Autoregulatory and Paracrine Control of Synaptic and Behavioral Plasticity by Dual Modes of Octopaminergic Signaling

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ABSTRACT

Synaptic plasticity – the ability of a synapse to change – is fundamental to basic brain function and behavioral adaptation. Studying the mechanisms of synaptic plasticity benefits our understanding of the formation of neuronal connections and circuitry, which has great implications in the field of learning and memory and the studies of numerous human diseases.

The *Drosophila* larval neuromuscular junction (NMJ) system is a powerful system for studying synaptic plasticity. The NMJ consists of at least two different types of motorneurons innervating the body wall muscles. Type I motorneurons controls muscle contraction using glutamate as the neurotransmitter, while type II are modulatory neurons that contain octopamine. Octopamine is a potent modulator of behavior in invertebrates. Nevertheless, its function at the synapse is poorly understood.

In my thesis research, I investigated the role of octopamine in synaptic plasticity using the *Drosophila* NMJ system. Preliminary observations indicate that increased larval locomotion during starvation results in an increase of filopodia-like structures at type II terminals. These structures, which we termed as “synaptopods” in our previous studies, contain synaptic proteins and can mature into type II synapses. I demonstrated that this outgrowth of type II terminals is dependent on activity and octopamine. Mutations and genetic manipulations affecting the production of octopamine decrease synaptopods, whereas increase of type II activity or exogenous application of octopamine increase synaptopods. Interestingly, I found that the type II octopaminergic neurons have an absolute dependence on activity for their innervation of the muscles.

Blocking activity in these neurons throughout development results in no type II synapses at the NMJ, whereas blocking activity after the formation of synapses results in gradual degradation of type II terminals.

Next, I examined the autoregulatory mechanism underlying the octopamine-induced synaptic growth in octopaminergic neurons. I discovered that this positive-feedback mechanism depends on an octopamine autoreceptor, Octß2R. This receptor in turn activates a cAMP- and CREB-dependent pathway that is required in the octopamine-induction of synaptopods. Furthermore, I demonstrated that this octopaminergic autoregulatory mechanism is necessary for the larva to properly increase its locomotor activity during starvation.

Thirdly, I investigated the possibility that type II innervation might regulate type I synaptic growth through octopamine. We found that ablation, blocking of type II activity, or the absence of octopamine results in reduced type I outgrowth, and this paracrine signaling is mediated by Octß2R which is also present in type I motorneurons.

Lastly, the function of another octopamine receptor, Octß1R, was examined. In contrast to Octß2R, Octß1R is inhibitory to synaptic growth. I demonstrated that the inhibitory effect of this receptor is likely accomplished through the inhibitory G-protein Goα. Similar to Octß2R, Octß1R also regulates the synaptic growth of both type I and type II motorneurons in a cell-autonomous manner. The inhibitory function of this receptor potentially breaks the positive feedback loop mediated by Octß2R, allowing the animal to reset its neurons when the environment is favorable.

In summary, the research presented in this thesis has unraveled both autoregulatory and paracrine mechanisms in which octopamine modulates synaptic and behavior plasticity through excitatory and inhibitory receptors.

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LIST OF ABBREVIATIONS

Brp – Bruchpilot

cAMP – Cyclic Adenosine Monophosphate

ChR2 – Channelrhodopsin2

CNS – Central nervous system

CREB – cAMP response element binding protein

Dnc – Dunce

Dp – Duplication chromosome

Eag – Ether a go-go

EJP – Excitatory Junctional Potential

FasII – FasciculinII

GluR – AMPA-type glutamate receptor

GPCR – G-Protein-Coupled Receptor

HEK293 – Human Embryonic Kidney 293 cells

Hid – Head Involution Defective

LTD – Long Term Depression

LTP – Long Term Potentiation

mEJP – Miniature Excitatory Junctional Potential

NMJ – Neuromuscular Junction

OA – Octopamine

PACα – Photoactivatable Adenylate Cyclase

PKA – Protein Kinase A

Rut – Rutabaga

ShDN – Dominant-negative Shaker K+ channel subunit

$ShiDN^{ts}$ – Temperature-sensitive Shibire (Dynamin)

Syt1 – Synaptotagmin1

TA – Tyramine

TBH – Tyramine ß Hydroxylase

TDC – Tyrosine Decarboxylase

+RT – with reverse transcriptase

-RT – without reverse transcriptase

CHAPTER 1

General Introduction

The human brain is comprised of over one hundred billion neurons, making countless synapses. These synaptic connections allow communication between neurons, which are crucial to brain function. Synapses are dynamically changing throughout a person's lifetime (Kandel et al., 2000). This process, which is called synaptic plasticity, refers to the ability of neurons to undergo changes in response to external factors or internal factors (Griffith and Budnik, 2006). Synaptic plasticity constitutes the basis of neuronal circuitry formation during development. It allows flexibility and variation in the circuitry, depending on the organism's experiences. Therefore, even genetically identical organisms can vary in developmental patterns and behaviors. The goal of this doctoral thesis was to study the molecular mechanisms of synaptic plasticity regulated by the biogenic amine, octopamine, using molecular genetic strategies in the fruit fly *Drosophila melanogaster*.

Until the twentieth century, the general dogma was that the brain is a relatively fixed structure after passing a critical period during early childhood. However, this belief was later challenged by discoveries of plasticity in the central nervous system, including those of Santiago Ramón y Cajal, revealing that many aspects of the brain remain plastic even into adulthood (Rakic, 2002). Learning is the acquisition of new knowledge and skills. For us to successfully learn or memorize, certain changes must occur in the brain to represent the new knowledge or skills. These changes include formation of new synapses and strengthening of existing synapses. The capacity of the brain to change with learning is plasticity (Black et al., 1997). In fact, many genes that affect learning and memory also affect synaptic growth. For instance, in *Drosophila*, learning mutants like *dunce* (phosphodiesterase) and *rutabaga* (adenylate cyclase) have been shown to have

altered number of synapses or synaptic strength (these mutants will be discussed below) (Zhong et al., 1991; Zhong et al., 1992; Davis et al., 2005). Therefore, studying synaptic plasticity can potentially facilitate our understanding of the mechanisms of learning and memory. Moreover, synaptic plasticity also plays an important role in numerous human diseases. For instance, in the case of a stroke where many neurons are damaged or destroyed, it is believed that the recovery process requires a significant amount of plasticity such that new functions can be assigned to the uninjured neurons (Cheetham and Finnerty, 2007; Kleim and Jones, 2008). In the case of Alzheimer's disease, apart from the hallmarks of plaque accumulation and neuronal loss, synaptic loss in the hippocampus and neocortex is also a common phenomenon (Palop et al., 2006). Interestingly, these are the most plastic regions of the brain. Loss of synaptic plasticity in these regions may contribute to the cognitive deficits commonly seen in Alzheimer's patients. In other words, Alzheimer's disease can be considered to be a disorder of synapses (Selkoe, 2002). Furthermore, physical therapies such as sensorimotor retraining activities and proprioceptive stimulation that involve specific fingers motion program have been shown to stimulate plasticity and alleviate focal dystonia (Candia et al., 2003), which commonly develops in Alzheimer's patients. In summary, since plasticity plays a role in learning and memory and a number of human diseases, it is of utmost importance for us to study its mechanisms.

The Role of Synaptic Plasticity in Behavioral Adaptation and Survival

Since synaptic plasticity is critical in learning and memory, it greatly contributes to an animal's adaptation to the ever-changing environment. Studies have shown that

environmental changes could alter behavior by modifying existing synapses between neurons and by neurogenesis in the hippocampus and other parts of the brain (Rampon and Tsien, 2000; Ponti et al., 2008). For example, enriched environments have been shown to induce more basal dendrites of neurons in the visual cortex (Holloway, 1966) and higher synapse density in the CA3 hippocampal region (Altschuler, 1979) of mice. At the same time, these enrichments also leads to better performances in rodents' learning tasks such as the Hebb-Williams maze (Ravizza and Hershberger, 1966) and Morris water maze (Mohammed et al., 1990). Even in simple organisms like the fruit fly *Drosophila melanogaster*, experiences can still sculpt the synapses at both the structural and functional level. For example, rearing larvae at a higher temperature results in more synaptic growth at the larval neuromuscular junction (NMJ) (Sigrist et al., 2003), and locomotor increase during food-deprivation results in larger synaptic vesicles at the NMJ (Steinert et al., 2006). The experience-molded nervous system can in turn monitor behavioral changes according to the need of the animal, allowing adaptation to the environment and benefiting survival. It is believed that this kind of behavioral plasticity is a key factor which has been rigorously selected for in the evolution of organisms, where the loss of such flexibility in behavior to adapt may render a species extinct (Hazlett 1988).

Biogenic Amines Regulate Synaptic and Behavioral Plasticity

One of the most conserved methods of facilitating synaptic and behavioral changes in both vertebrates and invertebrates is the use of biogenic amines. Perhaps arguably the most prominent series of studies on this subject belong to Nobel Prize

laureate, Eric Kandel, and his studies on the gill-withdrawal reflex of the marine mollusk *Aplysia*. Kandel examined three forms of learning in *Aplysia* (habituation, sensitization, and classical conditioning) (Pinsker et al., 1970, 1973; Carew et al., 1972, 1981). The study of habituation was performed by repeatedly evoking the gill-withdrawal reflex using a light tactile stimulus to the siphon or mantle shelf of the animal. It was found that the amplitude of the gill-withdrawal response showed marked decrement (Pinsker et al., 1970). Sensitization was performed by presenting a noxious stimulus such as electrical shock to the head or tail of the animal, inducing a massive gill-withdrawal reflex. Animals with shock experiences enhance its defensive response by increasing its duration of gill-withdrawal when the same stimulus is presented again (Pinsker et al., 1973). In classical conditioning, the light tactile stimulus is paired with electrical shock for training. The trained animal then associates the light tactile stimulus to danger, and would respond with a stronger and longer gill-withdrawal reflex (Carew et al., 1981). By studying these different forms of learning in *Aplysia*, two stages of memory storage was observed in the gill-withdrawal reflex – short-term and long-term facilitation. Short-term facilitation stems from changes in synaptic strength between neurons (Castellucci et al., 1970), while the conversion of short-term to long-term facilitation requires the formation of new connections and protein synthesis (Castellucci et al., 1989). In studying the molecular mechanisms of short and long-term facilitation, Kandel and colleagues discovered that the biogenic amine serotonin plays an important role in both forms of memory. Serotonin is normally released *in vivo* by interneurons in *Aplysia* by stimulation of the tail. It was found that a single pulse of serotonin produces a transient enhancement of synaptic strength independent of protein synthesis, whereas five pulses produce long-term facilitation that could be blocked by protein- and RNA-synthesis inhibitors (Abel and

Kandel, 1998). Serotonin was found to increase cyclic adenosine monophosphate (cAMP) in sensory neurons (Ocorr and Byrne, 1985). cAMP has been shown to be an important second messenger in presynaptic facilitation via activation of Protein Kinase A (PKA). It was found that injection of cAMP, PKA or bath application of forskolin produces the similar facilitation as that produced by serotonin (Brunelli et al., 1976; Castellucci et al., 1980; Klein 1993). Moreover, in cell-free membrane patches from *Aplysia* sensory neurons, the purified catalytic subunit of PKA reduces the number of opened S-type K^+ channel, simulating most aspects of the action of serotonin (Shuster et al., 1985). This enhances membrane excitability, and contributed to short-term facilitation. On the other hand, experiments using fluorescent ratio imaging to track the catalytic subunit of PKA showed that PKA translocates to the nucleus in sensory neurons in response to multiple pulses of serotonin (Bacskai et al., 1993). PKA then phosphorylates cAMP response element binding protein (CREB), triggering the transcription of synaptic effector proteins. The synthesis of new proteins is necessary for long-term facilitation, which involves the formation of new synapses and the strengthening of existing synapses (Byrne and Kandel, 1996; Abel and Kandel 1998).

Apart from serotonin, the mammalian catecholamines, adrenaline (epinephrine) and noradrenaline (norepinephrine), are also key regulators of synaptic and behavioral plasticity. Adrenergic receptors are metabotropic G-protein-coupled receptors (GPCRs). There are two main groups of adrenergic receptors – α and β. α1-adrenergic receptors are Gq-coupled, and are capable of increasing intracellular Ca^{2+} through the phospholipase C pathway (Gibbs and Bowser 2010). α2-receptors are Gi-coupled and are inhibitory to the cAMP pathway (Ma et al., 2005). The β-receptors, including β1, β2

and β3, are in general Gs-coupled and increase cAMP and adenylate cyclase activity (with the exception that β2 is also Gi-coupled and can be inhibitory) (Xiao, 2001). Both adrenaline and noradrenaline are well-studied stress hormones responsible for the “fight or flight” effect in vertebrates. Noradrenaline increases heart rate, triggers the release of glucose from energy stores, suppresses immunity and even potentially mobilizes the release of lipids from mammalian adipocytes, presumably to provide a temporary source of energy for the animal to evade a dire situation (Roeder, 2005). In terms of synaptic plasticity, noradrenaline is involved in the retrieval of contextual memories (Murchison et al., 2004). In this study, mice were trained to associate footshock with both a tone (cue) and an apparatus (context) in a single training trial. Then either the cue or the context is given to the mice. Wild-type mice freeze upon exposure to either conditioned stimulus, while mice lacking noradrenaline exhibit normal cued memory but impaired contextual fear memory (Murchison et al., 2004). In contrast, mice injected with adrenaline showed enhanced contextual memory (Frankland et al., 2004). Noradrenaline has also been implicated to play a role in long-term potentiation (LTP). LTP is believed to be a cellular model for memory formation. When high frequency stimulation is performed on a presynaptic neuron, a significant increase in the postsynaptic response is produced (Sarvey et al., 1989; Bliss and Lomo, 1973). In hippocampal slices, perfusion of noradrenaline enhances the magnitude of LTP by approximately 50% (Hu et al., 2007). It has also been demonstrated by western blots from hippocampal slices that noradrenaline induces phosphorylation of the GluR1 subunit of the AMPA receptor. This in turn facilitates its delivery to the synapse, as demonstrated by a GFP-tagged GluR1 (Hu et al., 2007). This phosphorylation was likely mediated by PKA as it was abolished by PKA inhibitors. Injection of adrenaline *in vivo* also increases the phosphorylation of

GluR1 in hippocampal lysates (Hu et al., 2007). Adrenergic signaling is also involved in long-term depression (LTD). LTD is induced by a long duration of low frequency stimulations, resulting in decreased postsynaptic response (Ito et al., 1982). LTD can be induced in hippocampus slice preparations of rats subjected to hemorrhage (removal of 25% blood volume). Injection of α2-adrenoreceptor inhibitors blocked this LTD in hemorrhaged rats, whereas bath application of noradrenaline induces LTD in the hippocampi of normal rats (Kuzmiski et al., 2007).

In the fruit fly *Drosophila melanogaster*, dopamine and octopamine have been rigorously studied for their roles in learning and memory (Davis, 2005). Dopamine and octopamine receptors are also regulators of cellular cAMP levels, and the cAMP signaling pathway has been demonstrated to be critical for classical conditioning (Dubnau and Tully, 1998). In fruit flies, the Rutabaga gene encodes a major adenylate cyclase, responsible for cAMP production; whereas Dunce encodes a cAMP-specific phosphodiesterase, responsible for reduction of cAMP. Both *rutabaga* and *dunce* mutants are defective in learning (Davis, 2005). Other key molecules in the cAMP pathway like PKA and CREB are also involved in learning and memory. Expression of an inhibitory form of the regulatory subunit of PKA or a dominant negative form of CREB impairs long-term memory (Abel et al., 1997; Yin et al., 1994). Since dopamine and octopamine are important regulators of cAMP signaling. It is believed that these molecules are responsible for reinforcing the unconditioned stimulus in classical condition (Davis, 2005). Blocking of dopaminergic neurons during conditioning but not retrieval abolishes aversive memory (pairing shock with odor), whereas mutants lacking

octopamine are defective in appetitive memory (pairing sugar reward with odor) (Schwaerzel et al., 2003).

Tyramine, the precursor of octopamine, was original thought to be just a transient molecule in the process of octopamine biosynthesis. Although in recent years, studies have demonstrated that it can function independently from octopamine and act as a neurotransmitter in *Caenorhabditis elegans* (*C. elegans*), modulating egg-laying, reversal and head oscillation behaviors (Alkema et al., 2005). The role of this biogenic amine in synaptic and behavioral plasticity is just beginning to be examined.

Octopamine: A Powerful Ruler of Invertebrate Behavior

Octopamine, originally discovered in the octopus, is the invertebrate counterpart of mammalian adrenaline or noradrenaline. Its chemical structure is similar to these catecholamines as well as its precursor, tyramine (**Fig. 1-1**). In invertebrates, octopamine is a major neurotransmitter, neuromodulator and neurohormone. It is a very potent, multifunctional molecule that regulates many types of behavior and mediates diverse physiological processes in both the peripheral and central nervous systems (Roeder, 2005). Octopamine is the neurotransmitter for light production in the firefly lantern (Robertson and Carlson, 1976). In lobsters, injection of octopamine into the circulatory system results in the displaying of submissive postures (Livingstone et al., 1980), whereas in crayfish octopamine induces escape behavior (Glanzman and Krasne, 1983). Octopamine injection into the house cricket *Acheta domesticus* results in elevation of carbohydrate and fatty acid in the hemolymph (Fields and Woodring, 1991), and this elevation of lipid and sugar by octopamine involves cAMP production and can

be blocked by the α-adrenergic antagonist phentolamine (Fields and Woodring, 1991). In the blowfly *Phormia regina*, injection of octopamine induces hyperphagia. Blowflies injected with octopamine imbibe significantly more sucrose solution than control animals, and their body weight is at least doubled (Long and Murdock, 1983). In the honeybee *Apis mellifera*, injection of octopamine can be used as a substitution for sucrose reward in classical conditioning (Hammer and Menzel, 1998). In *C. elegans*, octopamine inhibits pharyngeal pumping by affecting the duration of pharyngeal muscle action potentials (Horvitz et al., 1982). Incubation of *C. elegans* with octopamine suppresses serotonin-dependent aversive behavior (Wragg et al., 2007). Octopamine also mediates CREB activity during starvation in *C. elegans*. CREB activity was monitored using a GFP reporter. Starvation induces an increase of GFP signal in control animals but not in octopamine receptor mutants (Suo et al., 2006).

Many advances were made in the study on the physiological changes mediated by octopamine using the locust *Schistocerca gregaria*, due to its large, easily accessible muscles which are ideal for electrophysiology. Numerous studies suggest that octopamine has excitatory modulatory functions in specific skeletal and visceral muscles and increases the efficiency of both muscle contraction and relaxation. Bath application of octopamine results in the enhancement of amplitude and speed of evoked contractions of muscles, followed by quicker relaxation (Whim & Evans, 1988; Orchard and Lange, 1985; Braunig et al., 1994). Octopamine levels in the locust hemolymph are raised during locomotor activity (Goosey and Candy 1980) and can be doubled under stressful starvation conditions (Davenport and Evans, 1984). This increase of octopamine remains in the hemolymph for approximately 4 hrs even after the starved

animals have been re-fed. It has been postulated that octopamine may temporarily increase the availability of carbohydrates and lipid substrates in the hemolymph, allowing a starved animal to increase its locomotor activity for food-seeking (Evans, 1985).

In fruit flies, octopamine is required for normal egg-laying in adult females (Monastirioti et al, 1996). Mutant female flies devoid of tyramine-ß-hydroxylase (TBH), the enzyme for biosynthesis of octopamine, retain their eggs in their oviduct. This egg-laying defect can be rescued by either feeding the flies with octopamine or expressing TBH in the oviduct, suggesting octopamine's modulatory role in oviductal muscle contraction. In adult males, octopamine is involved in courtship and aggressive behavior (Certel et al., 2007; Hoyer et al., 2008; Zhou et al., 2008). Octopamine is necessary for normal locomotor activity of both larvae and adult flies (Saraswati et al., 2003; Hardie et al., 2007). Octopamine is also involved in sleep-wake behavior via the activation of PKA (Crocker and Sehgal, 2008). Flies devoid of octopamine have increased sleep that is rescued by feeding with octopamine, whereas flies with hyperexcitable octopaminergic neurons have decreased sleep that can be suppressed with decreased PKA activity (Crocker and Sehgal, 2008). In terms of learning and memory, octopamine has been shown to be critical in appetitive memory in adult flies (Schwaerzel et al., 2003) and larvae (Schroll et al., 2006). Notably, in adult flies, this form of octopamine-dependent conditioning produces long-term memory much more readily than aversive memory which does not involve octopamine (Krashes et al., 2008). In aversive conditioning, five sessions of spaced training are required to induce long-term memory (Tully et al., 1994). In contrast, a single session of appetitive memory training produces long-term memory

(Krashes et al., 2008). Similar to aversive long-term memory, this appetitive long-term memory is also cAMP and protein synthesis dependent, as the expression of a dominant negative CREB or the feeding of protein synthesis inhibitor abolished it (Krashes et al., 2008).

Octopamine Receptors are Modulators of Synaptic and Behavioral Plasticity

Octopamine receptors are G-protein-coupled receptors (GPCRs) resembling adrenergic receptors, capable of increasing Ca^{2+} or modulating cAMP levels (Balfanz et al., 2005; Evans and Maqueira, 2005). There are a total of four octopamine receptors identified in the *Drosophila* genome – OAMB, Octß1R (OA2), Octß2R and Octß3R. OAMB shares amino acid sequence homology with α-adrenergic receptors, and is capable of increasing intracellular Ca^{2+} and cAMP (Han et al., 1998). It has high expression levels in the mushroom bodies, which are the center of learning and memory in fruit flies (Han et al., 1998). OAMB is necessary for normal ovulation via Ca^{2+} signaling (Lee et al., 2009) and sleep-wake behavior via cAMP (Crocker et al., 2010). However, it has been suggested that the activation of cAMP level by OAMB is relatively low. The remaining octopamine receptors that resemble ß-adrenergic receptors may elicit a much stronger cAMP activation response, placing them as potential modulators of synaptic and behavioral plasticity as well (Balfanz et al., 2005; Evans and Maqueira, 2005). Nevertheless, where these Octß receptors are expressed in *Drosophila* and how they function *in vivo* are largely unknown. Furthermore, despite the vast amounts of behavioral studies involving octopamine and its receptors performed in the past few

decades, the role of octopaminergic signaling in synaptic plasticity is still very poorly understood.

The Drosophila Larval Neuromuscular Junction (NMJ) as a Model System to Study Octopaminergic Control of Synaptic Plasticity

Despite our primary interest in the brain, it is relatively difficult to manipulate and visualize specific neurons and synapses without inflicting damage to it, partly due to the vast complexity of circuitries present. Brain slices and cultured neuronal preparations offer better accessibility and resolution, but they still raise the question of relevance *in vivo*.

The *Drosophila* larval neuromuscular junction (NMJ) is a powerful model system that has been used for decades for studying synaptic plasticity. The post-synaptic body wall muscles of the larva are arranged in a segmentally repeated manner. The muscles are innervated by the pre-synaptic motorneurons, whose cell bodies reside in the ventral ganglion, and project their axons to the NMJ to control muscle activities. During its development from first to third instar larval stages, the animal dramatically increases in size. The number of muscles does not increase, but the size of muscles can increase up to a hundred fold (**Fig. 1-2**). In order to maintain synaptic efficacy, motorneurons undergo continuous addition of varicosities called “boutons”, which are the sites of neurotransmitter release (Jan and Jan, 1976). This kind of synaptic plasticity is activity-dependent. Genetic increase of presynaptic activity using ion channel mutations results in significant increase of synaptic outgrowth at the NMJ (Budnik et al., 1990). Food-deprivation-induced increase of locomotor activity also increases presynaptic release.

Although, no structural changes were noted, possibly due to the short time scale (Steinert et al., 2006). Furthermore, NMJs are highly tractable and the synapses are easily quantifiable and accessible by electrophysiology. Thus, comparisons of the same NMJ can be easily performed across different animals. In addition, *Drosophila* has a relatively short life cycle of about ten days, allowing genetic manipulation within a short period of time. Genetic tools are often readily available in the *Drosophila* community, partially due to the well-establish Gal4/UAS bipartite driver/promoter system (Duffy, 2002), as well as the availability of mutants from forward genetic screens and multiple transgenic RNAi collections. Taken together, the larval NMJ is a prime system for studying the mechanisms of synaptic plasticity.

The larval NMJ contains at least four different types of motorneurons (Johansen et al., 1989; Budnik and Ruiz-Canada, 2006). Type Ib (type I big) and type Is (type I small) motorneurons innervate the body wall muscles with large synaptic boutons (~2-3 μm). Type I boutons are the most abundant class of boutons at the NMJ. They release glutamate, which is the major neurotransmitter at the NMJ. Type I innervate all 30 muscles per segment in approximately one neuron to one muscle ratio, and responsible for controlling individual muscle contractions. Type II and type III motorneurons are modulatory neurons. Type II contains octopamine (Monastirioti et al., 1995) and type III contains a variety of peptides (Cantera and Nassel, 1992; Gorczyca et al., 1993). Comparing to type I, type II motorneurons innervate the body wall muscles in the form of smaller synaptic boutons (~1 μm) (**Fig. 1-3**). They originate from only a small number of cell bodies in the ventral ganglion, but innervate almost the entire muscle field (see below) (Monastirioti et al., 1995). Thus, when these neurons fire, virtually the entire

muscle field would be affected. This architecture strongly suggests them as modulatory neurons that exert global regulation over most of the NMJs (Budnik and Ruiz-Canada, 2006). Since the NMJ system contains both glutamatergic type I and octopaminergic type II motorneurons neighboring to each other, and since the system gives us great resolution in visualization and access to these neurons independently, it is the ideal system for studying octopaminergic modulation of synaptic plasticity.

The Anatomy Octopaminergic Type II Motorneurons

There are three type II motorneurons per segment in the ventral ganglion. Each triplet of these ventral unpaired neurons extends axons towards the dorsal side, combines into one bundle and then bifurcates laterally, exiting the ventral ganglion in 2 separate nerves bilaterally (**Fig. 1-4**). Each of these nerves appears to contain 3 axons (**Fig. 1-5**) which lead to the corresponding hemisegment (half segment) of body wall muscles (**Fig. 1-2**). The 3 axons from each lateral nerve innervate approximately 23 out of 30 body wall muscles per hemisegment, with the exception of muscle 3 to 7, 25 and 28 (Monastirioti et al., 1995) (**Fig. 1-6**). Thus, 3 type II motorneurons per segment in the ventral ganglion innervate approximately 46 muscles per segment of the body wall. Notably, most muscles devoid of type II innervation are ventral interior muscles that are located near internal organs. It is possible that this architecture prevents high concentrations of octopamine secretion from affecting the functions of internal organs, which could be undesirable.

Tdc2-Gal4 is a driver fly line generated by fusing the promoter region of the tyrosine decarboxylase-2 (Tdc2) gene to Gal4 transcription factor (Cole et al., 2005).

Since Tdc2 is the enzyme responsible for the biosynthesis of tyramine (precursor of octopamine) (**Fig. S2-3A**), this driver line expresses Gal4 in all tyraminergic and octopaminergic neurons in the central nervous system (CNS). We found that this line labels all type II motorneurons at the NMJ (**Fig. 1-3**). Furthermore, all type II motorneurons contain tyramine-ß-hydroxylase (TBH), the enzyme for converting tyramine into octopamine (**Fig. S2-3A**), as indicated by anti-TBH immunostaining (**Fig. S2-3B**). Thus, Tdc2-Gal4 allows us to manipulate all octopaminergic type II motorneurons at the larval NMJ.

