic Modulation Enables Pathway-Specific Plasticity in a Developing Sensory Circuit in Drosophila. Neuron *95*, 722.

Kim, J.H., Wang, X., Coolon, R., and Ye, B. (2013). Dscam expression levels determine presynaptic arbor sizes in Drosophila sensory neurons. Neuron *78*, 827-838.

Kirkwood, A., Rioult, M.C., and Bear, M.F. (1996). Experience-dependent modification of synaptic plasticity in visual cortex. Nature *381*, 526-528.

Luo, L. (2015). Principles of neurobiology (New York, NY: Garland Science).

Luscher, C., and Malenka, R.C. (2012). NMDA Receptor-Dependent Long-Term Potentiation and Long-Term Depression (LTP/LTD). Cold Spring Harbor Perspectives in Biology *4*.

Malenka, R.C., and Bear, M.F. (2004). LTP and LTD: An embarrassment of riches. Neuron *44*, 5-21.

Marques, G., and Zhang, B. (2006). Retrograde signaling that regulates synaptic development and function at the Drosophila neuromuscular junction. Fly Neuromuscular Junction: Structure and Function, Second Edition *75*, 267-285.

Martin, K.C. (2002). Synaptic tagging during synapse-specific long-term facilitation of Aplysia sensory-motor neurons. Neurobiol Learn Mem *78*, 489-497.

Matsunami, M., Kitano, J., Kishida, O., Michimae, H., Miura, T., and Nishimura, K. (2015). Transcriptome analysis of predator- and prey-induced phenotypic plasticity in the Hokkaido salamander (Hynobius retardatus). Mol Ecol *24*, 3064-3076.

Ohyama, T., Schneider-Mizell, C.M., Fetter, R.D., Aleman, J.V., Franconville, R., Rivera-Alba, M., Mensh, B.D., Branson, K.M., Simpson, J.H., Truman, J.W.*, et al.* (2015). A multilevel multimodal circuit enhances action selection in Drosophila. Nature *520*, 633-639.

Peters, M.A. (2002). Patterning the neural retina. Current opinion in neurobiology *12*, 43-48.

Plas, D.T., Dhande, O.S., Lopez, J.E., Murali, D., Thaller, C., Henkemeyer, M., Furuta, Y., Overbeek, P., and Crair, M.C. (2008). Bone morphogenetic proteins, eye patterning, and retinocollicular map formation in the mouse. J Neurosci *28*, 7057-7067.

Robertson, J.L., Tsubouchi, A., and Tracey, W.D. (2013). Larval defense against attack from parasitoid wasps requires nociceptive neurons. PLoS One *8*, e78704.

Rose, J.K., Sangha, S., Rai, S., Norman, K.R., and Rankin, C.H. (2005). Decreased sensory stimulation reduces behavioral responding, retards development, and alters neuronal connectivity in Caenorhabditis elegans. J Neurosci *25*, 7159-7168.

Roy, S., Huang, H., Liu, S.M., and Kornberg, T.B. (2014). Cytoneme-Mediated Contact-Dependent Transport of the Drosophila Decapentaplegic Signaling Protein. Science (New York, NY *343*, 852-+.

Roy, S., and Kornberg, T.B. (2011). Direct Delivery Mechanisms of Morphogen Dispersion. Sci Signal *4*.

Ruiz-Canada, C., and Budnik, V. (2006). Introduction on the use of the Drosophila embryonic/larval neuromuscular junction as a model system to study synapse development and function, and a brief summary of pathfinding and target recognition. Int Rev Neurobiol *75*, 1-31.

Sachse, S., Rueckert, E., Keller, A., Okada, R., Tanaka, N.K., Ito, K., and Vosshall, L.B. (2007). Activity-dependent plasticity in an olfactory circuit. Neuron *56*, 838-850.

Schuster, C.M. (2006). Experience-dependent potentiation of larval neuromuscular synapses. Int Rev Neurobiol *75*, 307-322.

Sigrist, S.J., Reiff, D.F., Thiel, P.R., Steinert, J.R., and Schuster, C.M. (2003). Experience-dependent strengthening of Drosophila neuromuscular junctions. Journal of Neuroscience *23*, 6546-6556.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and Okane, C.J. (1995). Targeted Expression of Tetanus Toxin Light-Chain in Drosophila Specifically Eliminates Synaptic Transmission and Causes Behavioral Defects. Neuron *14*, 341-351.

Takagi, S., Cocanougher, B.T., Niki, S., Miyamoto, D., Kohsaka, H., Kazama, H., Fetter, R.D., Truman, J.W., Zlatic, M., Cardona, A*., et al.* (2017). Divergent Connectivity of Homologous Command-like Neurons Mediates Segment-Specific Touch Responses in Drosophila. Neuron *96*, 1373-+.

Takahashi, T., Svoboda, K., and Malinow, R. (2003). Experience strengthening transmission by driving AMPA receptors into synapses. Science (New York, NY *299*, 1585-1588.

Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. Molecular cell *5*, 59-71.

Tracey, W.D., and Honjo, K. (2018). BMP Signaling Downstream of the Highwire E3 Ligase Sensitizes Nociceptors. bioRxiv.

Turner, A.M., and Greenough, W.T. (1985). Differential rearing effects on rat visual cortex synapses. I. Synaptic and neuronal density and synapses per neuron. Brain Res *329*, 195-203.

Vogelstein, J.T., Park, Y., Ohyama, T., Kerr, R.A., Truman, J.W., Priebe, C.E., and Zlatic, M. (2014). Discovery of brainwide neural-behavioral maps via multiscale unsupervised structure learning. Science (New York, NY *344*, 386-392.

Volkmar, F.R., and Greenough, W.T. (1972). Rearing complexity affects branching of dendrites in the visual cortex of the rat. Science (New York, NY *176*, 1445-1447.

Wilkinson, M.H.F. (1998). Optimizing Edge Detectors for Robust Automatic Threshold Selection: Coping with Edge Curvature and Noise. Graphical Models and Image Processing *60*, 385-401.

Ye, B., Kim, J.H., Yang, L.M., McLachlan, I., Younger, S., Jan, L.Y., and Jan, Y.N. (2011). Differential Regulation of Dendritic and Axonal Development by the Novel Kruppel-Like Factor Dar1. Journal of Neuroscience *31*, 3309-3319.

Ye, B., Petritsch, C., Clark, I.E., Gavis, E.R., Jan, L.Y., and Jan, Y.N. (2004). Nanos and Pumilio are essential for dendrite morphogenesis in Drosophila peripheral neurons. Curr Biol *14*, 314-321.

Ye, B., Zhang, Y., Song, W., Younger, S.H., Jan, L.Y., and Jan, Y.N. (2007). Growing dendrites and axons differ in their reliance on the secretory pathway. Cell *130*, 717-729.

Yoshida, S., Soustelle, L., Giangrande, A., Umetsu, D., Murakami, S., Yasugi, T., Awasaki, T., Ito, K., Sato, M., and Tabata, T. (2005). DPP signaling controls development of the lamina glia required for retinal axon targeting in the visual system of Drosophila. Development *132*, 4587-4598.

Yoshino, J., Morikawa, R.K., Hasegawa, E., and Emoto, K. (2017). Neural Circuitry that Evokes Escape Behavior upon Activation of Nociceptive Sensory Neurons in Drosophila Larvae. Current Biology *27*, 2499-+.

Yuan, Q., Xiang, Y., Yan, Z., Han, C., Jan, L.Y., and Jan, Y.N. (2011). Light-induced structural and functional plasticity in Drosophila larval visual system. Science (New York, NY *333*, 1458-1462.