Uncovering the Mechanism of Octopaminergic Signaling and Its Impacts

My thesis research focused on the role of octopamine in synaptic plasticity at the *Drosophila* larval NMJ and larval behavioral plasticity in response to food-deprivation.

In the second chapter of my thesis, I will present compelling evidence that octopamine induces synaptic growth at its own type II octopaminergic arbors (autoregulatory signaling). I discovered that this positive feedback mechanism requires Octß2R receptor, which activates a cAMP- and CREB-dependent pathway. Notably, this autoregulation was necessary for the increase of larval locomotor activity during starvation, suggesting its importance to survival. Furthermore, octopamine also induces synaptic growth at neighboring type I glutamatergic terminals through the same receptor Octß2R present in type I motorneurons (paracrine signaling). Octopamine also increases synaptic strength in type I motorneurons in an Octß2R-dependent manner, strongly suggesting type II as modulatory neurons regulating global synaptic plasticity. In addition, I made the surprising discovery that the type II innervation of body wall

muscles requires activity. This absolute requirement for electrical activity for type II synaptogenesis is in contrast to the common belief that activity is only important for refinement of existing connections.

In the third chapter of my thesis, I will present an interesting finding that another octopamine receptor, Octß1R, functions in complete opposition to Octß2R which we described chapter 2. Octß1R, which is also present in both type I and II motorneurons, inhibits synaptic growth. My data strongly suggests that this is achieved via the inhibitory G-protein, Goα, which inhibits cAMP production. Intriguingly, the disruption in Octß1R results in near-saturation levels of cAMP and promotes synaptic outgrowth. This in turn blocks the octopamine-induced excitatory signals via Octß2R, resulting in no further synaptic outgrowth in type II arbors in response to octopamine. As a consequence of this loss of synaptic plasticity in the octopaminergic neurons, the starvation-induced locomotor increase was abolished. This loss of behavioral plasticity due to blockage of octopamine autoregulatory signals confirmed our findings in the previous chapter, and further emphasized the importance of plasticity in aminergic neurons.

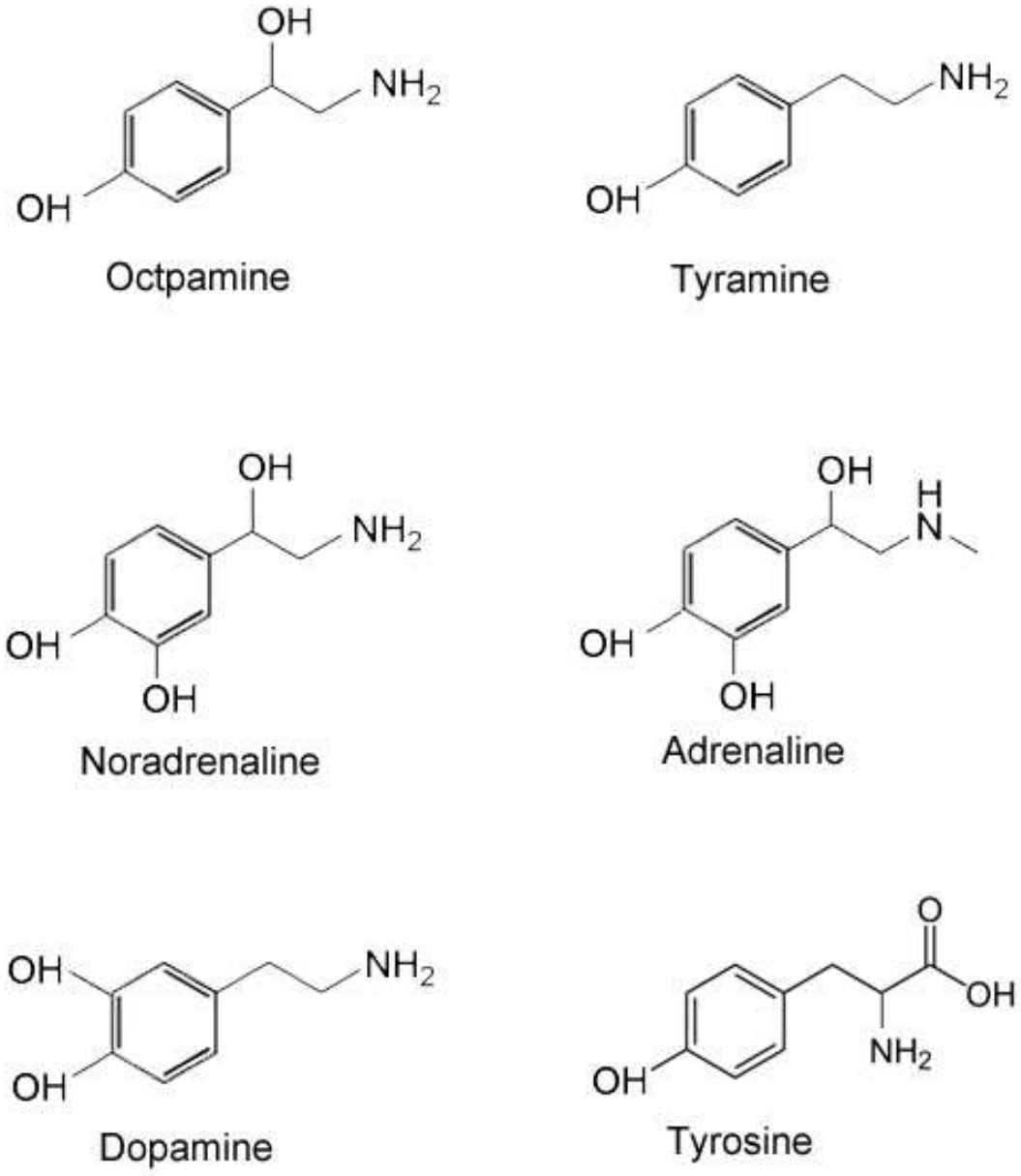
OH
NH2
OH
Octpamine
NH2
OH
Tyramine
OH
NH2
OH
OH
Noradrenaline
OH
H
N
OH
OH
Adrenaline
NH2
OH
OH
Dopamine
O
OH
NH2
OH
Tyrosine

Figure 1-1

Figure 1-1. The chemical structure of octopamine and related biogenic amines. Tyramine is the precursor of octopamine. Octopamine shares similarities in structure and function with mammalian noradrenaline (norepinephrine) and adrenaline (epinephrine). Dopamine is the precursor of noradrenaline and adrenaline. Both tyramine and dopamine are derived from the amino acid tyrosine.

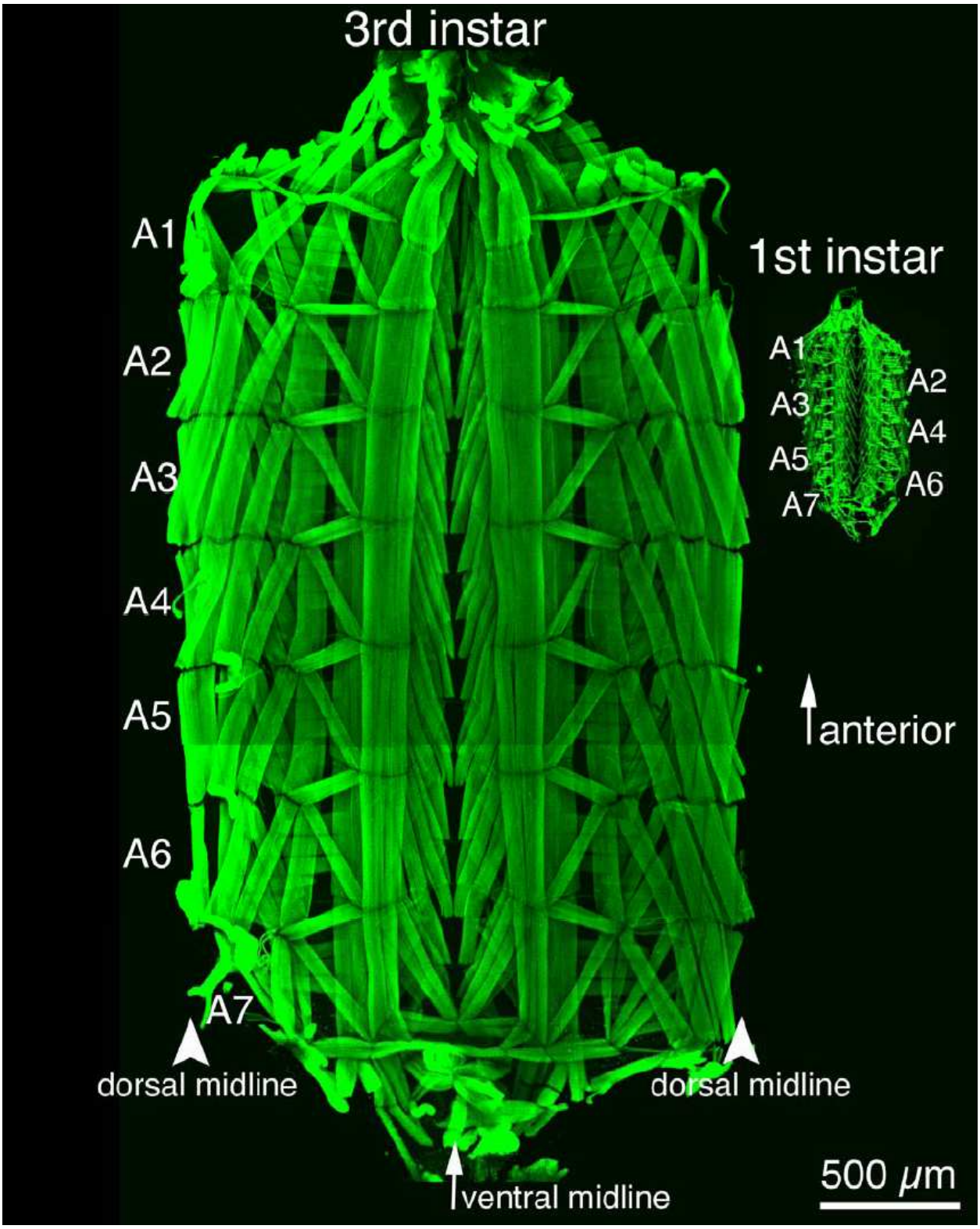
3rd instar
A1
A2
A3
A4
A5
A6
A7
dorsal midline
dorsal midline
ventral midline
1st instar
A1
A2
A3
A4
A5
A6
A7
anterior
500 μm

Figure 1-2

Figure 1-2. (Adapted from Budnik and Ruiz-Canada, 2006) Body wall muscles of the Drosophila larva. Third star wandering stage (left) and first instar (right) larval body wall muscles preparations labeled with FITC-conjugated phalloidin. During development, the number of muscles does not increase, but the size of each muscle can increase up to 100 fold. Abdominal segments 1-7 are labeled as A1 to A7. Up is anterior.

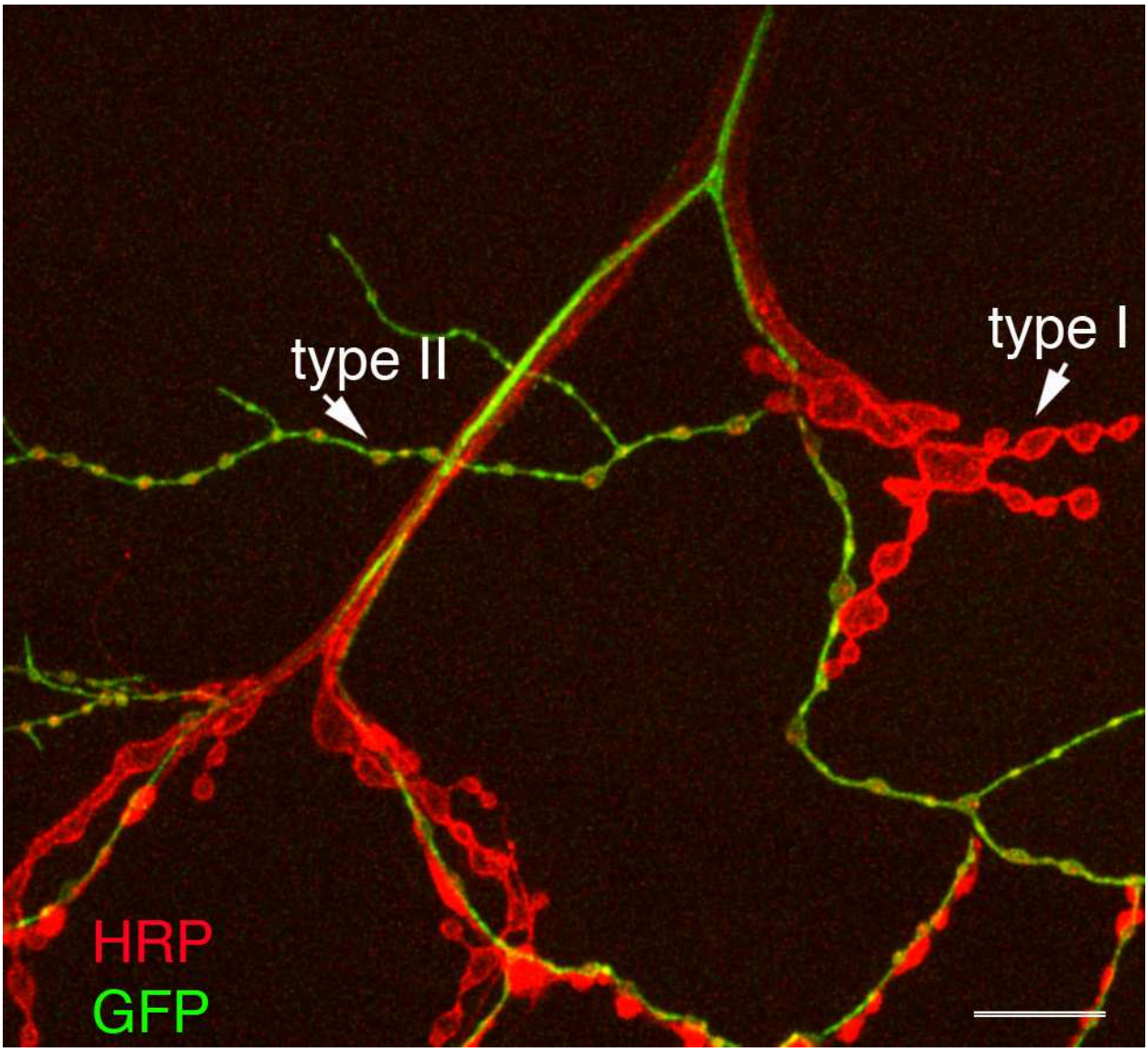

Figure 1-3

Figure 1-3. (Adapted from Koon et al., 2011) NMJs at muscles 12 and 13 of a third instar larva expressing mCD8-GFP in type II motorneurons using Tdc2-Gal4, showing type I and type II boutons (arrows). Immunostained with anti-HRP and anti-GFP. Anti-HRP labels all bouton types, whereas anti-GFP labels only type II boutons. Tdc2-Gal4 labels all tyraminergic and octopaminergic neurons of the nervous system. All Tdc2-labeled type II motorneurons at the NMJ contains tyramine-beta-hydroxylase (TBH), indicating that all type II motorneurons contain octopamine. Scale bar = 9 μm.

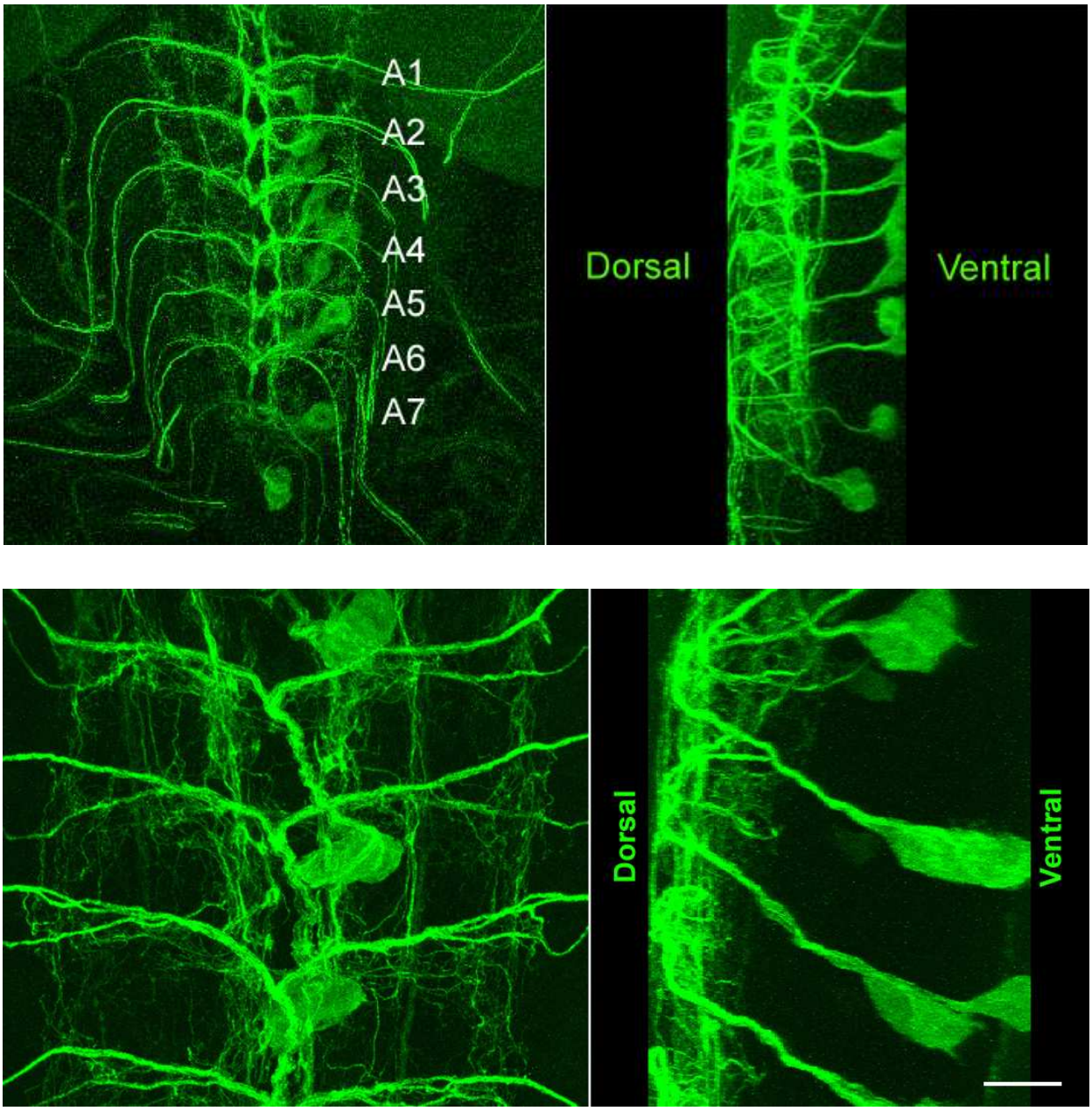

Figure 1-4

Figure 1-4.

Top panel: Ventral ganglion of a third instar larva expressing mCD8-GFP in type II motorneurons using Tdc2-Gal4. Labeled with anti-GFP. Similar to the body wall muscles, the ventral ganglion is also divided into 7 segments. Octopaminergic type II motorneurons from each segments of the ventral ganglion innervate corresponding segments of the larval body wall.

Bottom panel: Higher magnification of the ventral ganglion of a different animal of the same genotype. Scale bar = 50 μm in top panel and 20 μm in bottom panel.

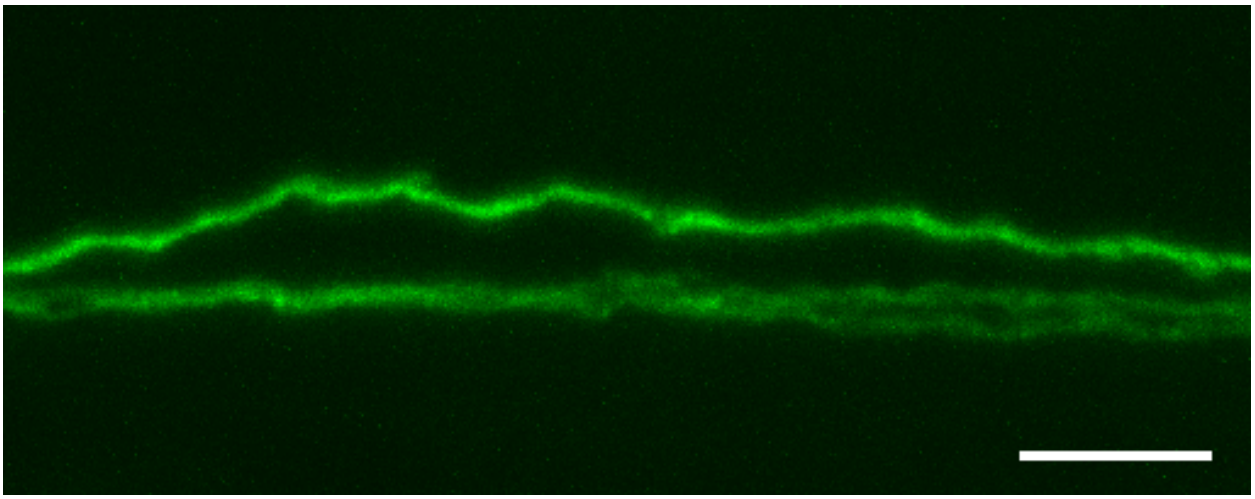

Figure 1-5

Figure 1-5. A single nerve of a third instar larva expressing mCD8-GFP in type II motorneurons using Tdc2-Gal4. Three type II motorneurons axons are observed per nerve. Scale bar = 7 μm

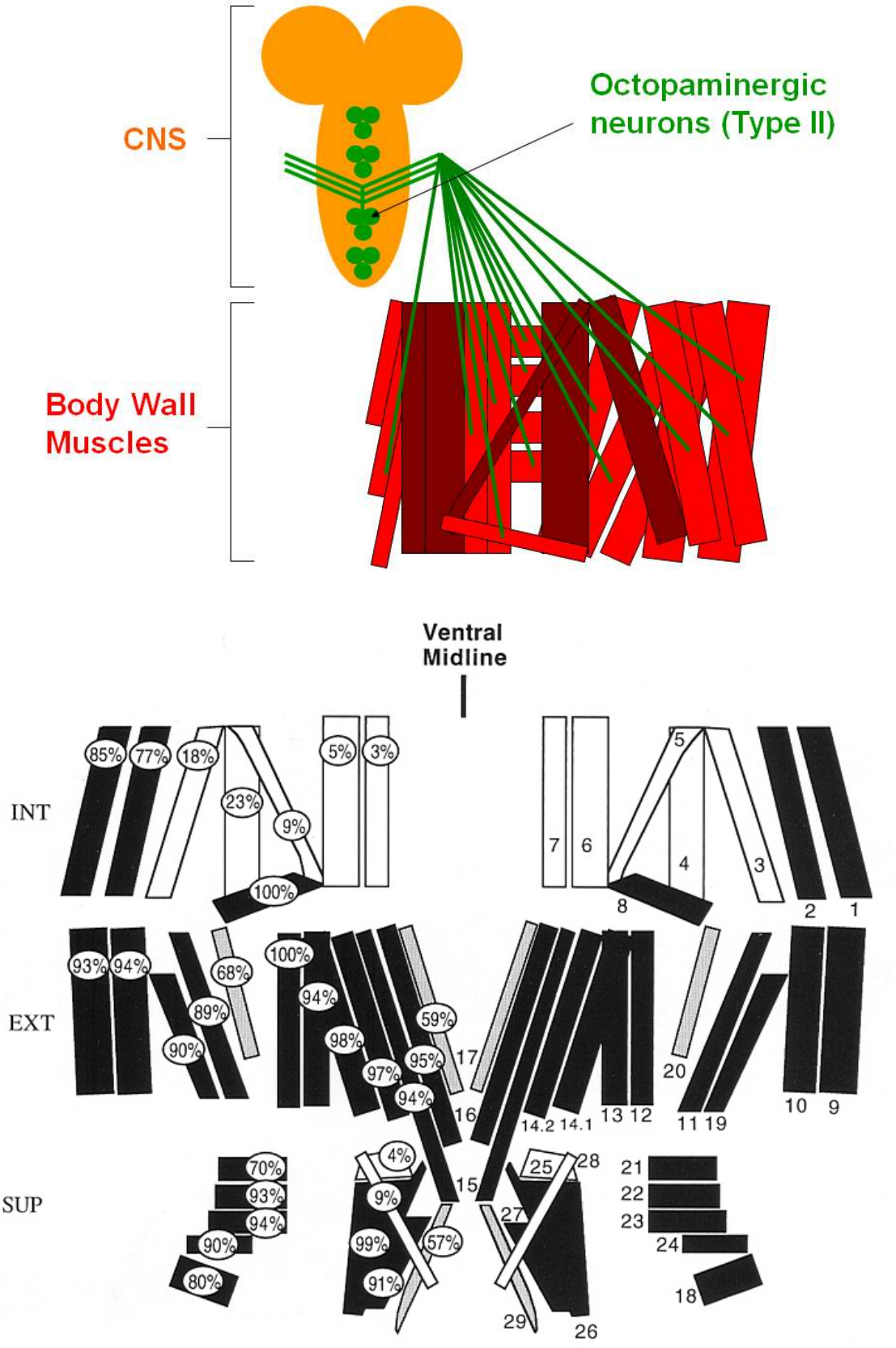
CNS
Octopaminergic
neurons (Type II)
Body Wall
Muscles
Ventral
Midline
INT
EXT
SUP
85%
77%
18%
23%
9%
100%
5%
3%
7
6
5
4
3
8
2
1
93%
94%
68%
89%
90%
100%
94%
98%
97%
94%
59%
95%
17
16
14.2
14.1
13
12
20
11
19
10
9
70%
93%
94%
90%
80%
4%
9%
99%
91%
57%
15
25
28
27
29
26
21
22
23
24
18

Figure 1-6

Figure 1-6.

Top panel: Schematic diagram of type II motorneurons innervating multiple body wall muscles. Green: Type II motorneurons originate from 3 cell bodies per segment in the ventral ganglion. Bright red muscles: Innervated by type II terminals. Maroon muscles (muscle 3 to 7): Not normally innervated by type II terminals.

Bottom panel: (Adapted from Monastirioti et al., 1995) Probability of type II innervation on each of the thirty body wall muscles. Notice the absence of type II on most of the internal muscles that are located near internal organs. INT: Internal muscles; EXT: External muscles; SUP: Superficial muscles.

CHAPTER 2

Autoregulatory and Paracrine Control of Synaptic and Behavioral Plasticity by Octopaminergic Signaling

Alex C Koon, James Ashley, Romina Barria, Shamik DasGupta, Ruth Brain, Scott Waddell, Mark J Alkema & Vivian Budnik

The following work is reprinted from the Nature Neuroscience article of the same name published in Feb 2011.

ABSTRACT

Adrenergic signaling has important roles in synaptic plasticity and metaplasticity. However, the underlying mechanisms remain poorly understood. Here we examined the role of octopamine, the invertebrate counterpart of adrenaline and noradrenaline, in synaptic and behavioral plasticity in *Drosophila*. We show that an increase in locomotor speed induced by food deprivation was accompanied by an activity- and octopamine-dependent extension of octopaminergic arbors, and that the formation and maintenance of these arbors required electrical activity. We found that octopaminergic arbor growth was controlled by a cAMP- and CREB-dependent positive feedback mechanism that required Octß2R octopamine autoreceptors. Importantly, this autoregulation was necessary for the locomotor response. In addition, octopamine neurons regulated the expansion of excitatory glutamatergic neuromuscular arbors, through Octß2Rs on glutamatergic motorneurons. These studies provide a mechanism of global regulation of excitatory synapses, presumably to maintain synaptic and behavioral plasticity in a dynamic range.

INTRODUCTION

Synaptic plasticity is fundamental for an organism's ability to adapt to a changing environment. Adrenergic receptors and their ligands are key regulators of plasticity. Noradrenaline is implicated in the retrieval of spatial and contextual memories[1], and it enhances LTP by promoting the synaptic delivery of AMPA-type glutamate receptors (GluR)[2]. Adrenergic signaling has also been implicated in the regulation of plasticity (a.k.a. metaplasticity) to reset a homeostatic circuit in response to acute perturbations, thus maintaining the circuit within a dynamic range [3]. However, the specific mechanisms by which adrenergic signals influence synaptic plasticity are still poorly understood.

Octopamine, the invertebrate counterpart of adrenergic ligands, activates receptors resembling adrenergic receptors[4]. Octopamine plays a key role in appetitive reinforcement in honeybees[5] and flies[6,7] and modulates behaviors such as aggression[8], egg-laying[9], food-seeking[10], and sleep[11], as well as synaptic functions[12].

The *Drosophila* larval neuromuscular junction (NMJ) is a powerful model system to investigate synaptic plasticity. While glutamate is the primary excitatory neurotransmitter, larval NMJs are also innervated by octopaminergic motorneurons[13]. Larval NMJs display several forms of synaptic plasticity, such as continuous expansion during larval development, to offset a massive increase in muscle size, as part of a homeostatic mechanism to maintain synaptic efficacy[14]. This process depends on signaling mechanisms such as the BMP[15] and Wnt pathways[16]. Larval NMJs can also respond to changes in the environment, such as food availability, by rapid increases in synapse strength[17,18]. In addition, genetic and physiological manipulations that increase presynaptic activity promote synaptic expansion[19,20]. To determine the significance of

octopaminergic innervation of body-wall muscles, we examined octopaminergic terminals during larval foraging behavior. Type II arbors responded to food deprivation by extending new endings. This depended both on activity levels and octopamine. Electrical activity at octopaminergic neurons was essential for initial and continued type II innervation of muscles. We uncovered a cAMP and CREB-dependent autoregulatory positive feedback mechanism that regulated the size of type II endings through the activation of Octß2R autoreceptors. Type II innervation also regulated the plasticity of glutamatergic type I motorneurons through Octß2Rs expressed in these neurons. Both the autocrine and paracrine mechanisms were required for the adaptive response to starvation.

RESULTS

Locomotor-increase associates with type II synaptic change

Larval NMJs respond to acute changes in presynaptic activity by modifications in synaptic structure[20]. However, the physiological conditions under which this mechanism is used by the intact organism are unknown. Larval foraging behavior is enhanced by food deprivation, which leads to long-lasting enhancement in evoked glutamate release from excitatory type I NMJs[17]. However, no gross changes in the structure of these endings were observed[17]. Most body-wall muscles are co-innervated by at least one additional class of motorneuron, the octopaminergic type II motorneurons[13] (**Fig. 2-1A**). Octopamine signaling has been implicated in appetitive behaviors and locomotion[6,10,21,22]. Thus, to determine if type II arbors changed structure during starvation, a physiological stimulus that increases locomotor activity, these arbors were labeled by expressing mCD8-GFP by using a *tyrosine decarboxylase-2* promoter fused to Gal4 (Tdc2-Gal4; **Fig. 2-1A**). NMJs from intact early 3rd-instar larvae were imaged live through the cuticle, subsequently deprived of food for 2 hrs, and the same NMJs were re-imaged.

Food-deprived wild-type larvae showed a significant increase in locomotor speed compared to fed-controls (**Fig. 2-1C**). Notably, type II endings from starved larvae showed dynamic filopodia-like extensions (synaptopods) that extended and retracted with a time course of minutes (**Fig. 2-1B,D**; Movie1). While synaptopods were also observed before food deprivation (a.k.a. “natural synaptopods”; **Fig. 2-1B**), the number of synaptopods was significantly increased upon starvation (**Fig. 2-1B,D**). Thus, changes in locomotor activity are accompanied by structural changes at type II endings.

We next investigated whether type II endings were necessary for behavior. We eliminated octopaminergic neurons by expressing the cell death protein Head Involution Defective (Hid; **Fig. S2-1**), which substantially reduced locomotion speed and the starvation response (**Fig. 2-1E,F**). A similar result was observed in *tyrosine-ß-hydroxylase (tbh^{nM18})* and *tdc2^{R054}* mutants, which are unable to synthesize octopamine (**Fig. 2-1E,F**)[9,23]. The defects in *tbh* mutants were specific, as they were significantly rescued by expressing a TBH transgene in octopaminergic neurons (**Fig. 2-1E,F**). Thus, the increase in locomotion elicited by food deprivation results in structural changes in octopaminergic endings, and octopamine innervation is necessary for this behavior.

We then examined whether octopamine was sufficient to increase locomotor activity in the absence of starvation. Channelrhodopsin-2 (ChR2) was expressed in octopaminergic neurons, and the neurons stimulated by blue light prior to the locomotion assay. Stimulated animals had a significant increase in locomotor speed and this effect was eliminated in *tbh* mutants (**Fig. 2-1G**). Thus, octopamine neurons are necessary and sufficient to increase locomotion.

To determine if octopaminergic innervation of body-wall muscles alone was sufficient for modifications in synaptic physiology, we applied exogenous octopamine to body-wall muscles devoid of central input. Bath application of 10μM octopamine elicited a 30% increase in the amplitude of excitatory junctional potentials (EJPs) without any change in the amplitude of miniature EJPs (mEJPs) (**Fig. 2-1H,J**). This was consistent with analysis of *tbh* mutants, in which EJP amplitude was significantly decreased (**Fig. 2-1I,J**). Thus, changes in locomotor activity are accompanied by growth of synaptopods and likely by an increase in synaptic strength.

Synaptopod extension precedes type II synapse formation

The physiological significance of synaptopods was determined by examining their dynamics (**Fig. 2-2A**; Movie1). Many synaptopods formed varicosity-like structures at their tip, measuring 0.6±0.04 μm in diameter (**Fig. 2-2B**; Movie2), smaller than mature type II boutons (1.57±0.05 μm). Upon new varicosity formation synaptopod motility halted (Movie2), the varicosity enlarged, and sometimes a new motile synaptopod emerged from that varicosity (secondary synaptopod; **Fig. 2-2C**; Movie 3). Thus, synaptopod formation could be a mechanism for type II arbor extension. This possibility was investigated by examining the same type II NMJ from 1st to 3rd-instar larval stage. First-instar type II arbors displayed synaptopods (**Fig. 2-2D**, e.g. red arrow; **Fig. S2-2A**) and synaptopods containing a varicosity at their tip (**Fig. 2-2D**, e.g. yellow arrowhead in inset; **Fig. S2-2A**). These structures developed into a completely new or extended a type II branch (**Fig. 2-2D**; **Fig. S2-2A**). To demonstrate that newly formed varicosities corresponded to new boutons, larvae expressing both mCherry and GFP-tagged Synaptotagmin-1 (Syt-GFP) were imaged as above. Syt-GFP accumulated within the newly formed varicosity (**Fig. 2-2E**). Thus, synaptopod extension is a mechanism to expand type II arbors, both during an acute increase in locomotor speed, and during larval development.

We also labeled preparations with antibodies to different synaptic markers. FasciclinII (FasII) was present at 100% of synaptopods from the earliest stages (**Fig. 2-2F**; **Fig. S2-2B**), and was maintained throughout subsequent stages of bouton maturation (**Fig. 2-2L,M**). In contrast, Synaptotagmin-1 (Syt1) was observed within

synaptopods only 40% of the time (**Fig. 2-2G,L,M**; **Fig. S2-2C**), while it was always present at the onset of varicosity formation (**Fig. 2-2L,M**; **Fig. S2-2D**), suggesting that vesicles begin to traffic into synaptopods even before type II varicosity formation.

The onset of octopamine synthesis was determined by using antibodies to TBH (**Fig. S2-3A-F**), which demonstrated that TBH was never observed before the onset of new varicosity formation (**Fig. 2-2H,L,M**; **Fig. S2-2B-F**), suggesting that the accumulation of another type of synaptic vesicle, marked by Syt1, preceded the accumulation of TBH-containing vesicles. Active zones were identified using anti-Bruchpilot (Brp; Elks/Cast/Erc homolog)[24], which was observed in 27% of enlarged varicosities and after the appearance of TBH (**Fig. 2-2I,L,M**; **Fig. S2-2E**)[20,25,26]. Lastly, the MAP1B-related protein, Futsch, was only observed after a secondary varicosity was formed, but the immunoreactivity was punctate (**Fig. 2-2J,L,M**; **Fig. S2-2F**). We were unable to determine the onset of postsynaptic GluRs, as the level of immunoreactivity was very low (**Fig. S2-4A**). The above data further support the notion that synaptopod extension constitutes a mechanism for the formation of new type II synaptic boutons. Indeed, even within intact larvae many of the synaptopods formed after starvation, developed varicosities (**Fig. 2-2K**). These observations also demonstrate that the formation of type II boutons follows a precise sequence of synaptic protein addition (**Fig. 2-2L**).

Acute activity and octopamine initiates type II outgrowth

The structural changes observed at type II boutons in intact larvae raised the possibility that octopaminergic neurons were activated during food deprivation or

increased locomotion, leading to the expansion of type II arbors. This hypothesis was tested by increasing motorneuron activity either with high-K^+-induced depolarization, or by blue light stimulation of ChR2 expressed in octopaminergic neurons. Preparations were subjected to spaced stimulation, in which each of 5 cycles of stimulation with either high-K^+ or blue light was separated by 15 min rest periods [20]. Identified type II endings were imaged before and after stimulation. Stimulated samples showed a significant increase in synaptopod number at type II endings after stimulation (**Fig. 2-3A,C**). As in intact larvae, natural synaptopods were also observed in unstimulated preparations, albeit at a lower frequency (**Fig. 2-3A,B**). Thus, similar to the starvation response, synaptopods at type II endings increased in frequency in response to spaced stimulation, and just stimulation of octopamine neurons alone was sufficient to elicit this response. This was confirmed by genetically increasing activity at octopaminergic neurons by expressing a dominant-negative Shaker K^+ channel subunit (ShDN)[27] in type II motorneurons of an *ether a go-go* (*eag*) K^+-channel subunit mutant, which resulted in an increase in natural synaptopods (**Fig. 2-3D**).

Next, we sought to determine if octopamine signaling could underlie the effects of activity. Eliminating octopamine in *tbh* null mutants resulted in a significant decrease in natural synaptopods (**Fig. 2-3D**). In contrast, bath application of octopamine for 15 min to wild-type preparations resulted in a dose-dependent increase in the number of synaptopods, while tyramine application was without effect (**Fig. 2-3B,E,F**). Induction of synaptopods by octopamine required normal (1.5 mM) Ca^{++} levels, as decreasing Ca^{++} to 0.1 mM, prevented this effect (**Fig. 2-3E**; sub-Ca^{++}).

The relationship between activity and octopamine was determined by stimulating the terminals with subthreshold parameters for induction of synaptopods by activity and octopamine. If the effect of activity was to increase octopamine release, then presenting both subthreshold stimuli together should elicit significant synaptopod formation. The subthreshold stimuli consisted of 3 cycles of spaced depolarization and application of 10 μM octopamine in 0.1 mM Ca^{++}, both of which were insufficient to induce synaptopods when presented alone (**Fig. 2-3G**). When applied together, however, they were sufficient to increase synaptopods to a level similar to 5 cycles of stimulation alone (**Fig. 2-3G**). Thus, exogenous octopamine can overcome the effect of insufficient activity for the induction of synaptopods, and *vice versa*, consistent with the notion that synaptopod formation is the result of activity-dependent octopamine release.

Surprisingly, octopamine failed to induce synaptopods in *tbh* mutants (**Fig. 2-3E**). Given that *tbh* mutants have an accumulation of tyramine[9] that might be developmentally deleterious, we also tested *tdc2* mutants, which lack tyramine accumulation. In *tdc2* mutants the response to octopamine was normal (**Fig. 2-3E**), suggesting that in *tbh* mutants the accumulation of tyramine renders the NMJs insensitive to exogenous octopamine.

We also examined synaptic growth by counting the number of type II boutons at the last stage of larval development. Increasing activity through expression of ShDN in octopaminergic neurons of *eag* mutants, led to a significant increase in the number of type II boutons and terminal branches (**Fig. 2-3H**; **Fig. S2-5A**). In contrast, in *tbh* mutants the number of type II boutons and branches was decreased (**Fig. 2-3H**; **Fig. S2-4B**). These phenotypes were specifically rescued by expressing a *tbh* transgene at

octopaminergic neurons (**Fig. 2-3H**; **Fig S2-4B**). The defect in *tbh* mutants was not due to the accumulation of tyramine in these mutants, since a null mutant in *tdc2*, which lacks tyramine in addition to octopamine, also showed a decrease in type II bouton number (**Fig. 2-3H**).

Type II synaptogenesis and maintenance requires activity

We also used transgenic approaches to block activity at octopaminergic neurons, including $ShiDN^{ts}$, which blocks vesicle recycling at restrictive temperatures[28], EKO, a hyperpolarizing Shaker potassium channel[29], and Kir2.1, which encodes an inward-rectifying K^+ channel that prevents membrane depolarization[30]. The efficiency of the blockade was tested by examining the ability of adult females to lay eggs, as octopamine function is required for egg-laying[9]. Only expression of Kir2.1 resulted in complete egg-laying block (data not shown). Strikingly, it also resulted in the complete elimination of type II innervation (**Fig. 2-4A,B**). This was not due to a pathfinding or cell death defect since type II motorneuron axons, labeled with mCD8-GFP, were always observed in the segmental nerves (**Fig. 2-4C,D**). In 69% of the nerves examined, these axons stalled within the segmental nerve. However, in 31% of the cases axons traveled the entire distance from the CNS and stalled close to the NMJ without innervating the muscles (**Fig. 2-4D**). Thus, in the absence of activity, type II endings are incapable of innervating body-wall muscles.

Although $ShiDN^{ts}$ did not completely eliminate octopaminergic function, expressing this transgene and rearing the animals at the restrictive temperature of 29^oC developmentally were sufficient to elicit marked abnormalities in the innervation of

muscles by type II endings. These included drastically reduced type II arbors (Fig. S5B), lack of innervation of muscles by type II arbors (**Fig. S2-5D**), thinning of type II neurites (**Fig. S2-5F,H**), and lack of TBH in some type II boutons (**Fig. S2-5H**).

To determine if there was a critical period in which activity was required for type II innervation, and whether the lack of innervation was the result of activity-dependent synaptogenesis or degeneration, first, we determined when type II innervation was established during the larval period, and the consequences of blocking activity at these stages. Type II varicosities were first observed during the 1st-instar stage (**Fig.2-4E,G**). Blocking activity eliminated type II boutons at any larval stage (**Fig.2-4F,H**), suggesting that the absence of activity in type II motorneurons prevents synaptogenesis.

As a second approach, we ubiquitously expressed a temperature-sensitive Gal80, which at 18˚C blocks Gal4-mediated expression[31], and Kir2.1 was simultaneously expressed at octopaminergic neurons. Larvae were raised at 18˚C, and then switched to 29˚C at different stages to permit expression of Kir2.1. Suppressing the activity of octopaminergic neurons 24 hrs before the 3rd-instar stage, did not elicit any abnormality in type II bouton morphology (**Fig. 2-4I**). In contrast, blocking activity starting from late 2nd-instar resulted in breaks in type II arbors (**Fig. 2-4J**). The severity of this phenotype was most pronounced when activity was blocked from late 1st-instar larval stage (**Fig. 2-4K,L,M**). Thus, activity in type II endings is required for synaptogenesis, whereas prolonged periods of inactivity after innervation leads to the degeneration of type II endings.

Octopamine-induced type II outgrowth requires cAMP and dCREB

The finding that octopamine induced the growth of type II arbors, suggested an autoregulatory mechanism controlling the formation of type II endings. Octopamine could be acting on autoreceptors at type II endings, or alternatively, octopamine might activate a retrograde signal that promotes the growth of type II boutons. Octopamine receptors are G-protein-coupled receptors, which can increase Ca^{++} and/or cAMP[4,32]. Previous studies had suggested that increasing cAMP levels by a mutation in the phosphodiesterase Dunce (Dnc) induced an enhancement of synaptic growth in all boutons[33]. Therefore we examined whether manipulating the levels of cAMP at type II motorneurons could influence synaptopod formation in response to octopamine. Mutations in *dnc* elicited significant enhancement in the number of naturally occurring synaptopods at type II endings (**Fig. 2-5A,B,D**). This phenotype was rescued by expressing Dnc exclusively in the octopaminergic neurons of *dnc* mutants or by a genomic duplication (Dp) of *dnc* (**Fig. 2-5D**). Thus, octopamine motorneurons contain a cAMP pathway that can promote synaptopod formation.

These observations were confirmed by using a mutation in *rutabaga* (*rut*) encoding an adenylate cyclase, and thus decreasing cAMP levels. rut^{2080} mutants had significantly fewer natural synaptopods and this phenotype was rescued by expressing Rut at octopaminergic neurons (**Fig. 2-5D**).

To determine if cAMP was acutely sufficient for synaptopod induction, we elevated cAMP levels using a photoactivatable adenylate cyclase (PACα)[34]. We expressed PACα at octopaminergic neurons and light-stimulated the preparation with blue light using a

spaced paradigm. A highly significant increase in the number of synaptopods was observed (**Fig. 2-5C,E**; Movie4), demonstrating that acute changes in cAMP levels are sufficient for the induction of synaptopods.

To determine if cAMP was downstream of activity and octopamine in the induction of synaptopods, we applied octopamine in *dnc* and *rut* mutants. As described above, dnc^{M14} mutants have an increased number of naturally occurring synaptopods, while rut^{2080} mutants show a decrease in this number. In *dnc* mutants we expected that the increase in synaptopods by octopamine would be occluded, as natural synaptopods are already saturated in this mutant. In the case of *rut* mutants, we expected that the decrease in adenylate cyclase activity would render NMJs unresponsive to octopamine. Consistent with these predictions, octopamine failed to increase the number of synaptopods in the two mutants (**Fig. 2-5F**). In contrast, increasing cAMP by activating PACα in a *tbh* mutant background still induced synaptopods (**Fig. 2-5E**). Thus the cAMP pathway is likely downstream of octopamine during synaptopod induction.

The cAMP pathway during long-term plasticity has been associated with the activation of CREB leading to the transcription of genes required for new synapse formation[35]. We sought to determine if the growth of type II endings in response to octopamine release required dCREB function. We expressed a dCREB dominant-negative transgene (*CREBdn/dCREB2-b*) previously shown to block CREB function[36], in octopamine neurons, which suppressed the increase in octopamine-induced synaptopod formation (**Fig. 2-5G**). Similarly, the translational inhibitor cycloheximide and the transcriptional inhibitor actinomycin-D completely suppressed octopamine-dependent synaptopod formation (**Fig. 2-5G**). Thus, the autoregulatory mechanism that initiates the

formation of new type II boutons activates a cAMP cascade that depends on CREB-mediated transcription.

As perturbations in the cAMP pathway altered synaptopod formation, and mutations preventing an increase in synaptopod formation in response to octopamine also showed behavioral defects, we predicted that similar behavioral defects would be observed in *dnc* and *rut* mutants. Interestingly, although *dnc*^{M14} mutants showed a decreased locomotor speed and *rut* mutants had normal locomotor speed (**Fig. 2-5H**), the response to starvation was blocked in both mutants, similar to *tbh* mutants (**Fig. 2-5I**). The behavioral defect in *rut* mutants was completely rescued by expressing a Rut transgene in octopaminergic neurons (**Fig. 2-5I**). Thus, normal cAMP levels in octopaminergic neurons are required for the response to starvation, and at least in the case of Rut, the starvation response can be separated from a defect in basal locomotion.

Octß2R autoreceptors mediate the autoregulatory mechanism

Presynaptic octopamine autoreceptors are plausible candidates for mediating autoregulation of synaptic structure. Four octopamine receptors have been identified in the *Drosophila* genome, OAMB, Octß1R (also called OA2), Octß2R, and Octß3R. OAMB receptors have homology to mammalian α-adrenergic receptors and can increase Ca^{2+} or cAMP levels[4,32]. Octß1R, Octß2R, and Octß3R receptors have been less studied, but share similarities with mammalian ß-adrenergic receptors and are thought to increase cAMP. We interfered with the function of OAMB and Octß2R.In the *oamb*^{584} genetic null allele[37], the number of natural synaptopods, the induction of

synaptopods by octopamine, and the number of type II boutons were normal (**Fig. 2-6A-C**).

In the case of Octß2R, we used a hypomorphic allele[11], as well as UAS-Octß2R-RNAi. We also verified that Octß2R was expressed in the nervous system and body-wall muscles (either pre- or postsynaptically) and the effectiveness of the RNAi transgene by RT-PCR (**Fig. S2-6A,B**). In *octß2R* mutants the number of natural synaptopods was significantly decreased (**Fig. 2-6A**) placing it as the likely mediator of the cAMP-dependent autoregulatory mechanism that controls the growth of type II endings. Accordingly, *octß2R* mutants did not show increased synaptopods in response to octopamine (**Fig. 2-6C**). This effect was cell autonomous, as expressing Octß2R-RNAi in octopaminergic neurons alone was sufficient to decrease the number of natural synaptopods (**Fig. 2-6A**) and to suppress the increase in synaptopods upon octopamine application (**Fig. 2-6C**). In addition, either the *octß2R* mutant or expression of Octß2R-RNAi in octopamine neurons resulted in a significant decrease in the number of type II boutons (**Fig. 2-6B**). Expressing Octß2R-RNAi in both type I and type II motorneurons by using C380-Gal4, also resulted in a significant reduction in the number of type II boutons (**Fig. 2-6B**). In contrast, expressing Octß2R-RNAi in muscles using C57-Gal4 was without effect. Taken together, these observations identify Octß2R receptors as the likely autoreceptor that regulates the growth of type II boutons.

Interestingly, the *octß2R* mutant had a reduction in evoked EJP amplitude similar to *tbh* mutants (EJP amplitude = 18.3±1 mV in wild type, vs 12.1±1 mV in *octß2R* mutants; N=7; $p<0.001$), suggesting that either removing octopamine, or the receptor decreases synaptic strength. As expected, bath application of octopamine to *octß2R*

mutants did not change EJP amplitude as observed in wild type (ratio EJP amplitude is 0.95 upon 10μM octopamine application vs 1.33 in wild type; N=5; $p<0.005$). Thus, Octß2R receptors are likely responsible for the octopamine induced changes in synaptic strength.

We predicted that removing the receptor would also eliminate the starvation response. *octß2R* mutants failed to respond to starvation by increasing locomotor speed (**Fig. 2-6D**). This defect was also observed when Octß2R was downregulated either in octopamine neurons or both in type I and type II motorneurons, but not in muscles (**Fig. 2-6D**).

Type II endings regulate type I synaptic bouton outgrowth

Excitatory transmission at the larval NMJ is mediated by the release of glutamate from type I NMJs. Similar to type II arbors, type I arbors continuously expand throughout larval development, in strong correlation to muscle size[38]. We considered the possibility that type II innervation might regulate this form of plasticity at type I boutons. To test this possibility, we first eliminated type II boutons by expressing Hid in octopaminergic neurons. Notably, the absence of type II innervation led to a substantial reduction in the number of type I boutons (**Fig. 2-7A**; **Fig. S2-6C**). Similar results were obtained by expressing Kir2.1 or in *tbh* mutants (**Fig. 2-7A**). The reduction in the number of type I boutons in *tbh* mutants was restored by expressing a *tbh* transgene in octopamine neurons (**Fig. 2-7A**). These results suggest that type II innervation regulates the plasticity of type I endings and therefore it is involved in a form of metaplasticity.

To further characterize the influence of type II endings on type I arbors, we examined *octß2R* mutants, and observed a reduction in the number of type I boutons (**Fig. 2-7B**). A potential mechanism by which type II arbors might regulate the growth of type I endings is through the presence of octopamine receptors at type I boutons. Therefore, we used the C380-Gal4-driver to downregulate Octß2R in both type I and type II motorneurons, and BG439-Gal4, which drives Gal4 expression in type I motorneurons but not in type II motorneurons (**Fig. 2-S7**). Both manipulations resulted in a highly significant decrease in the number of type I boutons (**Fig. 2-7B**). In contrast, downregulating Octß2R at octopamine neurons alone did not result in a significant decrease in the number of type I boutons. These results suggest that Octß2R is required in type I motorneurons for normal expansion.

DISCUSSION

Adrenergic signaling is involved in the regulation of synaptic plasticity[39,40]. However, the precise mechanisms for this regulation are poorly understood. We show that octopamine regulates behavioral and synaptic plasticity through an autoregulatory mechanism that promotes the growth of type II innervation and in turn the expansion of excitatory glutamatergic arbors. Importantly, this process appears associated with physiological stimuli that lead to increased locomotion. We propose that food deprivation elicits the release of octopamine by type II terminals. Octopamine binds to Octß2R receptors resulting in the increase of cAMP, which activates CREB-dependent regulation of transcription, leading to new type II synaptic growth (**Fig. S2-8A**). This autoregulatory mechanism might serve to control the amount of octopamine released by type II arbors. In turn, octopamine release stimulates the growth of type I arbors through Octß2R at type I motorneurons. This mechanism would therefore regulate, in a global fashion, excitatory transmission at the NMJ (**Fig. S2-8A**).

Increasing larval locomotion, type II motorneuron activity, or exogenous octopamine levels resulted in the extension of synaptopods. Taken together with the demonstration that synaptopod extension constitutes a mechanism for the formation of type II boutons, these results suggest that the above events serve to control the growth of octopaminergic endings in an acute manner. Analysis of mutations in octopamine receptors and components of the cAMP cascade demonstrated the presence of an autoregulatory mechanism to control this growth. First, type II motorneuron expression of Octß2R was required for type II synaptic growth. Second, altering cAMP levels by mutations in *dnc* or *rut* modified this response in a manner consistent with positive

regulation by cAMP. This regulation was cell autonomous in octopaminergic motorneurons, as the defects in synaptopod formation and type II synaptic growth were also elicited or rescued by transgene expression in octopaminergic motorneurons alone, in a chronic or acute manner. The presence of auto-octopamine receptors had been suggested in locusts[41], although the identity of the proposed autoreceptor was not known. However, it was proposed that the locust octopamine autoreceptors served to inhibit octopamine release. In contrast, our experiments are consistent with a positive feedback mechanism that enhances synaptic growth. Autoregulatory mechanisms that control the amount of neuromodulator release have been previously demonstrated for neuromodulators such as dopamine[42].

Intriguingly, as in other forms of synaptic plasticity, including late LTP and long-term memory[35], the autoregulatory mechanism required the function of CREB and new protein synthesis. This finding underscores the universality of mechanisms by which the nervous system modifies the efficacy of connections in a long-lasting manner. Octopamine receptor activation leading to CREB signaling has also been demonstrated in *C. elegans*[10].

Our studies revealed that this pathway regulated the structure of octopaminergic arbors in an autoregulatory fashion, and that this influenced the growth of type I excitatory arbors. The presence of a positive feedback that controls the growth of modulatory inputs in an acute manner is highly significant, as it provides a novel mechanism by which animal experience can modify circuitry and thus adapt to a changing environment.

Activity was absolutely required for innervation of body-wall muscles by type II arbors, as reduced activity perturbed type II synaptogenesis. This is in contrast to the widely held view that while activity is important for the refinement of connections, it is not required for initial synaptogenesis[43]. Part of this view arises from the examination of arbors mediating classical neurotransmission[43]. In contrast, the activity dependence of modulatory terminal growth has been less studied. Our studies provide compelling evidence that octopamine has an influence on both octopaminergic type II and type I bouton outgrowth. Studies of type I bouton outgrowth have identified local factors that influence the development of pre- and/or postsynaptic compartments, including Wnts and BMPs[15,16]. We suggest that octopamine release by type II arbors might put forth a more global regulation of outgrowth.

At the *Drosophila* larval NMJ, glutamatergic type Ib motorneurons innervate each muscle in an approximately 1:1 manner[44] (**Fig. S2-8B**; type Ib). In addition, 2 glutamatergic type Is motorneurons innervate the entire ventral or dorsal muscle field within each hemisegment[44,45] (**Fig. S2-8B**; type Is). In contrast, the 3 octopaminergic neurons per segment innervate the majority of the body-wall muscles in a bilateral fashion ([13] and this report; **Fig. S2-8B**; type II). The layout of this innervation suggests that type II synapses might establish global regulation of the plasticity of type I arbors. This might serve as a mechanism to set excitability levels in the entire body-wall, and thus maintain synaptic function within a dynamic range. Similarly, studies in mammalian systems have demonstrated that adrenergic signaling can affect glutamatergic synapse plasticity, either through changes in ionotropic GluR localization[2], or through regulation of metabotropic GluR, affecting the ability of a synapse to become potentiated

depending on its history[3]. Octopamine might regulate the ability of type I NMJs to trigger muscle contraction by long-term regulation of type I synaptic growth.

Two previous studies at the *Drosophila* larval NMJ have demonstrated an enhancement of synaptic transmission in response to octopamine[46,47]. However, another study suggests that octopamine may inhibit glutamatergic transmission in 1st-instar larvae[48]. Our studies suggest that blocking activity or interfering with octopamine signaling in type II neurons, leads to a decrease in type I synaptic outgrowth, consistent with octopamine release as a positive regulator of type I transmission. We suggest that in the short-term, octopamine enhances synaptic strength, as observed in our electrophysiology experiments, leading to the observed increase in crawling behavior after starvation. This would be consistent with studies showing that food deprivation induced increases in locomotor speed lead to an enhancement of synaptic efficacy[17].

Octopamine is a potent modulator of invertebrate behaviors and has been shown to be secreted during starvation in invertebrates[10,22]. Nevertheless, its function at the synaptic level is poorly understood. Our study, for the first time, demonstrates that octopamine can influence synapses at the structural level through the activation of Octβ2R autoreceptors in octopamine neurons and through the presence of these receptors in type I motorneurons.

An important question is whether octopamine is simply involved in locomotion, and the lack of starvation response in mutants unable to synthesize octopamine is an indirect effect from defective locomotion. It is not possible to answer this question in *tbh* mutants, since basal locomotion was reduced in these mutants. However, our experiments revealed conditions in which changes in basal activity could be genetically

separated from changes in the starvation response. One such case is *rut* mutants, which have normal locomotion, but lack starvation response. This effect appeared to be due to the function of Rut in octopamine neurons, as the defective starvation response was completely rescued by expressing a Rut transgene in octopamine neurons. A second, although somewhat less clear observation is regarding *octß2R* mutants. While baseline locomotion was much less altered in these mutants, compared to *tbh* mutants, these animals were still unable to mount a starvation response (**Fig. 2-6D,E**). Thus, it is quite likely that octopamine neurons are not only involved in locomotion, but also in the response to starvation.

Octopamine is also known to be required for appetitive memory in adult fruit flies[7]. Notably, the appetitive memory paradigm requires starvation prior to the assay, and *tbh* mutants are unable to learn in this paradigm. Octopamine has been proposed to mediate the reinforcing effects of sugar in appetitive memory formation[5,7]. Our studies raise the possibility that this mechanism may involve structural changes at synaptic sites.

Although our studies focused on structural changes at type II NMJs, many of our manipulations affected all octopamine neurons, as the Tdc2-Gal4 drives Gal4 in all octopaminergic neurons. Thus, our studies cannot rule out an influence from other octopaminergic neurons, besides motorneurons, in the changes observed and in the behavior. However, the finding that the manipulations resulted in specific changes in type II NMJ terminals, and that octopamine modulates synaptic strength at the NMJ argues that at least some of the effects observed are likely to be due to the peripheral octopamine innervation.

In summary, our studies reveal important mechanisms by which activity regulates the ability of motorneurons to scale the release of regulatory signals, which is important for the adaptation of the organism to the environment. In addition, they provide a mechanism by which excitatory synapses are regulated in a global manner, presumably to maintain synaptic plasticity in a dynamic range.

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MATERIALS AND METHODS

Fly strains. Flies were reared in standard *Drosophila* medium at 25 °C except where indicated. Animals used in RNAi experiments were reared at 29 °C to increase knockdown efficiency. The following stocks were used: the wild-type strain Canton-S (CS), Tdc2-Gal4 (Bloomington Stock Center), UAS-Hid[49], *tbh*^{nM18}, *tdc2*^{RO54} (ref. 23), UAS-Kir2.1 (ref. 30), tubP-Gal80^{ts} (Bloomington), UAS-ShDN[27], *eag*[1] (Bloomington), UAS-mCD8-GFP (Bloomington), *y dnc*^{M14} *cv f* (Bloomington) *y w dnc*^{ML} *f*^{36a} (Bloomington), UAS-Dnc (remobilized to the second chromosome)[50], *rut*^{2080} (Bloomington), UAS-Rut (Bloomington), UAS-dCREB2-b (Bloomington), *oamb*^{584} (ref. 37), Pbac{WH}Octβ2R[f05679] (Bloomington), Dp(1;2)51b (duplication of *dnc*; Bloomington), UAS-Syt1-GFP (Bloomington), C380-Gal4 (Budnik et al., 1996), BG439 (V. Budnik, unpublished), UAS-Dicer-2 (Bloomington), UAS-Octβ2R-RNAi (8486 and 104524; Vienna *Drosophila* RNAi Center) and UAS-PACα (see below).

Generation of PACα flies. *Euglena gracilis* PACα cDNA was provided by M. Watanabe. A 3,104-bp EagI fragment encompassing the full-length PACα cDNA with 5′ leader sequence was ligated into the EagI site of the *Drosophila* transformation vector pUAST. The insert was verified to be in the appropriate orientation by PCR and end sequencing, and transformed into flies by germline transformation.

Generation of TBH antibodies. TBH antibodies were raised against amino acids 277–670 of TBH, which is the following peptide sequence:
QETTYWCHVQRLEGNLRRRHHIVQFEPLIRTPGIVHHMEVFHCEAGEHEEIPLYNGDC
EQLPPRAKICSKVMVLWAMGAGTFTYPPEAGLPIGGPGFNPYVRLEVHFNNPEKQSGL

VDNSGFRIKMSKTLRQYDAAVMELGLEYTDKMAIPPGQTAFPLSGYCVADCTRAALPA TGIIIFGSQLHTHLRGVRVLTRHFRGEQELREVNRDDYYSNHFQEMRTLHYKPRVLPGD ALVTTCYYNTKDDKTAALGGFSISDEMCVNYIHYYPATKLEVCKSSVSEETLENYFIYMK RTEHQHGVHLNGARSSNYRSIEWTQPRIDQLYTMYMQEPLSMQCNRSDGTRFEGRS SWEGVAATPVQIRIPIHRKLCPNYNPLWLKPLEKGDCDLLGECIY. The specificity of the antibody was demonstrated by the lack of immunoreactivity in null *tbh*^{nM18} mutants (Supplemental Fig. 2-S1C,F).

Immunocytochemistry. Larval body-wall muscles were dissected and fixed for 15 min in 4% paraformaldehyde. For TBH immunocytochemistry samples were fixed in Bouin's fixative. Antibodies and their concentrations were: anti-TBH 1:400, anti-mCD8a 1:100 (Invitrogen), anti-HRP-Texas Red 1:200 (Jackson ImmunoResearch), anti-FasII 1:2 (Developmental Studies Hybridoma Bank, DSHB), anti-Syt1 1:100 (gift from T. Littleton), anti-Bruchpilot 1:100 (nc82; DSHB), anti-Futsch 1:50 (22C10, DSHB), anti-GluRIIA 1:10 (8B4D2; DSHB). Secondary antibodies conjugated to FITC, Texas Red (Jackson) or Alexa 647 (Invitrogen) were used at a concentration of 1:200. Imaging of fixed preparations was as described[20].

Animal rearing conditions for synaptopod analysis. All animals used in synaptopod analysis carried a copy of Tdc2-Gal4 and a copy of UAS-CD8:GFP. Egg collection was done in standard 25-mm diameter cornmeal/agar/molasses food vials at 25 °C with ~60% humidity, and larvae were kept at low density. Wandering late third-instar larvae were used for experiments.

Stimulation procedures and live imaging of dissected preparations. Synaptopods were imaged from live preparations as described[20]. Both the spaced high K^+ depolarization procedure and the ChR2 stimulation procedure were as described[20]. Briefly, the high K^+ procedure consisted of 5 incubation cycles with 90 mM K^+-containing saline, each lasting 2 min and separated by 15-min resting intervals. For the ChR2 stimulation procedure, animals were placed inside a drop of HL3 saline (~300 µl) containing 1.5 mM Ca^{2+}. The procedure consisted of five cycles of blue light stimulation from four 491-nm 1-W LEDs placed 1.2 meters away from the animals. Each cycle consisted of a repeating 5-min procedure of 2 s on and 3 s off, separated by 15-min resting intervals. Saline was exchanged once during each resting interval. Animals were imaged 15 min after stimulation or subjected to the crawling assay. For octopamine stimulation, larvae were dissected in HL3 saline containing 0.1 mM Ca^{2+} and preparations gently glued onto a custom-made glass imaging chamber using surgical glue. Then, identified NMJs were imaged on an Improvision spinning disc confocal microscope (PerkinElmer) with a C9100-13 Hamamatsu cooled EM-CCD camera and using a 40× 1.2 NA objective, with a 2.4× optical zoom. After imaging for less than 30 min, animals were partially unglued to allow muscles to contract freely, and 10 µM octopamine in HL3 containing 1.5 mM Ca^{2+} was then applied for 15 min followed by 5 × 15-min washes with 0.1 mM Ca^{2+} HL3 saline before imaging again. In some experiments actinomycin D (Sigma) 5 µM in HL3, and cycloheximide (Sigma) 100 µM in HL3 were applied throughout octopamine incubation and washes. The final concentration of DMSO in these solutions was approximately 0.03%. For PACα experiments, NMJs were imaged as above, then stimulated with a broad spectrum blue light dental gun placed ~2 cm away. Stimulation consisted of 5 × 5-s light exposures, each separated by 2 min rest.

Live imaging of intact (undissected) larvae. For live-imaging through the cuticle of intact larvae, animals were anesthetized using Sevoflurane (Baxter) and identified NMJs (muscle 9, A4) were imaged for not longer than 30 min. Larvae were allowed to recover for 1.5 h on food plates and then used for food deprivation experiments (see below) before imaging. Larvae that did not recover in 1.5 h after the first imaging session or after starvation were discarded. For non-starved controls, larvae were placed in food between imaging sessions.

Crawling assay. Synchronized larvae were grown at 25 °C in 28.5-mm diameter standard food vials at low density until mid third-instar larval stage. After washing with water, individual larvae were loaded onto a 24 × 24-cm 3% agar plates and allowed a pre-run of 25 s on the agar before recordings were made. Larval tracks were then recorded manually for 1 min on transparency paper over the plate lid, and the distances crawled were measured using ImageJ. All behavioral experiments were carried out in a 25 °C, 60% humidity behavioral room. *n* represents one animal. Percentage increase in locomotor speed of individual animals was calculated by: (locomotor speed of individual animals after starvation minus mean locomotor speed before starvation) × 100 divided by the mean locomotor speed before starvation. Mean percentage and s.e.m. were calculated. For RNAi experiments, food vials rearing animals at 29^{o}C were incubated at 25^{o}C for 1 hour prior to crawling assay.

Starvation assay. Larvae were maintained in food or food-free moisturized 35-mm Petri dishes for 2 h and then either assayed for synaptopod formation or subjected to the crawling assay. For RNAi experiments, food vials rearing animals at 29^{o}C were incubated at 25^{o}C for 1 hour prior to starvation assay.

Quantification of boutons and synaptopod number. The number of type I boutons was obtained at muscles 6 and 7 of abdominal segment A3, and the number of type II boutons was measured at muscle 12 in A3. For muscle area measurements the muscle length and width were measured using an ocular scale bar. Measurements of synaptopod number were from muscle 12 (A4) in dissected preparations and those from intact larvae were from muscle 9 (A4). Numbers of synaptopods in the histograms represent the total number of synaptopods per 100 μm of each arbor. Synaptopods were defined as such if they measured 0.5 μm or more. *n* represents number of NMJs. At most two NMJs (segment A3) were quantified in each animal.

Statistical analysis. For comparisons between more than two sample groups an analysis of variance (ANOVA) with Tukey post-hoc test was performed. For pair-wise comparisons a Student *t*-test was used. Numbers in histograms represent mean ± s.e.m. Unless otherwise noted, sample number (*n*) represents one synaptic arbor for anatomical measurements, or one animal for behavioral analyses. Statistical analysis for animals reared at 25^{o}C and animals reared at 29^{o}C are calculated separate.

Genotype abbreviations. Type II driver control, Tdc2/+; Type I+II driver control, C380-Gal4/+; muscle driver control, C57-Gal4/+; [transgene]-type II, Tdc2-Gal4>[transgene]; [transgene]-type I+II, C380-Gal4>[transgene]; [transgene]-muscle, C57-Gal4>[transgene] unless otherwise indicated ; Dcr, UAS-Dicer-2.

Electrophysiology. Membrane potential recordings were performed on dissected third-instar larvae as described[20]. Briefly, larvae were dissected in a custom magnetic chamber in 0.3 mM Ca^{2+} HL3 saline, and the A3 segmental nerves carefully cut close to

the ventral ganglion. The chamber was then moved to the recording setup where it was perfused with 0.5 mM Ca^{2+} HL3. Muscle 6 in segment A3 was impaled with a 15–20-MΩ electrode, and voltage recordings were collected with an Axoclamp2A amplifier (Molecular Devices), using Heka Pulse software (Heka). Only samples with resting membrane potentials between −60 and −63 mV were considered. For EJP recordings the segmental nerve was stimulated with a suction electrode at 0.3 Hz, with a stimulus of 0.3 ms and sufficient voltage to evoke responses from both type I boutons. Four minutes of both mEJP and EJP data were recorded for each sample. Data was analyzed using Minianalysis software (Synaptosoft), and statistical analysis was done with Origin software (OriginLab). Bath application of octopamine was performed by changing the perfusion solution from 0.5 mM Ca^{2+} HL3 to 0.5 mM Ca^{2+} HL3 containing 10 μM octopamine. Samples were allowed to equilibrate for 2 min in the new saline, and then evoked and spontaneous events were recorded again. Statistical analysis was done as above.

RT-PCR. Total mRNA was extracted from dissected larval body wall muscles or larval brains using a combination of Trizol (Invitrogen) and the RNeasy kit (Qiagen). Synthesis of cDNA for +RT reactions was performed using the Superscript III kit (Invitrogen), where −RT reactions lacked reverse transcriptase. The +RT and −RT reactions were then diluted and used for PCR using the forward primer CATGCTGATGCACCGACCATC and the reverse primer CACTCCTCGCAGGTCATGGAG. These primers were specifically designed to recognize all known splice variants of *octβ2R*, and across two exon-intron junctions to avoid false signals from any contaminating genomic DNA. For semiquantitative RT-PCR,

we determined the linear range of Octβ2R amplification for the amount of starting cDNA (50 ng) and number of PCR cycles for wild type, and then amplified cDNA made from RNA isolated from wild type and C380>UAS-Octβ2R-RNAi.

Quantification of branch-points. Type II arbors were examined in an epifluorescence microscope and each branch bifurcation was counted as a single branch point. For these quantifications *n* is the number of total type II arbors (segment A3) quantified for each genotype.

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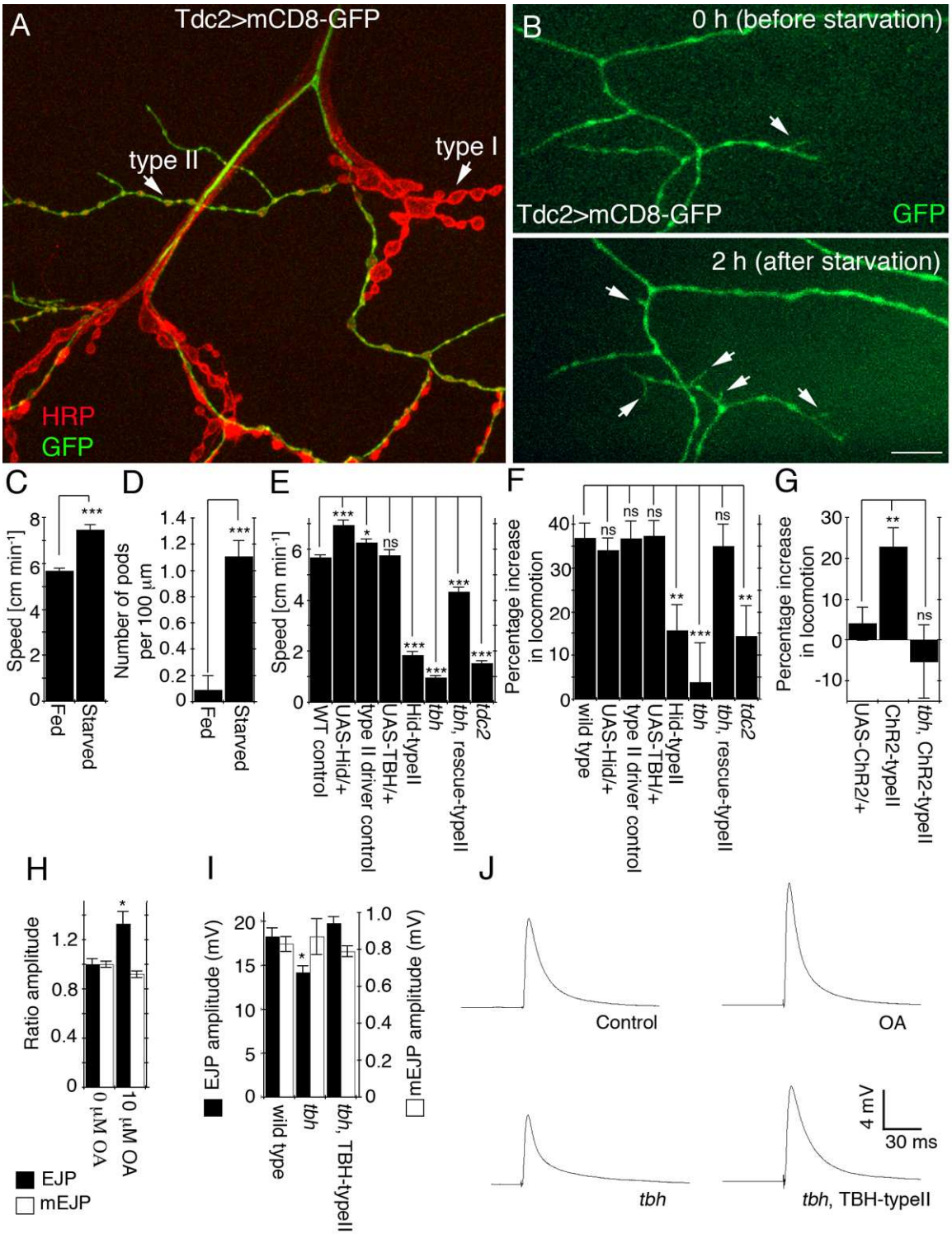

A
Tdc2>mCD8-GFP
type II
type I
HRP
GFP
B
0 h (before starvation)
Tdc2>mCD8-GFP
GFP
2 h (after starvation)
C
Speed [cm min-1]
Fed
Starved
D
Number of pods per 100 µm
Fed
Starved
E
Speed [cm min-1]
WT control
UAS-Hid/+
type II driver control
UAS-TBH/+
Hid-typeII
tbh
tbh, rescue-typeII
tdc2
F
Percentage increase in locomotion
wild type
UAS-Hid/+
type II driver control
UAS-TBH/+
Hid-typeII
tbh
tbh, rescue-typeII
tdc2
G
Percentage increase in locomotion
UAS-ChR2/+
ChR2-typeII
tbh, ChR2-typeII
H
Ratio amplitude
0 µM OA
10 µM OA
EJP
mEJP
I
EJP amplitude (mV)
mEJP amplitude (mV)
wild type
tbh
tbh, TBH-typeII
J
Control
OA
tbh
tbh, TBH-typeII
4 mV
30 ms

Figure 2-1

Figure 2-1. Food-deprivation increase in larval locomotion is correlated with synaptopod formation at type II arbors. (**A**) NMJs at muscles 12 and 13 of a 3rd-instar larva expressing mCD8-GFP in type II motorneurons, showing type I and type II endings (arrows), labeled with anti-HRP and anti-GFP. (**B**) Live imaging of type II endings through the cuticle of intact larvae before and after 2-hr starvation. Arrows point to synaptopods. (**C**) Locomotor speed of wild-type (Canton-S) larvae before and after 2-hr starvation (N=31,26). (**D**) Number of synaptopods (pods) in fed and 2-hr starved intact Tdc2>mCD8-GFP larvae (N=10,10). (**E**) Locomotor speed in the indicated genotypes (N=31,23,29,15,34,18,25). (**F**) Percentage increase in locomotor speed in response to starvation in the indicated genotypes (N=26,25,15,14,38,14,25). (**G**) Percentage increase in locomotor speed in response to light stimulation in the indicated genotypes (N=20,20,16). (**H**) Ratio of EJP and mEJP amplitude upon bath application of 10 μM octopamine (N is 10 animals) (**I**) EJP and mEJP amplitude in *tbh* mutants (N is 6, 5, and 5 animals respectively) (**J**) Representative EJP traces in the indicated conditions. Calibration bar is 8.5μm for **A**, 7.5μm for **B**. Error bars represent SEM.

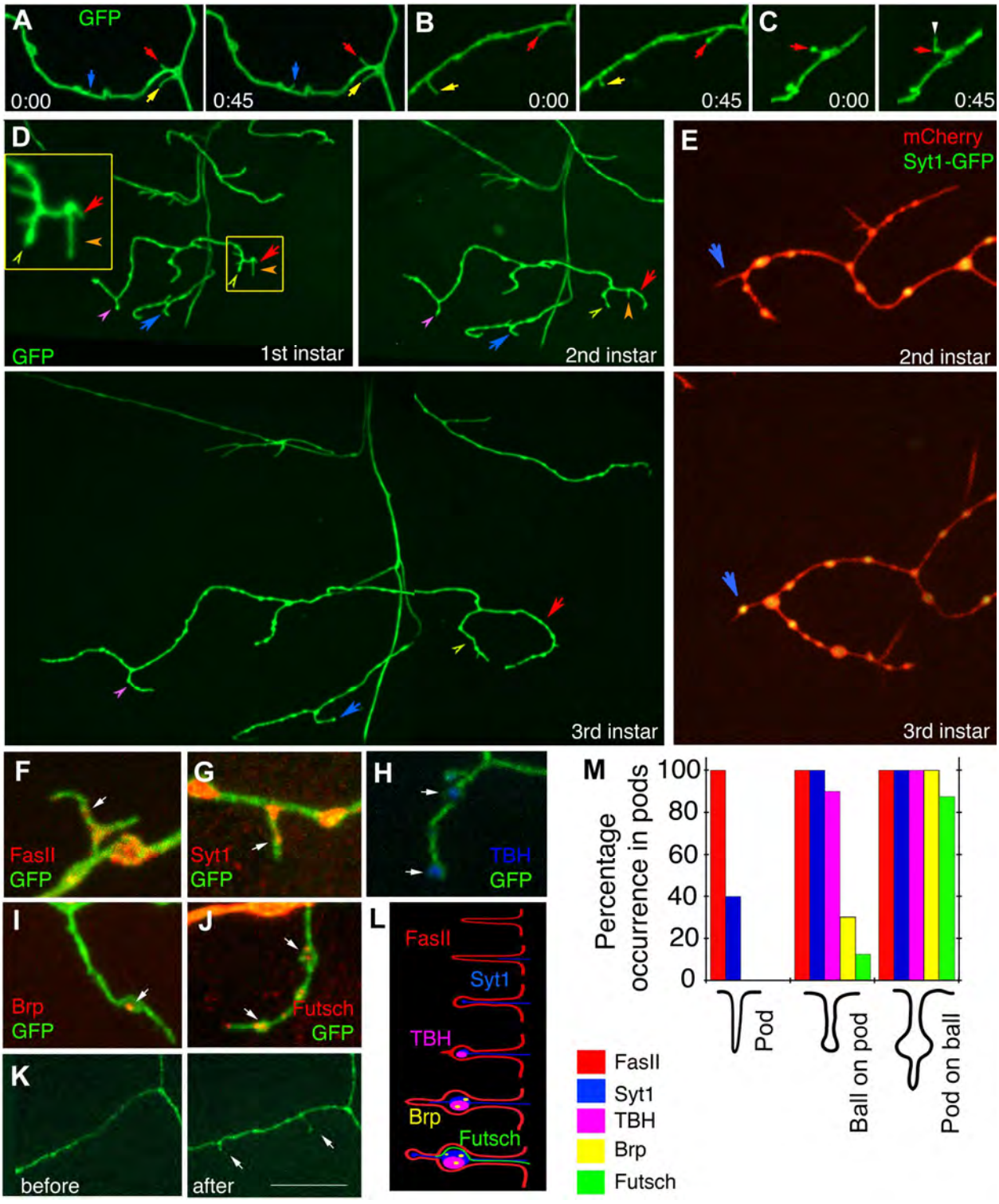

A
GFP
0:00
0:45
B
0:00
0:45
C
0:00
0:45
D
GFP
1st instar
2nd instar
E
mCherry
Syt1-GFP
2nd instar
3rd instar
3rd instar
F
FasII
GFP
G
Syt1
GFP
H
TBH
GFP
I
Brp
GFP
J
Futsch
GFP
K
before
after
L
FasII
Syt1
TBH
Brp
Futsch
M
Percentage occurrence in pods
100
80
60
40
20
0
Pod
Ball on pod
Pod on ball
FasII
Syt1
TBH
Brp
Futsch

Figure 2-2

Figure 2-2. Stepwise development of synaptopods. (**A, B, C**) Time lapse imaging of synaptopods in Tdc2>mCD8-GFP larvae showing (**A**) the extension of synaptopods (arrows), (**B**) the formation of varicosities at the tip of synaptopods (arrows), (**C**) the formation of a secondary synaptopod (arrowhead) from a newly formed varicosity (arrow). Images of the same NMJs were taken 45 sec apart. (**D**) Developmental time-lapse imaging of the same NMJ through the cuticle of 1st, 2nd and 3rd-instar. Red arrow: a synaptopod developed into an entire branch. Orange arrow: a synaptopod was eliminated at 3rd-instar. Blue arrow: a synaptopod developed into a varicosity. Yellow and purple arrow: a varicosity developed into a new branch. (**E**) Time lapse imaging as in (D) in animals expressing mCherry and Syt-GFP in type II endings in 2nd and 3rd-instar. Arrow points to a synaptopod that acquired Syt1-containing varicosity. (**F**-**J**) Sequence of protein addition to an extending type II branch in Tdc2>mCD8-GFP preparations triple labeled with anti-GFP, anti-TBH and antibodies to (**F**) FasII, (**G**) Syt1, (**H**) TBH, (**I**) Brp, and (**J**) Futsch. Arrows point to sites of protein localization at synaptopods or newly formed varicosities. (**L**) Diagrammatic representation of developing type II endings showing the sequence of synaptic protein addition. (**M**) Percentage of synaptopods showing the presence of the indicated proteins at the stages of synaptopod, varicosity at the tip of a synaptopod (ball on pod), and secondary synaptopod (pod on ball). N (number of pod structures) is FasII=30, Syt1=30, TBH=20, Brp=20, and Futsch=10. (**K**) Live imaging of synaptopods through the cuticle of intact Tdc2>mCD8-GFP larvae before and after starvation, showing the formation of varicosities (arrows) on top of synaptopods after starvation. Calibration bar is 10 μm for **A-C**, 22μm for **D**, 8.5μm for **E**, 2.5 μm for **F-J**, 12 μm for **K**.

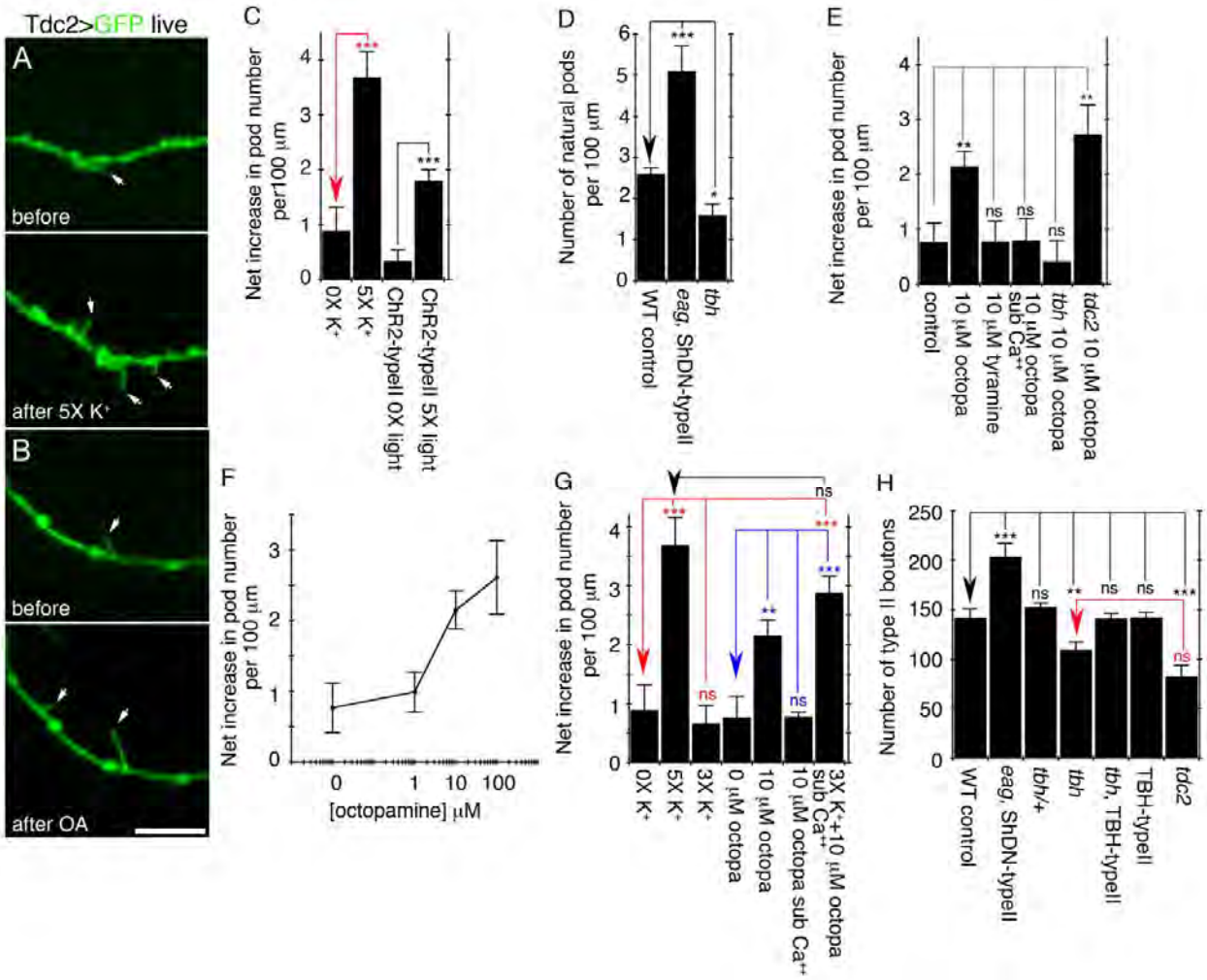

Tdc2>GFP live
A
before
after 5X K+
B
before
after OA
C
Net increase in pod number per100 µm
0X K+
5X K+
ChR2-typeII 0X light
ChR2-typeII 5X light
D
Number of natural pods per 100 µm
WT control
eag, ShDN-typeII
tbh
E
Net increase in pod number per 100 µm
control
10 µM octopa
10 µM tyramine
10 µM octopa sub Ca++
tbh 10 µM octopa
tdc2 10 µM octopa
F
Net increase in pod number per 100 µm
[octopamine] µM
G
Net increase in pod number per 100 µm
0X K+
5X K+
3X K+
0 µM octopa
10 µM octopa
10 µM octopa sub Ca++
3X K++10 µM octopa sub Ca++
H
Number of type II boutons
WT control
eag, ShDN-typeII
tbh/+
tbh
tbh, TBH-typeII
TBH-typeII
tdc2

Figure 2-3

Figure 2-3. Electrical activity and octopamine regulate the extension of synaptopods. (**A, B**) Live imaging of synaptopods before and after stimulation with (**A**) high K^+, or (**B**) octopamine, in Tdc2>mCD8-GFP larvae. (**C**) Net increase in synaptopod number, approximately 2 hr after the indicated K^+ or ChR2 stimulation paradigms (see Materials & Methods for details; N= 8,10,10,11). (**D**) Number of natural synaptopods in Tdc2>mCD8-GFP (WT control), and indicated genotypes (N=31,11,31). (**E**) Net increase in synaptopod number in Tdc2>mCD8-GFP control (no drug) and preparations exposed to the indicated drugs (N=11,13,8,18); control= no drug. (**F**) Net increase in the number of synaptopods in response to different concentrations of octopamine in Tdc2>GFP larvae (N=11,7,9,11). (**G**) Net increase in synaptopod number at subthreshold concentration of octopamine or K^+ depolarization in the presence of 0.1 mM Ca^{++} (sub-Ca^{++}) in Tdc2>mCD8-GFP larvae (N=8,10,11,11,13,18,13). (**H**) Number of type II boutons at muscle 12 (A3) of 3rd-instar larvae in the indicated genotypes (N=16,10,15,14,12,6); wild-type= Canton-S. (**I**) Percentage increase in locomotor speed in response to food deprivation in the indicated genotypes (N=31,20,34,18,25); WT control= Canton-S. Calibration bar is 7 μm in A, B. Error bars represent SEM.

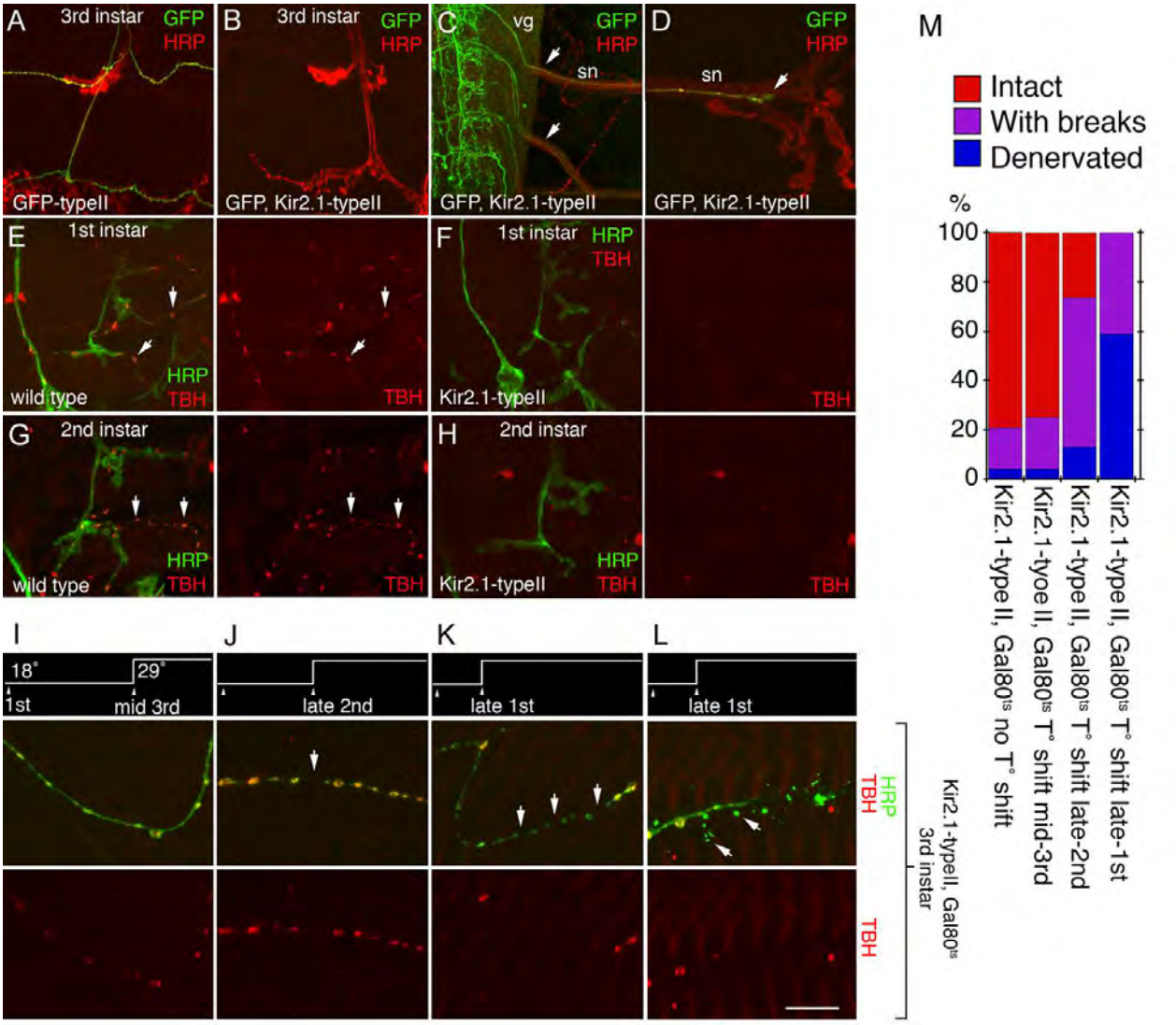

A
3rd instar
GFP
HRP
GFP-typeII
B
3rd instar
GFP
HRP
GFP, Kir2.1-typeII
C
vg
GFP
HRP
sn
GFP, Kir2.1-typeII
D
GFP
HRP
sn
GFP, Kir2.1-typeII
E
1st instar
HRP
TBH
wild type
TBH
F
1st instar
HRP
TBH
Kir2.1-typeII
TBH
G
2nd instar
HRP
TBH
wild type
TBH
H
2nd instar
HRP
TBH
Kir2.1-typeII
TBH
I
18°
29°
1st
mid 3rd
J
late 2nd
K
late 1st
L
late 1st
HRP
TBH
TBH
Kir2.1-typeII, Gal80ts
3rd instar
M
Intact
With breaks
Denervated
%
100
80
60
40
20
0
Kir2.1-typeII, Gal80ts no T° shift
Kir2.1-tyoeII, Gal80ts T° shift mid-3rd
Kir2.1-typeII, Gal80ts T° shift late-2nd
Kir2.1-typeII, Gal80ts T° shift late-1st

Figure 2-4

Figure 2-4. Innervation and maintenance of type II arbors depends on activity. (**A, B, E-H**) NMJs at muscles 12 and 13 in preparations expressing mCD8-GFP in octopamine neurons and either double labeled with (**A, B**) anti-GFP and anti-HRP antibodies or (**E-H**) anti-HRP and anti-TBH antibodies in (**A**) Tdc2>mCD8-GFP**,** (**B**) Tdc2>mCD8-GFP, Kir2.1, (**E, G**) wild type, and (**F, H**) Tdc2>Kir2.1 at the (**A, B**) third, (**E, F**) first, and (**G, I**) 2nd-instar larval stage. Arrows point to type II boutons. (**M**) Percentage of intact, broken or absent (denervated) type II NMJs in Tdc2>Kir2.1, TubP-Gal80^{ts} (Kir2.1-type II, Gal80^{ts}) shifted to 29˚C at the indicated stages (N=25,24,46,22). (**C, D**) Type II motorneuron axons labeled with anti-GFP and anti-HRP (**C**) emerging from the CNS at the segmental nerves (arrows) and (**D**) terminating close to the NMJ (arrow) in GFP, Kir2.1-type II preparations stained with antibodies to HRP and GFP. (**I-L**) 3rd-instar type II NMJs from Kir2.1-type II, Gal80^{ts} shifted to 29˚C at the stages indicated in the time scale at the top of each panel, double stained with anti-HRP and anti-TBH. Arrows point to breaks in the arbors or debris. Calibration bar is 24 μm in A-B, 12 μm in D, E-H, 38 μm in C, and 10 μm in I-L.

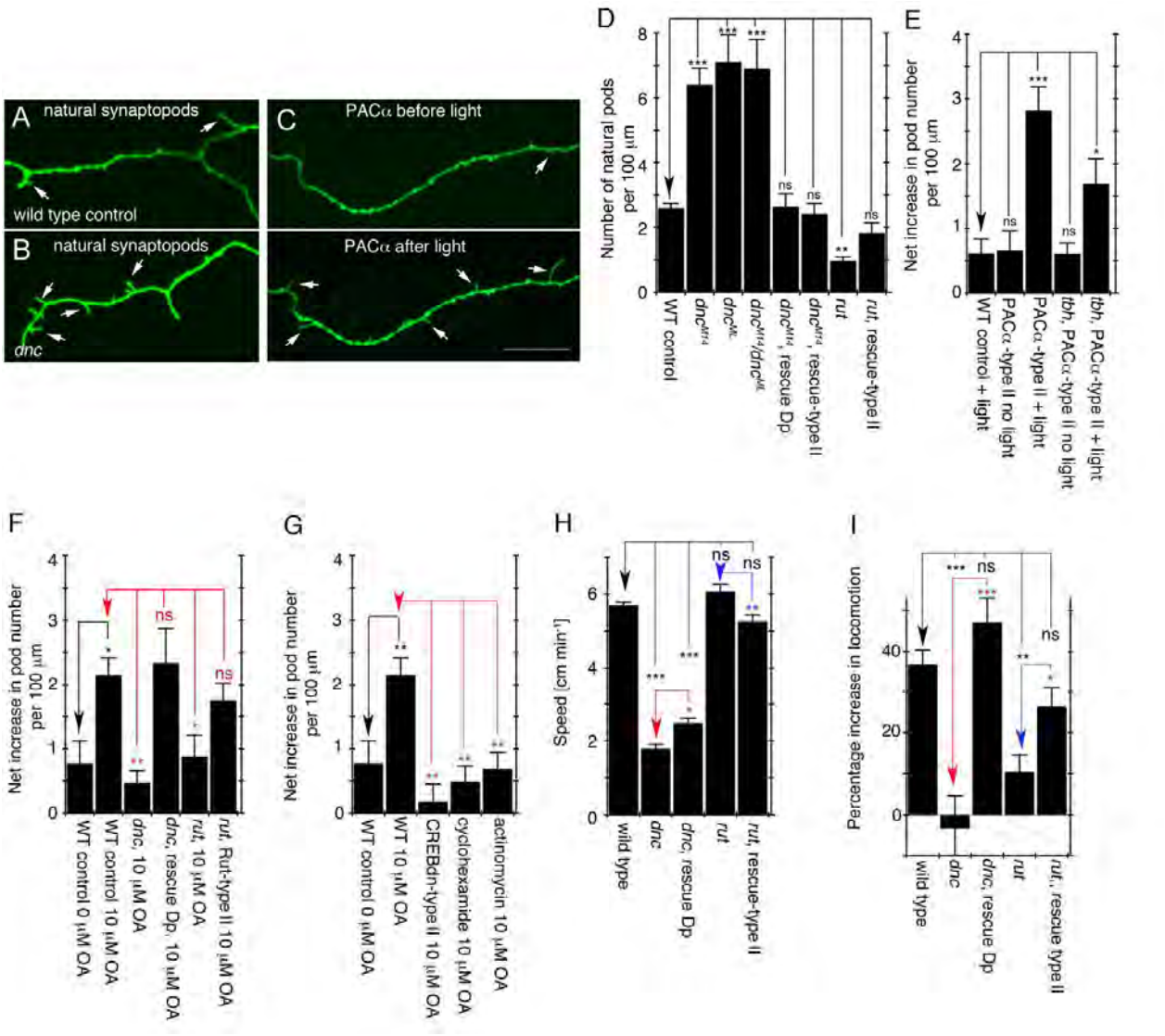

A
natural synaptopods
wild type control
B
natural synaptopods
dnc
C
PACα before light
PACα after light
D
Number of natural pods per 100 µm
WT control
dnc^M14
dnc^KK
dnc^M14/dnc^KK
dnc^M14, rescue Dp
dnc^M14, rescue-type II
rut
rut, rescue-type II
E
Net increase in pod number per 100 µm
WT control + light
PACα -type II no light
PACα -type II + light
tbh, PACα-type II no light
tbh, PACα-type II + light
F
Net increase in pod number per 100 µm
WT control 0 µM OA
WT control 10 µM OA
dnc, 10 µM OA
dnc, rescue Dp, 10 µM OA
rut, 10 µM OA
rut, Rut-type II 10 µM OA
G
Net increase in pod number per 100 µm
WT control 0 µM OA
WT 10 µM OA
CREBdn-type II 10 µM OA
cyclohexamide 10 µM OA
actinomycin 10 µM OA
H
Speed [cm min-1]
wild type
dnc
dnc, rescue Dp
rut
rut, rescue-type II
I
Percentage increase in locomotion
wild type
dnc
dnc, rescue Dp
rut
rut, rescue type II

Figure 2-5

Figure 2-5. Synaptopod extension is regulated by the cAMP pathway, and requires new protein synthesis, and CREB. (**A-C**) Live imaging of synaptopods (arrows) in (**A**) Tdc2>mCD8-GFP (WT control), (**B**) *dnc*^{M14}, Tdc2>mCD8-GFP, and (**C**) Tdc2>mCD8-GFP, PACα before and after light stimulation. (**D**) Number of natural synaptopods in the indicated genotypes (N=175,21,11,8,17,11,24,11). (**E-G**) Net increase in synaptopod number (**E**) in preparations expressing PACα in wild type and *tbh* mutant background and subjected to the indicated light paradigms (N=10,12,12,11,12), (**F**) upon application of octopamine (OA) in the indicated genotypes (N=11,13,10,11,12,8) and (**G**) in Tdc2>mCD8-GFP preparations treated with actinomycin or cycloheximide, or expressing Tdc2>CREBdn and exposed to octopamine as indicated (N=11,13,18,11,13; see Materials & Methods for details of PACα and octopamine stimulation paradigms). (**H**) Locomotor speed of the indicated genotypes (N=31,18,15,21,15). (**I**) Percentage increase in locomotor speed in response to food deprivation in the indicated genotypes (N= 26,18,15,21,15). Calibration Bar in A-C is 14 μm. Error bars represent SEM.

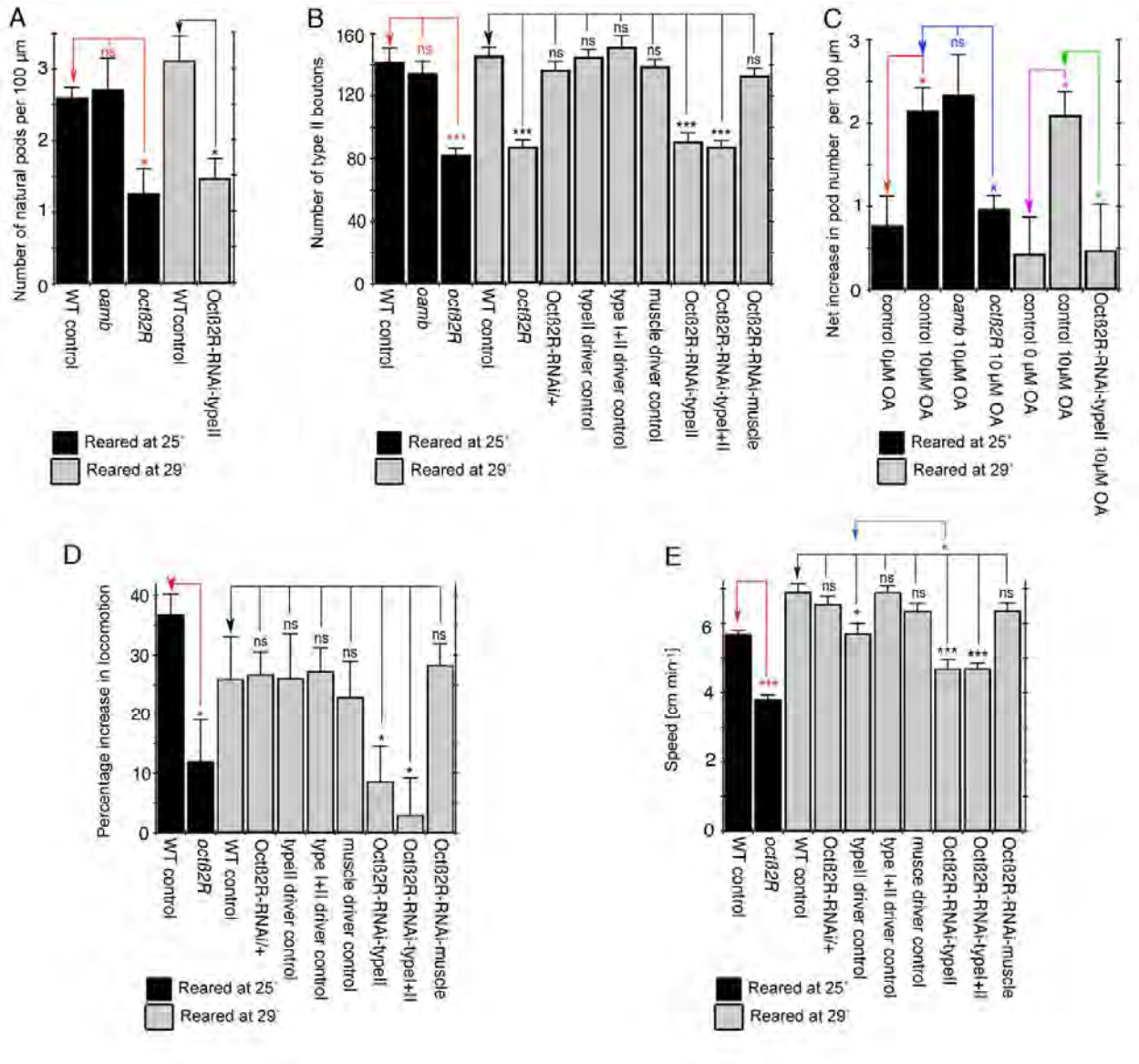

A
Number of natural pods per 100 µm
WT control
oamb
octβ2R
WTcontrol
Octβ2R-RNAi-typeII
Reared at 25°
Reared at 29°
B
Number of type II boutons
WT control
oamb
octβ2R
WT control
octβ2R
Octβ2R-RNAi/+
typeII driver control
type I+II driver control
muscle driver control
Octβ2R-RNAi-typeII
Octβ2R-RNAi-typeI+II
Octβ2R-RNAi-muscle
Reared at 25°
Reared at 29°
C
Net increase in pod number per 100 µm
control 0µM OA
control 10µM OA
oamb 10µM OA
octβ2R 10 µM OA
control 0 µM OA
control 10µM OA
Octβ2R-RNAi-typeII 10µM OA
Reared at 25°
Reared at 29°
D
Percentage increase in locomotion
WT control
octβ2R
WT control
Octβ2R-RNAi/+
typeII driver control
type I+II driver control
muscle driver control
Octβ2R-RNAi-typeII
Octβ2R-RNAi-typeI+II
Octβ2R-RNAi-muscle
Reared at 25°
Reared at 29°
E
Speed [cm min^{-1}]
WT control
octβ2R
WT control
Octβ2R-RNAi/+
typeII driver control
type I+II driver control
musce driver control
Octβ2R-RNAi-typeII
Octβ2R-RNAi-typeI+II
Octβ2R-RNAi-muscle
Reared at 25°
Reared at 29°

Figure 2-6

Figure 2-6. Presynaptic Octß2R autoreceptors, but not OAMB, regulate the growth of type II arbors. (**A**) Number of natural synaptopods in the indicated genotypes (N=175,12,13,25,14); WT control= Tdc2>mCD8-GFP. (**B**) Number of type II boutons in the indicated genotypes (N=16,12,16,16,15,11,11,10,12,12,13,12); WT control= Canton-S; typeII driver control=Tdc2>Dcr; Octß2R-RNAi –typeII=Tdc2>Dcr, Octß2R-RNAi. (**C**) Net increase in the number of synaptopods in the indicated conditions and genotypes (N=11,13,11,10,12,11,14; WT control= Tdc2>mCD8-GFP). (**D**) Percentage increase in locomotor speed in the indicated genotypes in response to food deprivation (N=26,16,13,15,13,17,15,13,13,15); (**E**) Locomotor speed in the indicated genotypes (N=31,20,17,17,15,18,17,13,13,17). For (**D, E**), WT control= Canton-S; type II driver control= Tdc2>Dcr; type I+II driver control= C380>Dcr; muscle driver control= C57>Dcr; Octß2R-RNAi-type II= Tdc2>Dcr, Octß2R-RNAi; Octß2R-RNAi-type II+II= C380>Dcr, Octß2R-RNAi; Octß2R-RNAi-muscle= C57>Dcr, Octß2R-RNAi. Error bars represent SEM.

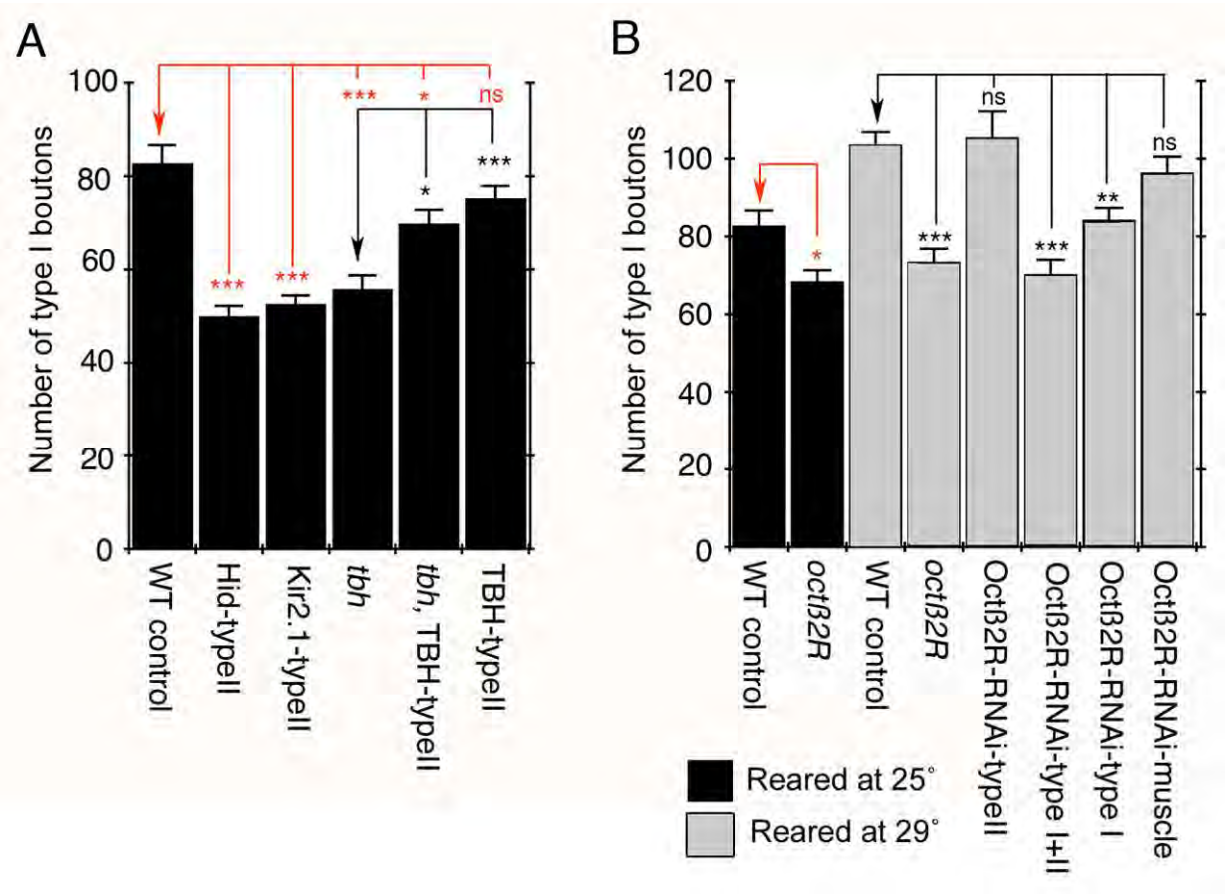
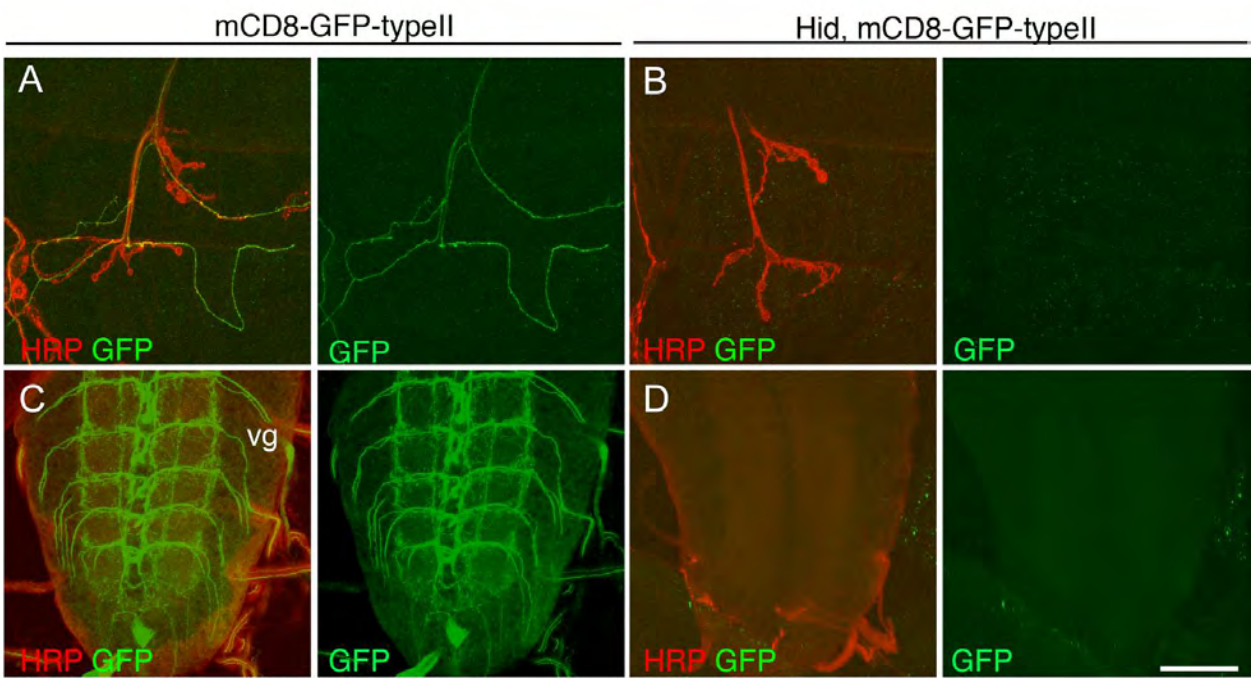

A
Number of type I boutons
100
80
60
40
20
0
***
***
***
*
ns
WT control
Hid-typeII
Kir2.1-typeII
tbh
tbh, TBH-typeII
TBH-typeII
B
Number of type I boutons
120
100
80
60
40
20
0
*
***
ns
***
**
ns
WT control
octß2R
WT control
octß2R
OctB2R-RNAi-typeII
OctB2R-RNAi-type I+II
OctB2R-RNAi-type I
OctB2R-RNAi-muscle
Reared at 25°
Reared at 29°

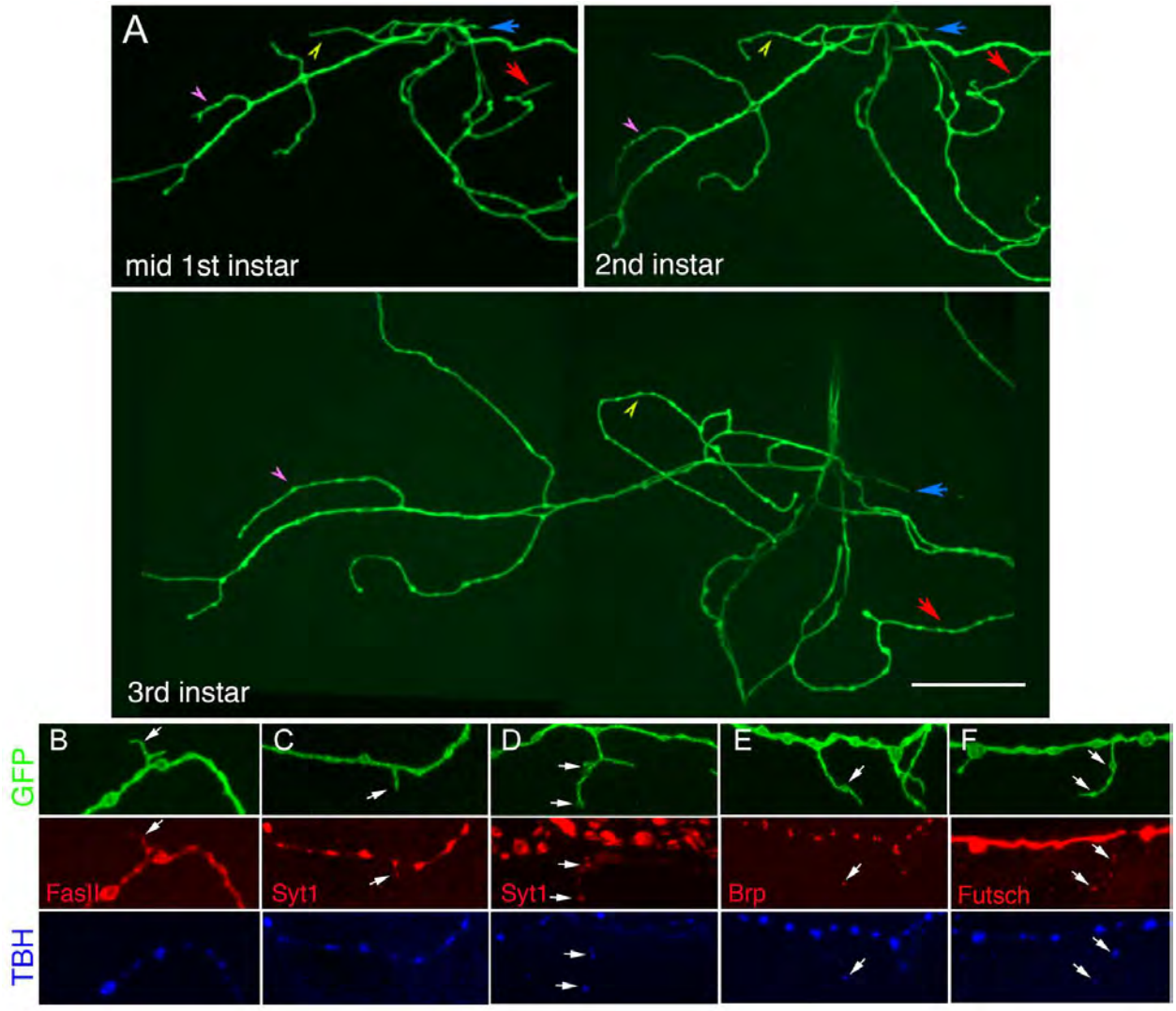
Figure 2-7

Figure 2-7. Type II motorneurons regulate the growth of type I arbors. (**A, B**) Number of type I boutons at muscle 6 and 7 (A3) in 3rd-instar larvae (**A**) upon eliminating either type II motorneurons, or octopamine production from type II motorneurons (N=12,17,21,18,20,13), and (**B**) in larvae with decreased Octß2R levels (N=12,36,17,18,12,13,11). WT control in A, B= Canton-S. Error bars represent SEM.



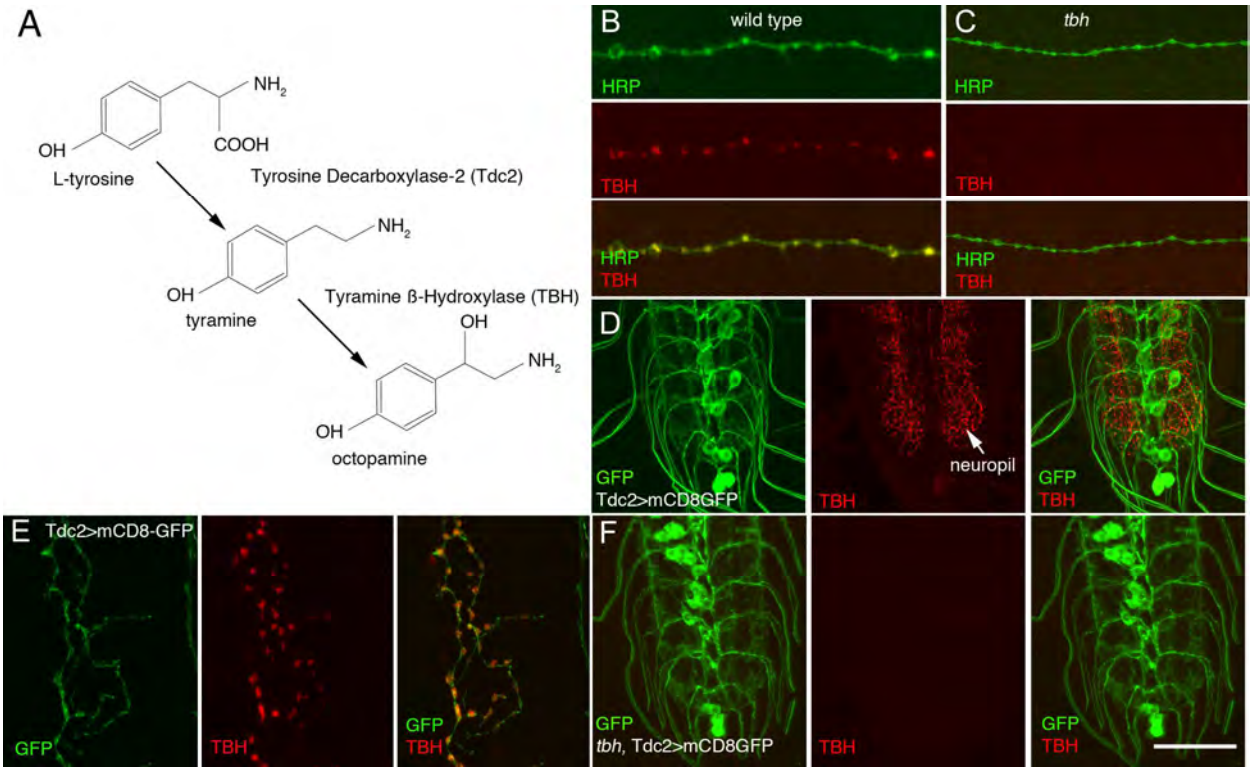
Supplemental Figure 2-1

Supplemental Figure 2-1. Hid ablation of octopamine neurons. Larval preparations labeled with anti-HRP and anti-GFP of either (**A, B**) NMJs at muscle 12 and 13 or (**C, D**) larval CNS. (**A, C**) Control animals expressing Tdc2>mCD8GFP and (**B, D**) animals expressing Tdc2>Hid. The lack of GFP signal in (**B**, **D**) demonstrates the complete loss of octopamine neurons. Calibration bar is 40 μm for **A, B**, and 50 μm for **C, D**.



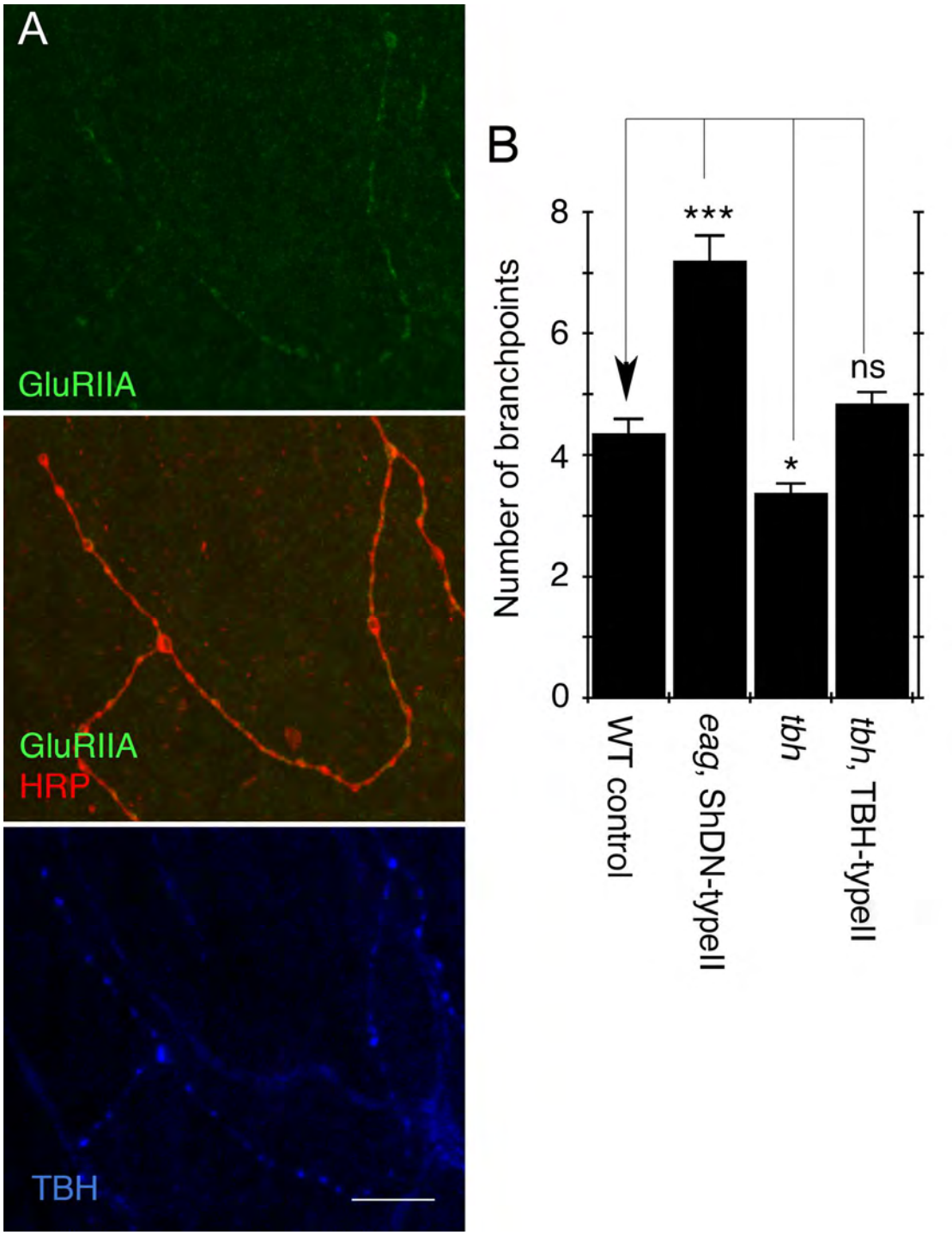
Supplemental Figure 2-2

Supplemental Figure 2-2. Imaging of synaptopods and their fate during larval development. (**A**) Time-lapse imaging of an NMJ in 1st, 2nd, and 3rd instar from an intact larva, showing synaptopods maturing into branches (arrows and arrowheads). (**B**-**F**) protein localization in maturing synaptopods in preparations triple labeled with anti-GFP, anti-TBH and antibodies to (**B**) FasII, (**C, D**) Syt1, (**E**) Brp, and (**F**) Futsch. Arrows point to sites of protein localization at synaptopods or newly formed varicosities. Calibration bar is 30 μm for **A** and 10 μm for **B-F**.



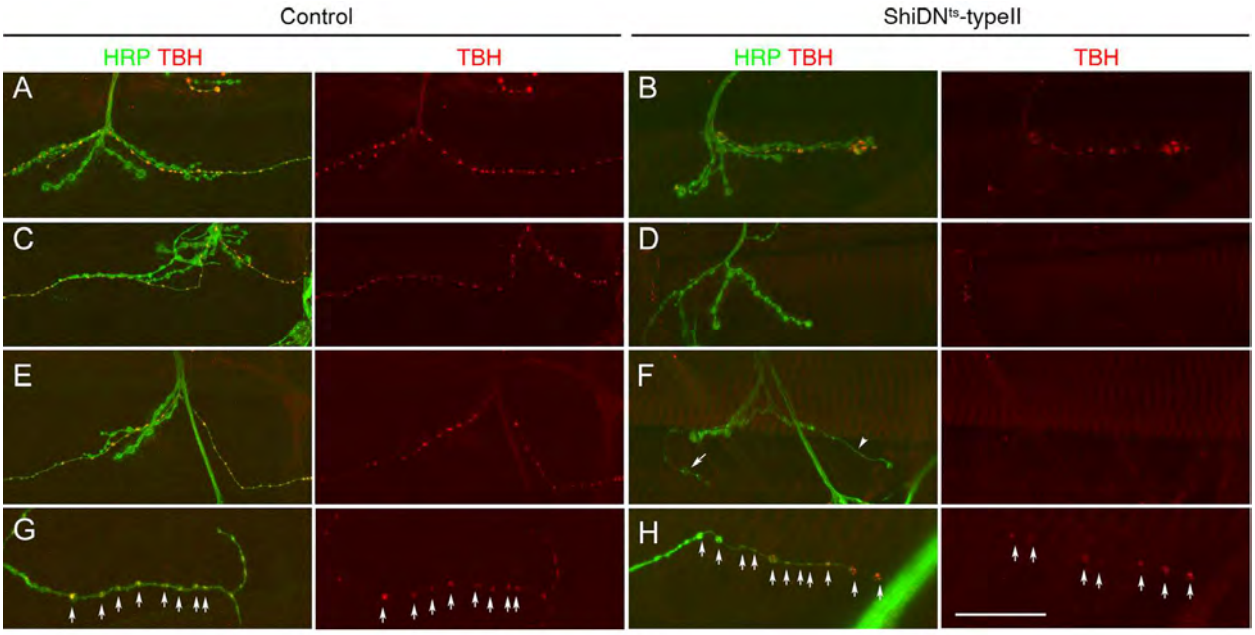
Supplemental Figure 2-3

Supplemental Figure 2-3. Tdc2- and TBH-expressing neurons in the CNS and at the NMJ. (**A**) Octopamine biosynthetic pathway. (**B, C**) Larval NMJs at muscle 12 labeled with anti-HRP and anti-TBH in (**B**) wild-type and (**C**) *tbh* mutants. (**D-F**) Larval CNS preparations double labeled with anti-TBH and anti-GFP from Tdc2>mCD8-GFP larvae in (**D**) wild-type and (**F**) *tbh* mutant. (**E**) High magnification view of the neuropil of wild-type. Calibration bar is 15 μm for **B**, **C**, **E**, and 70 μm for **D**, **F**.



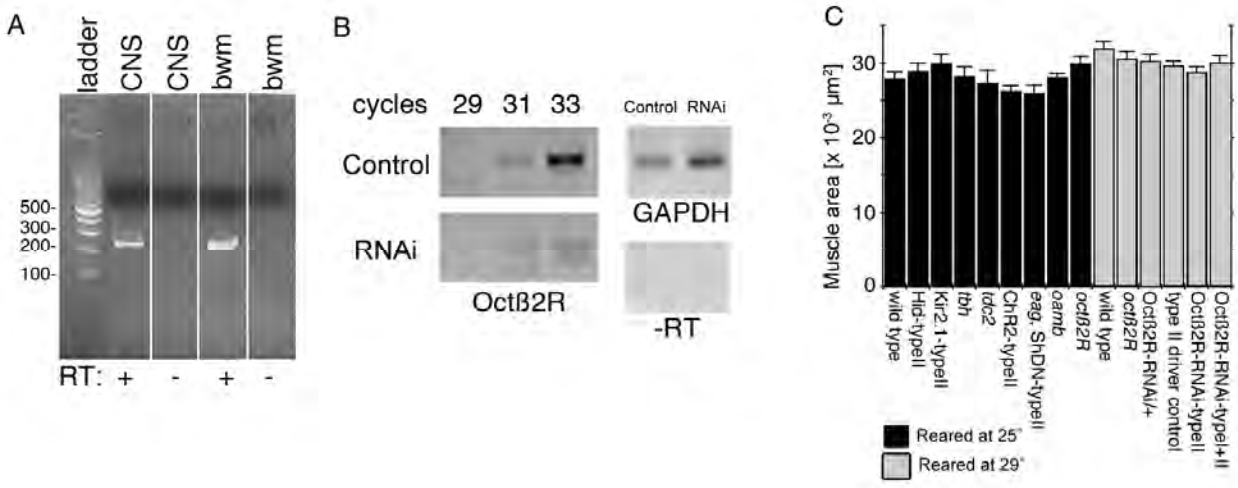
Supplemental Figure 2-4

Supplemental Figure 2-4. GluRIIA immunoreactivity around type II boutons, and quantification of branch-points in genotypes with altered synaptopod numbers. (**A**) Wild-type type II arbor labeled with anti-GluRIIA, anti-HRP, and anti-TBH. (**B**) Quantification of type II branch-points on muscle 12. For (**B**) N is 14, 10, 13, 26 respectively. Calibration bar is 10 μm.



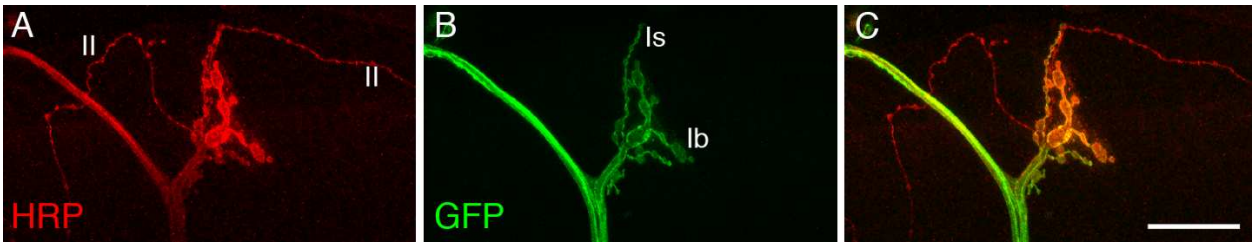
Supplemental Figure 2-5

Supplemental Figure 2-5. Expression of ShiDN^{ts} in octopamine arbors. Images of 3rd instar larval NMJs from larvae reared at 29^{o}C labeled with anti-HRP and anti-TBH at (**A-D,G-H**) muscle 12 and (**E,F**) muscle 13. (**A,C,E,G**) UAS-ShiDN^{ts} without Gal4 driver; (**B,D,F,H**) Tdc2>ShiDN^{ts} animals. Abnormal phenotypes include (**B**) shortened arbors, enlarged boutons with fragmented TBH signals, (**D**) strongly reduced innervation and lack of TBH signal, (**F**) thinned (arrowhead), shortened arbors, enlarged boutons (arrow) and lack of TBH signal, (**H**) thinned arbors and lack of TBH signals (missing arrows in H2). Calibration bar is 43 μm for **A-F**, 20 μm for **G, H**.



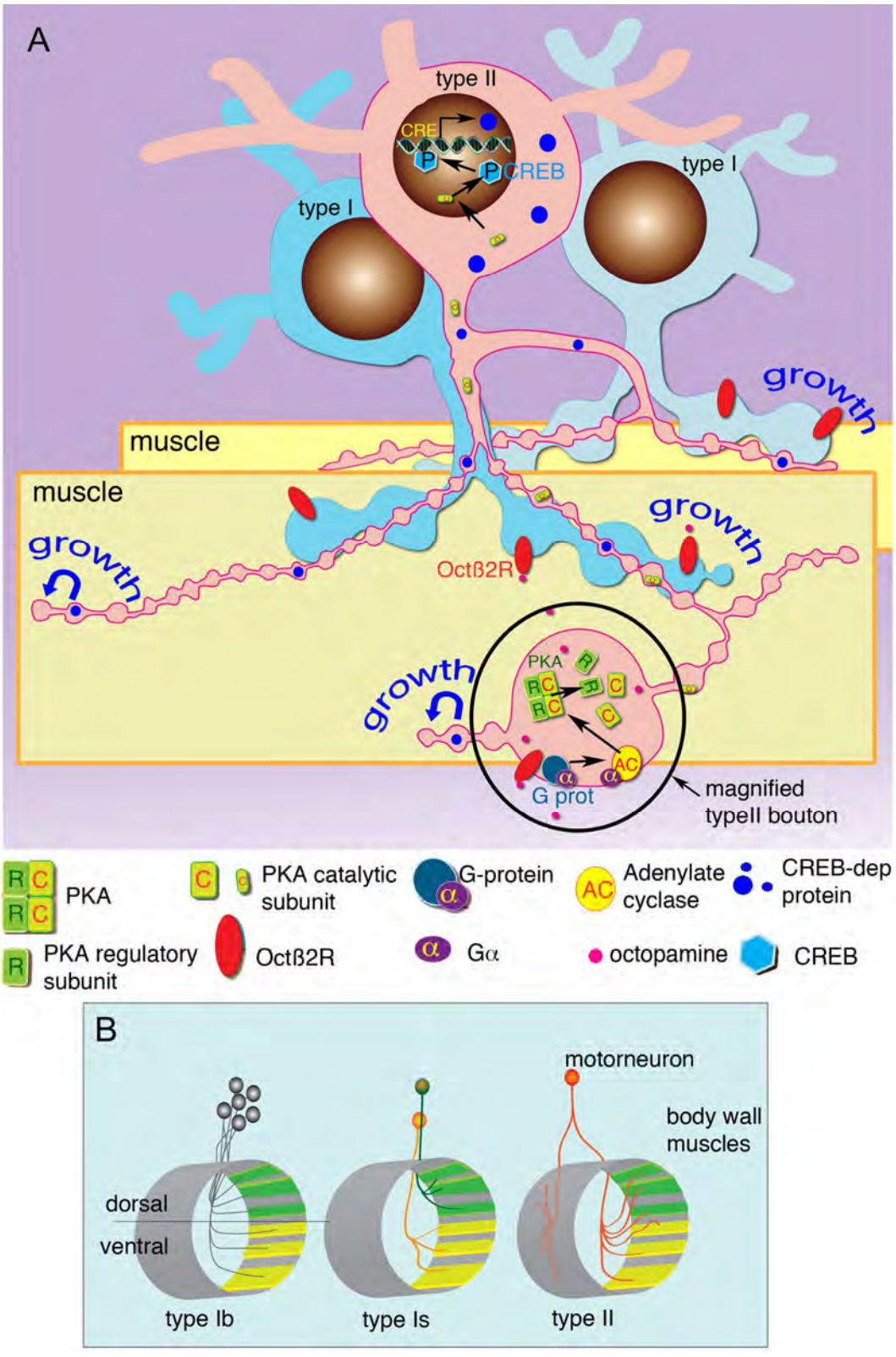
Supplemental Figure 2-6

Supplemental Figure 2-6. Expression of Octβ2R in CNS and body wall muscles, efficacy of Octβ2R-RNAi and muscle size. (**A**) PCR amplifications from +RT (with reverse transcriptase) and –RT (without reverse transcriptase) of wild-type cDNA synthesized from either dissected brains or body wall muscles. Numbers to the left of the gel are in base pairs. (**B**) Effectiveness of the Octβ2R-RNAi transgene demonstrated by semi-quantitative RT-PCR. Numbers represent number of PCR amplification rounds. Control = UAS-Octβ2R-RNAi without Gal4 driver, RNAi= C380>Octβ2R-RNAi. GAPDH is used as a cDNA loading control. (**C**) Muscle area in indicated phenotypes. N= 12, 10, 10, 10, 7, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10 respectively.



Supplemental Figure 2-7

Supplemental Figure 2-7. Expression pattern of BG439-Gal4. 3rd-instar larval NMJs from muscle 13 of segment A4 of BG439>mCD8-GFP animals co-labeled with anti-HRP and anti-GFP. Calibration bar is 27 μm.



Supplemental Figure 2-8

Supplemental Figure 2-8. Model and cartoon of different motorneuron innervations. (**A**) Model of the autoregulatory mechanism in type II arbor growth and its influence on type I arbor growth (see text for details). (**B**) Cartoon depicting different modes of body-wall muscle innervation by different motorneuron types (see text for details).

Supplemental Movie 1 (20K)

Dynamics of natural synaptopods at type II arbors.

http://www.nature.com/neuro/journal/v14/n2/extref/nn.2716-S2.mov

Supplemental Movie 2 (256K)

Synaptopods develop ball-shaped varicosities.

http://www.nature.com/neuro/journal/v14/n2/extref/nn.2716-S3.mov

Supplemental Movie 3 (112K)

Secondary synaptopod formation on a varicosity.

http://www.nature.com/neuro/journal/v14/n2/extref/nn.2716-S4.mov

Supplemental Movie 4 (1M)

Induction of synaptopod formation upon acute increase in cAMP levels.

http://www.nature.com/neuro/journal/v14/n2/extref/nn.2716-S5.mov

CHAPTER 3

Inhibitory Control of Synaptic and Behavioral Plasticity by Octopaminergic Signaling

Alex C Koon, James Ashley, Ceren Korkut & Vivian Budnik

ABSTRACT

Adrenergic receptors and their ligands are important regulators of synaptic plasticity and metaplasticity. However, the development and plasticity of the aminergic terminals for these catecholamines are poorly understood. Octopamine, the invertebrate homolog of mammalian adrenaline or noradrenaline, plays important roles in modulating behavior and synaptic functions. We previously demonstrated the excitatory effect of octopamine on synaptic growth and locomotor activity during starvation through the autoreceptor Octß2R, resulting in a positive feedback mechanism. Here, we examined the role of another autoreceptor Octß1R. We found that Octß1R is inhibitory to arbor extension, likely through Goα. The fact that it has antagonistic downstream effects to Octß2R suggests that octopamine may offer a braking mechanism to its own positive feedback effect on synaptic growth. Interestingly, disruption of Octß1R or Goα function results in defective octopamine-induced neuronal outgrowth and starvation-induced larval locomotor increase, possibly due to near-saturated cAMP levels and outgrowth mechanism. These results demonstrate the dual action of octopamine on both synaptic growth and behavioral plasticity.

INTRODUCTION

Synaptic plasticity plays an important role in behavioral changes of animals and their adaptations to the environment. Understanding the mechanism that mediates plasticity is therefore crucial. Adrenaline and noradrenaline are key regulators of synaptic plasticity and metaplasticity (Murchison et al., 2004; Hu et al., 2007; Kuzmiski et al., 2009). However, the development and plasticity of the aminergic neurons are poorly understood, and the underlying mechanisms of how adrenergic signals function at synapses are also unclear.

Octopamine, the insect counterpart of mammalian adrenaline or noradrenaline activates receptors resembling adrenergic receptors (Balfanz et al. 2005). Octopamine is a powerful behavior modulator, involves in diverse physiological processes, such as appetitive behavior (Long and Murdock, 1983; Suo et al., 2006), aggression (Hoyer et al., 2008; Zhou et al., 2008) and synaptic function (Breen and Atwood, 1983).

The *Drosophila* larval neuromuscular junction (NMJ) is a prime model system for studying synaptic plasticity (Griffith and Budnik, 2006). Apart from the type I motorneurons, which use glutamate as the primary excitatory neurotransmitter (Jan and Jan, 1976), the larval NMJ is also innervated by type II motorneurons containing octopamine (Monastirioti et al., 1995). Larval NMJs display several forms of functional and structural synaptic plasticity, such as continuous addition of varicosities during development to cope with the dramatic increase in muscle size (Schuster et al., 1996). This process depends on both retrograde and anterograde signals such as BMP (Marques and Zhang, 2006) and Wnt (Korkut and Budnik, 2009). Larval NMJs are sensitive to environmental changes for conditions such as deprivation of food, which

induces rapid increase in synaptic strength (Sigrist et al., 2003; Steinert et al., 2006). Furthermore, genetic and physiological manipulations that increase presynaptic activity promote synaptic expansion (Ataman et al., 2008; Budnik et al., 1990).

In one of our previous studies, we investigated the significance of octopaminergic type II innervation at the NMJ (Koon et al., 2011). We demonstrated that octopaminergic terminals are critical for normal synaptic growth and larval foraging behavior. Octopamine, which is present in type II terminals, is secreted in an activity-dependent manner during starvation. Octopamine binds to autoreceptor Octß2R and activates a cAMP second messenger pathway that leads to CREB activation and transcription of synaptic proteins, which in turn allows the extension of new type II endings that we termed “synaptopods”. Type II innervation also regulated the plasticity of glutamatergic type I motorneurons through Octß2Rs expressed in these neurons. However, activation of this feed forward loop could eventually lead to exponential increase of the synaptic growth and excitation, which is undesirable and unsustainable. To prevent excessive growth, a braking mechanism is necessary to reset the system.

In this study, we sought an inhibitory octopamine receptor that could play a role in this braking mechanism. We found that octopamine receptor Octß1R (also called OA2), which is also present in motorneurons, is inhibitory to synaptic growth. Octß1R likely interacts with the inhibitory G-protein Goα, which upon activation, suppresses cAMP production by adenylate cyclase. Abolishing Octß1R function or disrupting Goα function leads to synaptic overgrowth in both type I and type II motorneurons in a cell-autonomous manner. In addition, we demonstrate a genetic interaction between Octß1R and Goα, suggesting that the two molecules functions in the same pathway. The

synaptic overgrowth in *octß1r* mutants is likely due to near-saturation levels of cAMP, since it can be fully suppressed by disrupting cAMP production using either an adenylate cyclase mutation (rut^{2080}) or by overexpressing the phosphodiesterase (Dnc). Interestingly, disruption of Octß1R or Goα functions in type II motorneurons alone results in defective octopamine-induced synaptopods. This is possibly due to saturation of the system, since *octß1R* animals already have an overgrowth phenotype, and forskolin application fails to induce more synaptopods on their type II arbors. Furthermore, disrupting Octß1R or Goα functions in type II alone is sufficient to disrupt starvation-induced locomotor increase in larvae. These results are consistent with our previous findings that synaptic plasticity in type II terminals is critical for this type of behavioral plasticity.

RESULTS

Octß1R receptors are present in motorneurons

We have recently demonstrated that at the larval NMJ, octopamine-containing synaptic terminals (type II terminals) undergo structural changes in response to behavioral states that induce an increase in locomotion (Koon et al., 2011). Underlying this structural change is the activation of a positive feedback mechanism, in which octopamine release by type II synapses activates Octß2R autoreceptors (Koon et al., 2011). In turn, Octß2R turns on a cAMP- and CREB-dependent signaling cascade at type II endings, which induces synaptic growth. This positive control mechanism does not only promote the growth of type II synaptic boutons, but also functions in a paracrine fashion to stimulate the growth of type I boutons (Koon et al., 2011), primary mediators of excitatory transmission at the NMJ.

Analysis of Gal4 transcriptional reporters, 19H07-Gal4, 21E03-Gal4, 20C11-Gal4, and 20E11-Gal4, generated by fusing Gal4 to four different intronic regions of the Octß1R (a.k.a OA2) octopamine receptor in *Drosophila* (Pfeiffer et al., 2008), suggested that like Octß2R, Octß1R is also expressed in motorneurons (**Fig. 3-1A-E**). mCD8-GFP reporter signal could be observed in all bouton types, including type I and type II boutons (**Fig. 3-1A-D**). To determine if Octß2R and Octß1R serve redundant roles at the NMJ, we generated an *octß1r* mutant by FRT mediated recombination of two P-element insertions (PBac [WH]oa2[f02819] and PBac[WH]w[f06195]) (**Fig. 3-1E**). Analysis of the mutant strain demonstrated that most of the *octß1r* coding region had been removed (**Fig. S3-1**), excluding a fragment encompassing the 5' UTR and the first exon of *octß1r*, which is predicted to encode the N-terminal extracellular domain, the first transmembrane domain, and 4

amino acids of the first intracellular loop of the receptor. However, RT-PCR demonstrated the virtual absence of the predicted transcript fragment (**Fig. 3-1F**), suggesting that *octß1r* is likely a null mutant.

Octß1R receptors are negative regulators of synaptic growth

We previously demonstrated that type II NMJs expand by extending natural synaptopods, motile filopodia-like extensions observed during the expansion of type II terminals through larval development (Koon et al., 2011). Further, we found that the number of natural synaptopods is reduced in *octß2r* mutants due to an autonomous function of Octß2R in these neurons (Koon et al., 2011). To determine if Octß1R receptors had a redundant role in type II boutons, we examined natural synaptopods in *octß1r* mutants by expressing mCD8-GFP in type II motorneurons using Tdc2-Gal4 (Cole et al., 2005). Notably, the number of natural synaptopods was substantially increased in this mutant (**Fig. 3-2A, B, C**), in complete opposition to the phenotype found in *octß2r* mutants. A similar phenotype was found when Octß1R receptor was downregulated in octopaminergic neurons alone by expressing Octß1R-RNAi in octopaminergic neurons (**Fig. 3-2C**), suggesting a cell-autonomous function of the receptor. Thus, Octß1R receptors appear to negatively regulate the formation of synaptopods at type II terminals. Consistent with this role, the number of type II boutons was also increased both in *octß1r* mutants and in larvae expressing Octß1R-RNAi in octopaminergic neurons (**Fig. 3-2D, E, F**).

The inhibitory function of Octß1R is likely mediated by Goα

Synaptopod formation is downstream of elevated cAMP levels, mediated by octopamine-dependent activation of Octß2Rs (Koon et al., 2011) a G-protein-coupled receptor. Octß1R is also predicted to function as a G-protein-coupled receptor (Balfanz et al., 2005; Evans and Maqueira, 2005). Therefore we wondered whether the negative regulation of type II synaptic growth by Octß1R could be mediated through activation of G-protein inhibitory subunits, such as Goα or Giα (Johnston et al., 2003; El-Armouche et al., 2003). This hypothesis was first examined by bath applying pertussis toxin (PTX), which in *Drosophila* specifically inhibits Goα (Thambi et al., 1989), and determining its consequences on octopamine-dependent synaptopod formation. As previously reported, application of 10 µM octopamine to wild type control NMJs resulted in a significant increase in the number of synaptopods (**Fig. 3-3A, C**). Application of 10 µM octopamine in conjunction with 1.5µg/ml PTX dramatically enhanced this effect (**Fig. 3-3B, C**). This is consistent with the idea that activation of Goα partially inhibits octopamine-dependent synaptopod formation. In support of this interpretation, the number of natural synaptopods was substantially increased in larvae expressing UAS-PTX in octopamine neurons throughout larval development (**Fig. 3-3D, E, G**).

To corroborate an involvement of Goα in inhibiting synaptopod formation, we expressed two different Go-RNAi constructs in octopaminergic neurons, Go-RNAi1 and Go-RNAi2, and examined the number of natural synaptopods at type II arbors in 3rd instar larvae. In agreement with our model, downregulating Goα by either RNAi resulted in significant increase in the number of natural synaptopods (**Fig. 3-3F, G**).

In contrast, downregulating Giα by using two different Gi-RNAi constructs was without effect (**Fig. 3-3G**).

Since synaptopods are precursors of type II boutons (Koon et al., 2011), we also expected that inhibiting or downregulating Goα function should result in abnormal type II bouton growth. Indeed, expressing PTX or the two Go-RNAi constructs in octopaminergic neurons resulted in significant increase in the number of type II boutons (**Fig. 3-3H**).

To further test the hypothesis that Octß1R mediates inhibition via Goα, we looked for a genetic interaction between the two molecules. We found that heterozygous *octß1R* and *goα* (*go[007]*) mutations result in a synergistic effect of synaptic overgrowth in both natural synaptopods and type II boutons (**Fig. 3-3I,J**). Together, these results suggest that Octß1R receptors inhibit the growth of type II endings via inhibitory G-protein Goα. Disrupting Goα or Octß1R leads to increased cAMP production, which in turn results in increased synaptopods and boutons.

<u>Octß1R function is antagonized by changes in cAMP levels but its function is partially independent from Octß2R</u>

If Octß1R receptors inhibit cAMP production, consistent with the above model, then decreasing cAMP levels by an independent approach should suppress the effects of *octß1r* mutants. This hypothesis was tested by examining the effect of synaptopod formation in animals lacking the adenylate cyclase, Rutabaga (Rut), or by overexpressing the phosphodiesterase, Dunce (Dnc) in the *octß1r* mutant background. Consistent with the above hypothesis, both conditions prevented the increase in synaptopods elicited by mutations in *octß1r* (**Fig. 3-4A**). Similarly, they

also prevented the increase in the number of type II boutons (**Fig. 3-4B**). Since the resulting phenotypes are no difference from *rut* alone or overexpressing Dnc alone, it is likely that Rut and Dnc are downstream components of the same pathway.

We previously demonstrated that Octß2R receptors promote the formation of synaptopods and the expansion of type II terminals by facilitating cAMP production (Koon et al., 2011). However, other G-protein-coupled receptors, in addition to Octß2R receptors, may also regulate cAMP levels. To determine if Octß1R receptors function by inhibiting the action of Octß2R receptors, we generated *octß1r;octß2r* double mutants by recombination on the third chromosome. The double mutation was verified by genomic PCR (data not shown). If Octß1R and Octß2R antagonize each other, then null mutations in *octß2r* should completely suppress the effect of mutations in *octß1r* in synaptopod formation. However, we found that in the double mutant, the number of natural synaptopods was intermediate, and significantly different between the reduced number of natural synaptopods in *octß2r* mutants and the increased number of natural synaptopods in *octß1r* mutants (**Fig. 3-4C**). Similar results were obtained in the number of type II boutons (**Fig.3-4D**). These observations suggest that Octß1R receptors function, at least in part, independently from Octß2R receptors.

octß1r mutants likely have near-saturated levels of cAMP

We also examined the effect of octopamine application in synaptopod formation at type II endings in *octß1r* mutants and in larvae expressing Go-RNAi, Gi-RNAi or UAS-PTX in octopaminergic neurons. No response to octopamine was observed at type II boutons in *octß1r* mutants, when Goα was downregulated, or when PTX was

expressed throughout larval development in octopaminergic neurons (**Fig. 3-5A**). In contrast, the response to octopamine when Giα was downregulated was normal (**Fig. 3-5A**). A likely explanation for the lack of response to octopamine in *octß1r* mutants or when expressing either Go-RNAi or UAS-PTX, is that in these conditions the number of natural synaptopods is substantially increased (**Fig. 3-4A**). It is possible that synaptopod formation has reached saturation in these animals, which would occlude a further increase in number by octopamine application. This interpretation is supported by studies of *dunce* mutants. *Dunce* encodes a cAMP-specific phosphodiesterase, and thus when mutated it results in significant increase in cAMP levels and thus a drastic increase in the number of natural synaptopods, likely to saturation (Koon et al. 2011). As in the above strains, in *dnc* mutants there was not significant response to octopamine (**Fig. 3-5A**).

If it is true that *octß1r* has near-saturated levels of cAMP, then increasing cAMP levels in these mutants should not result in further increase of synaptopods. To test this hypothesis, we bath-applied opened living larval preparations of control animals and *octß1r* mutants in 10 μM forskolin to increase their cellular cAMP. In control animals, forskolin resulted in an increase of synaptopods similar to that produced by octopamine. In contrast, this increase is abolished in *octß1r* mutants (**Fig. 3-5B**). In our previous study, we demonstrated that *octß2r* mutants are also defective in octopamine-induced synaptopod increase, similar to that of *octß1r* mutants (Koon et al., 2011). However, *octß2r* mutants have an undergrowth phenotype, which is opposite to that of *octß1r*. In addition, in contrast to *octß1r* mutants, *octß2r* mutants have normal response to forskolin. These results further support the notion that *octß1r* mutants have near-saturated levels of cAMP.

octß1r mutants are defective in starvation-induced locomotor increase

Since *octß1r* mutants have near-saturated levels of cAMP, Octß2R in these mutants apparently cannot further increase cAMP in response to octopamine. In our previous study, we showed that the autoregulatory mechanism of octopamine neurons through Octß2R is necessary for normal locomotor increase in response to starvation (Koon et al., 2011). We therefore hypothesized that *octß1r* may have similar behavioral deficits as those of *octß2r*. As we expected, *octß1r* failed to respond to starvation by increasing locomotor speed (**Fig. 3-5C**). The same defect was also observed in animals expressing UAS-PTX or Go-RNAi1 in octopaminergic neurons, but not Gi-RNAi1 (**Fig. 3-5C**). This result indicates that defective Octß1R signaling likely leads to defective Octß2R signaling, which is required for normal starvation-induced behavior in larva. It also confirms our previous findings that octopaminergic signaling in octopaminergic neurons is necessary for this type of behavioral plasticity. Interestingly, *octß1r; octß2r* double mutants are still defective in the starvation response (**Fig. 3-5C**), even though they have wild type levels of natural synaptopods and boutons. This is probably because they still lack the excitatory Octß2R, which is the receptor necessary for this behavioral response.

Octß1R receptors cell-autonomously inhibit type I synaptic growth

We have previously demonstrated that the undergrowth of type II boutons result in parallel defects in the growth of type I boutons, implicating type II terminals regulating type I growth (Koon et al., 2011). Therefore, we seek to determine if overgrowth of type II would result in overgrowth of type I. We examined type I bouton

numbers in *octß1r* mutants and animals with disrupted Goα functions in type II motorneurons by expressing UAS-PTX or Go-RNAi. *octß1r* indeed has a type I overgrowth phenotype (**Fig. 3-6A, B**). But disrupting Goα functions in type II alone did not result in increased type I boutons (**Fig. 3-6B, C**). Nevertheless, hampering Goα functions in type I & II using a type I- & II-specific driver (C380-Gal4) did result in increase of type I boutons. This suggests that Octß1R functions in a cell-autonomous fashion at type I boutons to regulate synaptic growth, similar to our previous finding with Octß2R, but in the opposite manner (Koon et al., 2011). In addition, similar to our findings in synaptopods and type II boutons, the genetic interaction between Octß1R and Goα was also observed in type I, suggesting that the Octß1R inhibits synaptic growth through Goα in these terminals as well, similar to the mechanism in type II.

DISCUSSION

We previously demonstrated that octopamine regulates synaptic and behavioral plasticity through an autoregulatory positive feedback mechanism involving Octß2R that promotes both type I and type II outgrowth (Koon et al., 2011). In this study, we have identified an octopamine receptor, Octß1R, with antagonist functions to Octß2R, which may serve to break the positive feedback mechanism. We demonstrated that this receptor inhibits the cAMP pathway via the inhibitory G-protein Goα. Loss of Octß1R or Goα function results in synaptic overgrowth in type I and type II motorneurons in a cell-autonomous manner. Defective Octß1R signaling apparently results in near-saturation of cAMP levels, and this obscures the function of Octß2R of increasing cAMP. Thus, the loss of Octß1R function results in insensitivity of octopaminergic neurons to octopamine stimulation. This in turn abolishes starvation-induced behavioral changes that require Octß2R signaling.

Octopamine receptors have been shown to elicit intracellular Ca^{2+} and/or cAMP increase (Han et al, 1998; Balfanz et al., 2005). OAMB, the α-adrenergic-like receptor in *Drosophila*, has been implicated to function via Ca^{2+} signaling in the *Drosophila* oviduct (Lee et al., 2009). Interestingly, OAMB was not found to be expressed in the oviduct muscle cells, but in the oviduct epithelium (Lee et al., 2009). Since octopamine induces relaxation in the dissected oviduct, it was suggested that another octopamine receptor could be present in the oviduct muscles, eliciting an inhibitory effect on cAMP production (Lee et al., 2009). Our work here demonstrates that the inhibitory octopamine receptor

Octß1R is capable of decreasing cAMP levels in motorneurons. It is possible that Octß1R is also expressed in oviduct muscle cells, mediating muscle relaxation.

In apparent contradiction to our findings, a previous study has shown that Octß1R (a.k.a. OA2) is capable of increasing cAMP (Balfanz et al., 2005). In this study, HEK293 cells transfected with Octß1R were exposed to different concentrations of octopamine and assayed for changes in cAMP levels (Balfanz et al., 1005). It was found that Octß1R enhanced cAMP synthesis. A likely problem of the above experimental design is that the overexpression of Octß1R might alter its binding to G-proteins. For instance, mammalian ß2-adrenergic receptors are known to couple to both Gs and Gi/o proteins (Xiao, 2001). The disruption of inhibitory G-proteins by pertussis toxin significantly enhances ß2-adrenergic receptors-mediated contractile response in mammalian cardiomyocytes, suggesting that this receptor is capable of pairing with inhibitory G-protein Gi or Go (Xiao et al., 1995). However, overexpression of ß2-adrenergic receptors in the murine heart constitutively couples the receptor to Gs (Milano et al., 1994; Bond et al., 1995) but not to Gi/o protein. High or medium overexpression of ß2-adrenergic receptors in transgenic mice was demonstrated to co-precipitate with Gs but not Gi/o proteins in the absence of agonist (Gurdal et al., 1997). Thus, overexpression might significantly alter the normal physiological condition of these receptors. In addition, HEK293 cells originate from human embryonic kidneys, whereas Octß1R is from *Drosophila*. The coupling of an insect octopamine receptor to mammalian G-proteins does raise questions of physiological relevance.

Goα (a.k.a Brokenheart) is expressed in the nervous system of *Drosophila*, with markedly increased during the development of axonal tracts (Guillen et al., 1991). The level of Goα proteins is altered in memory mutants including *dunce* and *rutabaga* (Guillen et al., 1990). Furthermore, Goα was found to be necessary in associative learning (Ferris et al., 2006). When PTX is overexpressed in the mushroom bodies of adult *Drosophila*, memory formation is severely disrupted (Ferris et al., 2006), suggesting a role of Goα in synaptic plasticity. However, as the name “Brokenheart” suggests, homozygous *goα* mutants are lethal due to defective development of the heart (Fremion et al., 1999), rendering this mutant difficult to be used in studies of the larval NMJ. Moreover, overexpression of inhibitory G-proteins has been shown to sequester available Gß and Gγ subunits, resulting in undesired downregulation of other G-protein signaling (Katanayeva et al., 2010). Therefore, overexpression is not a desirable method to study the function of Goα. Fortunately, the use of pertussis toxin and multiple RNAi constructs allowed us to downregulate Goα functions cell-specifically, developmentally or acutely in the larva, permitting us to examine synaptic overgrowth. Moreover, genetic interaction was also found between heterozygous *octß1r* and heterozygous *goα* mutations, indicating that the two molecules act in the same signaling pathway to inhibit synaptic growth. These results provided strong evidence of Goα’s involvement in synaptic plasticity.

In summary, our studies reveal that, apart from excitation, octopamine can also elicit inhibition on synaptic growth. Having the flexibility to convert from excitatory effects

to inhibitory effects allows octopamine to exert a broader level of control over synaptic functions and behavior.

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MATERIALS AND METHODS

Fly strains. Flies were reared in standard *Drosophila* medium at 25°C except where indicated. Animals used in RNAi experiments were reared at 29°C to increase knockdown efficiency, but were incubated at 25°C for 1 hour prior to experiments. The following stocks were used: the wild-type strain Canton-S (CS), Tdc2-Gal4 (Bloomington Stock Center), 19H07-Gal4, 20C11-Gal4, 20E11-Gal4, 21E03-Gal4 (Pfeiffer et al., 2008), UAS-mCD8-GFP (Bloomington), UAS-PTX (Ferris et al., 2006), *y dnc^{M14} cv v f* (Bloomington), UAS-Dnc (remobilized to the 2nd chromosome) (Cheung et al., 1999), *rut^{2080}* (Bloomington), *goα [007]* (Fremion et al., 1999), UAS-Dcr2 (Bloomington), Pbac{WH}oa2[f02819] (Bloomington), Pbac{WH}w[f06195] (Harvard Exelixis), Pbac{WH}Octß2R[f05679] (Bloomington), w[1118]; Df(3R)Exel6191, P{w[+mC]=XP-U}Exel6191 (Bloomington), C380-Gal4 (Budnik et al., 1996), UAS-Octß1R-RNAi (110537; Vienna *Drosophila* RNAi Center), UAS-G(o)-RNAi (110552 & 19124; Vienna *Drosophila* RNAi Center) (110552 is Go-RNAi1 and 19124 is Go-RNAi2), UAS-G(i)-RNAi (28510; Vienna *Drosophila* RNAi Center. JF01608; Transgenic RNAi Project, Harvard Medical School) (28510 is Gi-RNAi1 and JF01608 is Gi-RNAi2).

Generation and analysis of octβ1R mutants. octβ1r mutants were generated using Pbac{WH}oa2[f02819] and Pbac{WH}w[f06195] which contain piggyback-based transposons in the same orientation on the third chromosome. A heat-shock Flipase was crossed into the first chromosome to induce recombination between the two transposons and to excise the flanked DNA. The heat-shock processes were performed at 37°C for 30 min during 1st instar and 2nd instar larval stages respectively. The heat-shock Flipase was then crossed out of the first chromosome to obtain a stable mutant.

Genomic PCR was performed to verify the desired deletion. Primers used to verify the deletion were GTCATGCGGCACCGGAAATTG (against genomic DNA) paired with CCTCGATATACAGACCGATAAAAC (WH3'); and CTAAAGTGCATTGCACCTGG (against genomic DNA) paired with TCCAAGCGGCGACTGAGATG (WH5'). Negative control primers (against Octβ1R genomic sequence) to verify deletion were ACAGGAGCGTCTGGTGTAC paired with CGGAGTGATGCAACTATCGC, TGTCAAGCGCACAGAACTC paired with GCGTTGGTTGGTTCCAAGG, and AGTGCTGTACAGTAGCGAGC paired with CCTGACTCCATGACACCTAAATATG. RT-PCR was performed by extracting total mRNA from dissected larval body wall muscles and larval brains using a combination of Trizol (Invitrogen) and the RNeasy kit (Qiagen). Synthesis of cDNA for +RT reactions was performed using the Superscript III kit (Invitrogen), where –RT reactions lacked reverse transcriptase. The +RT and –RT reactions were then used for PCR using forward primer CCGCCTGGCAACGAGTAAC and reverse primer CTCGTCGATGAGCCCGTC. These primers were specifically designed to recognize all known splice variants of Octß1R, and across an exon-intron junction to avoid false signal from any contaminating genomic DNA.

Immunocytochemistry: Larval body-wall muscles were dissected and fixed for 15 minutes in 4% paraformaldehyde. For tyramine-β-Hydroxylase (TBH) immunocytochemistry, samples were fixed in Bouin's fixative. Antibodies and their concentrations were: anti-TBH 1:400 (Koon et al., 2011), anti-HRP-Dylite594 1:500 (Jackson Immunoresearch). Secondary antibodies conjugated to either FITC or Dylite594 (Jackson) were used at a concentration of 1:200. Imaging of fixed preparations was described (Ataman et al., 2008).

Animal rearing conditions for synaptopod analysis: All animals used in synaptopod analysis carried a copy of Tdc2-Gal4 and a copy of UAS-CD8:GFP to visualize the type II terminals. Egg collection was done in standard 25 mm diameter cornmeal/agar/molasses food vials at 25^{o}C with approximately 60% humidity. Larvae were grown at low density. Wandering late 3rd-instar larvae were used for experiments.

Stimulation procedures and live imaging of dissected preparations: Synaptopods were imaged from live preparations as described in (Koon et al., 2011). For octopamine stimulation, larvae were dissected in HL3 saline (Stewart et al., 1994) containing 0.1 mM Ca^{2+} and preparations gently glued onto a custom-made glass imaging chamber using surgical glue. Then, identified NMJs were imaged on an Improvision spinning disc confocal microscope (PerkinElmer) with a C9100-13 Hamamatsu cooled EM-CCD camera and using a 40X; 1.2 NA objective, with a 2.4X optical zoom. After imaging for less than 30 min, animals were partially unglued to allow muscles to contract freely, and 10 µM octopamine in HL3 containing 1.5 mM Ca^{2+} was then applied for 15 min followed by 5 x 15 min washes with 0.1 mM Ca^{2+} HL3 saline before imaging again. For experiments involving pertussis toxin (PTX) application, dissected living larval prep was pre-incubated in HL3 containing 0.1 mM Ca^{2+}, 0.03% DMSO, 30 µM ATP and 1.5 µg/ml PTX (purchased from Sigma) for 2 hours. Then, HL3 containing 1.5 mM Ca^{2+}, 10 µM octopamine, 0.03% DMSO, 30 µM ATP and 1.5 µg/ml PTX was applied for 15 min, followed by 5 x 15min washes with the pre-incubation HL3. Control animals were pre-incubated, stimulated and washed in the same way but without PTX.

Crawling assay and starvation assay: Details of crawling and starvation assays were described in (Koon et al., 2011). Synchronized mid 3rd-instar larvae were washed with

water, and individually loaded onto a 3% agar plate. Animals were allowed a pre-run of 25 sec on the agar before manual recordings were made for 1 minute. Experiments were carried out in a 25˚C, 60% humidity behavioral room under red light. For starvation assay, larvae were maintained in food or food-free moisturized 35 mm Petri dishes for 2 hrs, and then subjected to the crawling assay. N represents one animal. For RNAi experiments, food vials rearing animals at 29^{o}C were incubated at 25^{o}C for 1 hour prior to crawling assay or starvation assay.

Quantification of boutons and synaptopod number: Type I boutons number was obtained at muscles 6 and 7 of abdominal segment A3, while type II bouton number was quantified at muscle 12 in A3. For muscle area measurements the muscle length and width was measured using an ocular scale bar. Measurements of synaptopod number were from muscles 12 of segment A4 in dissected preparations. Number of synaptopods in the histograms represents the total number of synaptopods per 100 μm of each arbor. Synaptopods were defined as such if they measured 0.5 μm or more. N represents number of NMJs. At most two NMJs were quantified per animal.

Statistical analysis For comparisons between more than 2 sample groups an analysis of variance (ANOVA) with Tukey post-hoc test was performed. For pair-wise comparisons a Student t-test was used. Numbers in histograms represent mean± SEM. *** is p< = 0.0001, ** is p< = 0.001, * is p<0.05. Unless otherwise noted, sample number (N) represents one synaptic arbor for anatomical measurements, or one animal for behavioral analyses. Statistical analysis for animals reared at 25^{o}C and animals reared at 29^{o}C are calculated separate.

Genotype abbreviations: Type II driver control is Tdc2/+. Type I+II driver control is C380-Gal4/+. [transgene]-type II is Tdc2-Gal4>[transgene]. [transgene]-type I+II is C380-Gal4>[transgene], unless otherwise indicated.

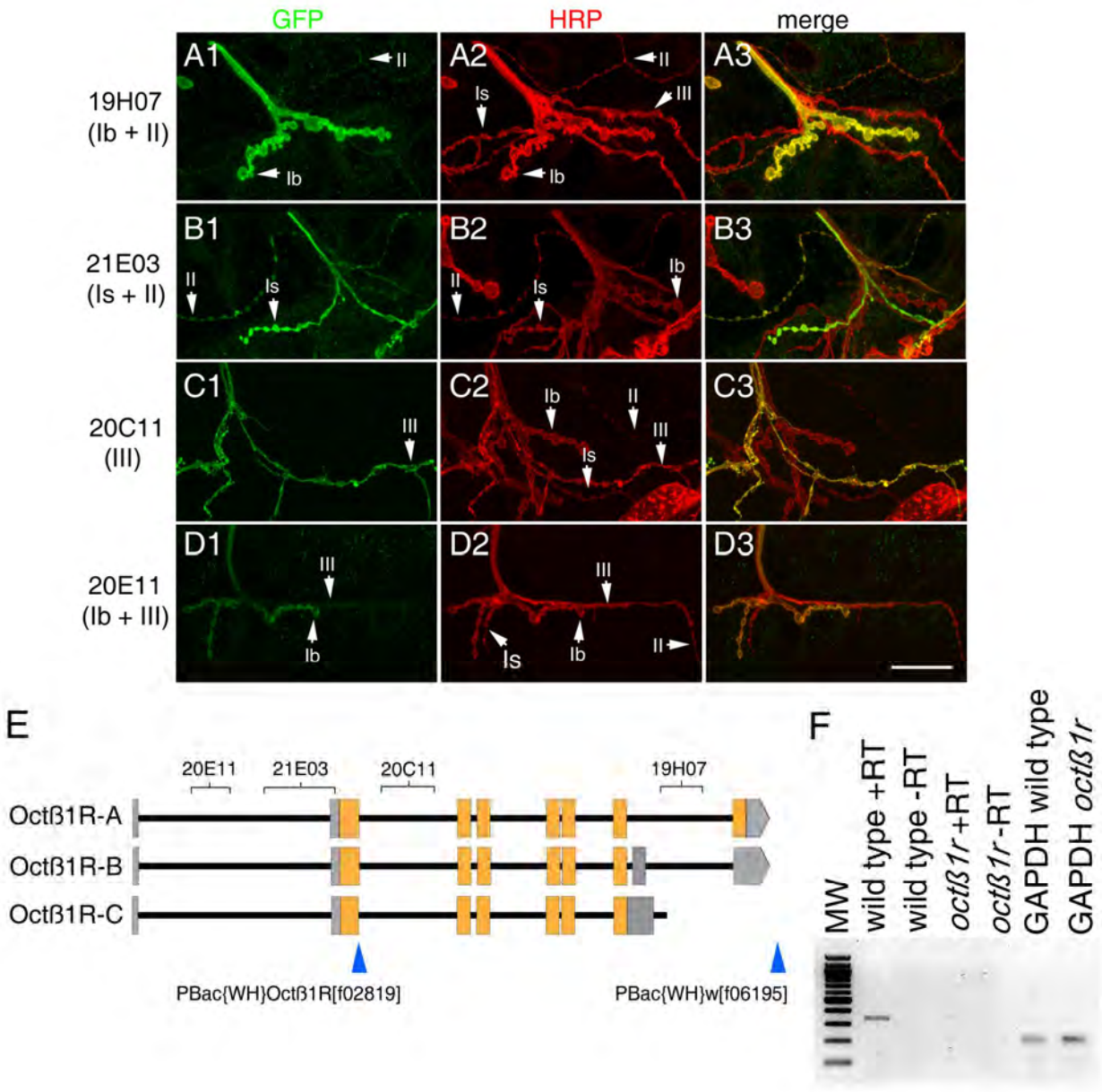

GFP
HRP
merge
A1
A2
A3
19H07
(Ib + II)
II
Is
III
Ib
B1
B2
B3
21E03
(Is + II)
C1
C2
C3
20C11
(III)
D1
D2
D3
20E11
(Ib + III)
E
20E11
21E03
20C11
19H07
OctβR-A
OctβR-B
OctβR-C
PBac{WH}Octβ1R[f02819]
PBac{WH}w[f06195]
F
MW
wild type +RT
wild type -RT
octβ1r+RT
octβ1r-RT
GAPDH wild type
GAPDH octβ1r

Figure 3-1

Figure 3-1. Expression patterns of Octß1R-Gal4 transcriptional reporters.
(**A-D**) NMJs at muscle 12 of a third instar larva expressing mCD8-GFP using four different Octß1R-Gal4s generated from different intronic regions of the Octß1R locus. As shown by white arrows, (**A**)19H07-Gal4 has expression in type Ib and type II boutons, (**B**) 21E03-Gal4 – type Is and type II, (**C**) 20C11-Gal4 – type III, and (**D**) 20E11-Gal4 – type Ib and type III. Labeled with anti-HRP (**A2, B2, C2, D2**) and anti-GFP (**A1, B1, C1, D1**). Calibration bar is 23 µm. (**E**) Schematic diagram of the Octß1R locus showing the locations of different intronic regions used to generate the Gal4 transcriptional reporters (Pfeiffer et al., 2008). The diagram also shows the location of the two Pbac insertions used for generating the *octß1r* mutant. Orange = exon. Grey = non-coding UTRs. Thin black line = intron. (**F**) RT-PCR of whole wild type animals versus *octß1r* mutants. Minimal expression of the remaining 5'UTR and exon was detected in *octß1r* mutants. +RT: reverse transcription reactions with reverse transcriptase added; -RT: reverse transcriptase absent.

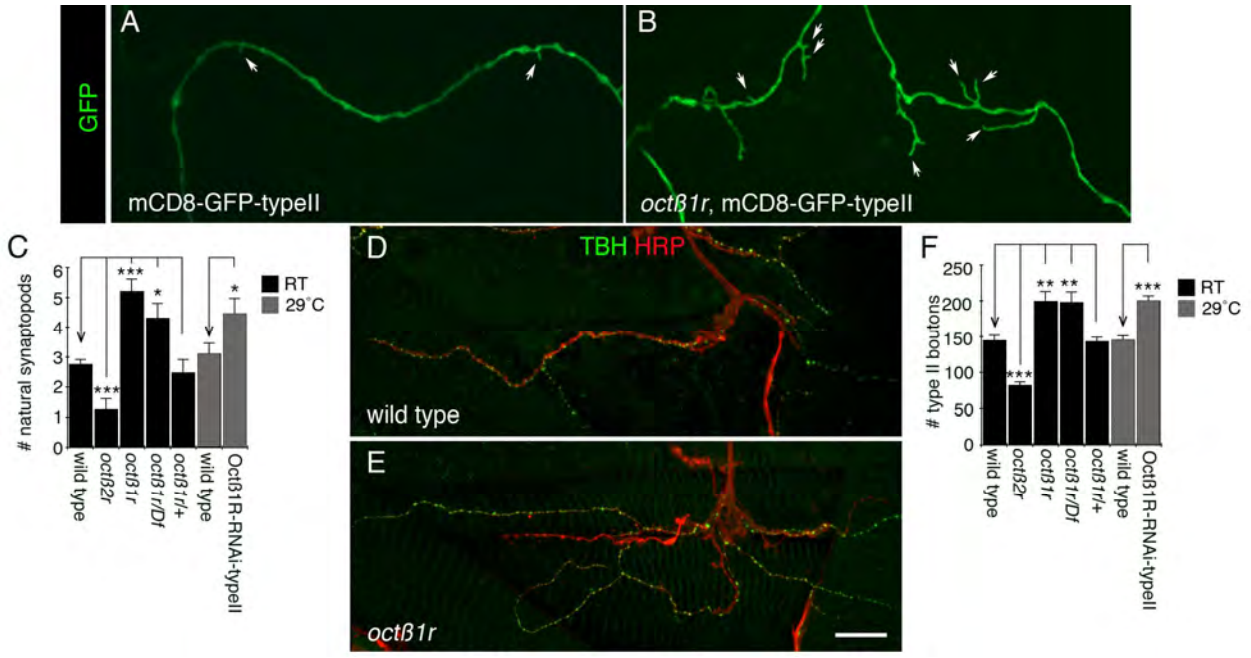

A
GFP
mCD8-GFP-typeII
B
octβ1r, mCD8-GFP-typeII
C
natural synaptopods
RT
29°C
wild type
octβ2r
octβ1r
octβ1r/Df
octβ1r+
wild type
Octβ1R-RNAi-typeII
D
TBH HRP
wild type
E
octβ1r
F
type II bouton
RT
29°C
wild type
octβ2r
octβ1r
octβ1r/Df
octβ1r+
wild type
Octβ1R-RNAi-typeII

Figure 3-2

Figure 3-2. *octß1r* mutants have a synaptic overgrowth phenotype.
(**A**) type II arbors of a control animal (**B**) type II arbors of an *octß1r* animal showing marked increase of synaptopods (white arrows). (**C**) Quantification of number of natural synaptopods per 100 µm arbor in *octß1r* mutants and animals expressing Octß1R-RNAi in type II motorneurons (N=175,13,21,10,11,25,17). NMJs at muscles 12 of a third instar larva of (**D**) wild type and (**E**) *octß1r* . Labeled with anti-HRP (red) and anti-TBH (green). (**F**) Quantification of the number of type II boutons on muscle 12 of the above animals (N=22,17,15,11,16,20,11). RT: Reared at 25^{o}C. 29^{o}C: Reared at 29^{o}C. Animals used in RNAi experiments were reared at 29^{o}C to increase knockdown efficiency. Calibration bar is 8 µm in (**A,B**) and 20 µm in (**D,E**).

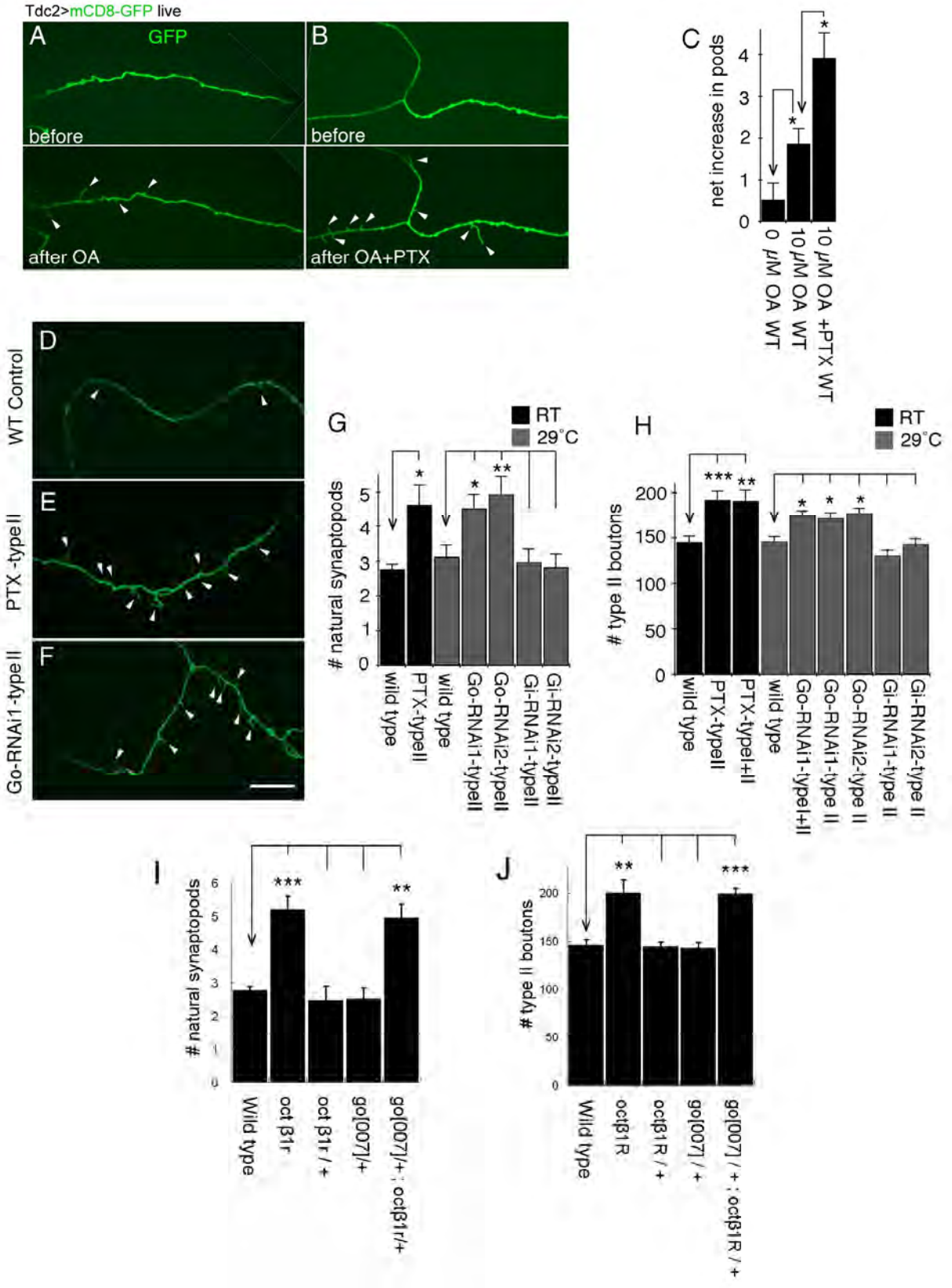

Tdc2>mCD8-GFP live
A
GFP
before
after OA
B
before
after OA+PTX
C
net increase in pods
0 μM OA WT
10 μM OA WT
10 μM OA +PTX WT
D
WT Control
E
PTX -type II
F
Go-RNAi1-type II
G
RT
29°C
natural synaptopods
wild type
PTX-typeII
wild type
Go-RNAi2-typeII
Go-RNAi1-typeII
Gi-RNAi1-typeII
Gi-RNAi2-typeII
H
RT
29°C
type II boutons
wild type
PTX-typeII
PTX-typeI+II
wild type
Go-RNAi1-type II
Go-RNAi2-type II
Gi-RNAi1-type II
Gi-RNAi2-type II
I
natural synaptopods
Wild type
oct β1r
oct β1r / +
go[007]/+
go[007]/+ ; octβ1r/+
J
type II boutons
Wild type
octβ1R
octβ1R / +
go[007] / +
go[007] / + ; octβ1R / +

Figure 3-3

Figure 3-3. Disruption of Goα phenocopies synaptic overgrowth of *octß1r*

(**A**) Bath application of octopamine increases the number of synaptopods on type II arbors. (**B**) The presence of pertussis toxin (PTX) results in significantly higher increase of synaptopods by octopamine stimulation. (**C**) Quantification of the net increase of synaptopods in (**A**) and (**B**) per 100µm arbor (N=18,13,16). (**D-F**) Natural synaptopods in (**D**) control, (**E**) animals expressing PTX in type II, and (**F**) animals expressing Go-RNAi1. (**G**) Quantification of natural synaptopods in animals expressing PTX, Go-RNAi or Gi-RNAi (N=175,1219,10,11,10). (**H**) Quantification of type II boutons in animals expressing PTX, Go-RNAi or Gi-RNAi (N=22,16,12,20,24,16,16,10,16). (**I**) Quantification of natural synaptopods of transheterozygotes of *octß1r* and *goα* (N=175,21,11,10,11), (**J**) Quantification of type II boutons of the animals in (**I**) (N=22,15,16,12,16). RT: Reared at 25^{o}C. 29^{o}C: Reared at 29^{o}C. Animals used in RNAi experiments were reared at 29^{o}C to increase knockdown efficiency. Calibration bar is 12 µm in (**A,B,D-F**).

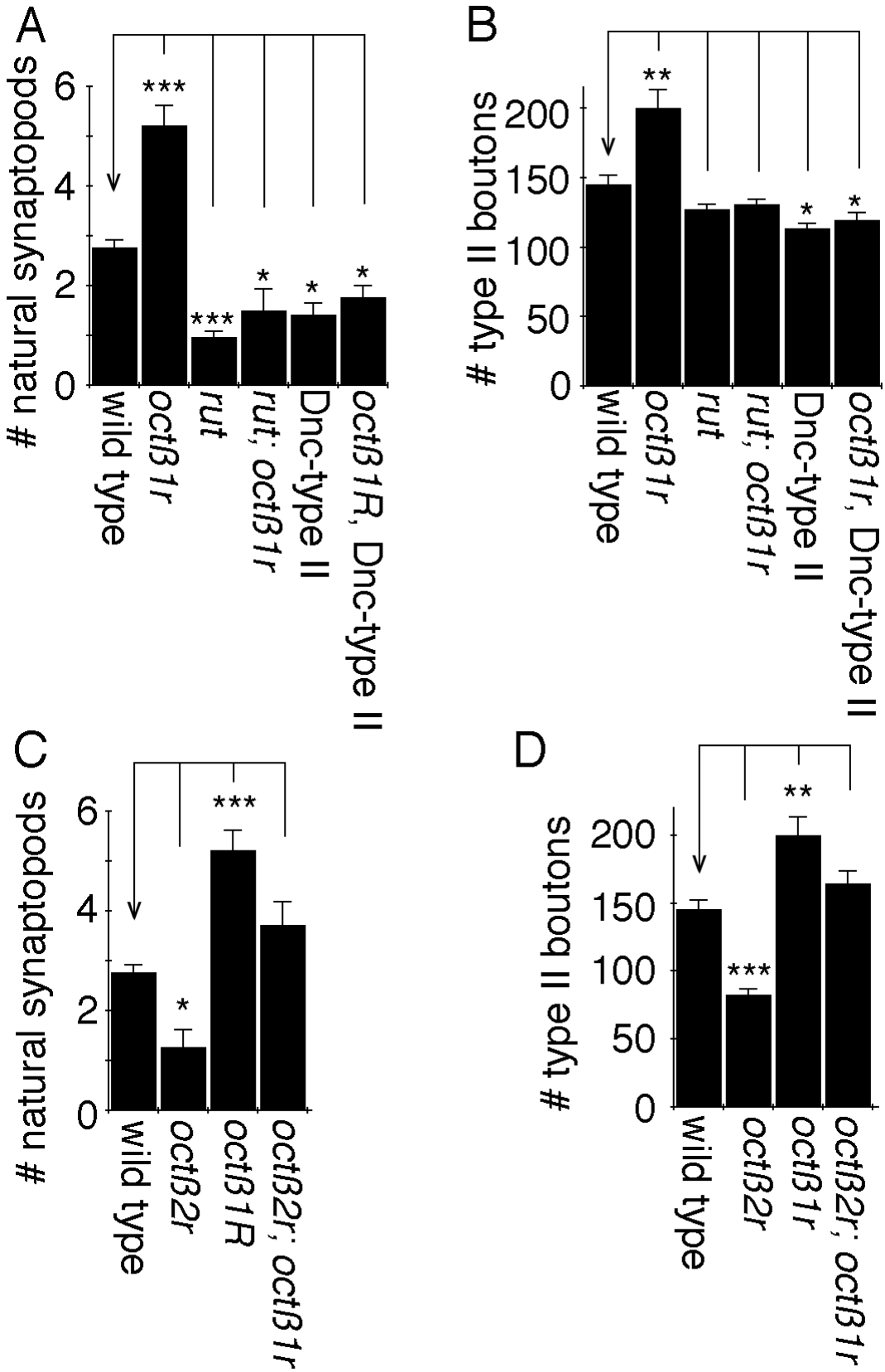

A
natural synaptopods
0
2
4
6
***
***
*
*
*
wild type
octβ1r
rut
rut; octβ1r
Dnc-type II
octβ1R, Dnc-type II
B
type II boutons
0
50
100
150
200
**
*
*
wild type
octβ1r
rut
rut; octβ1r
Dnc-type II
octβ1r, Dnc-type II
C
natural synaptopods
0
2
4
6
*
***
wild type
octβ2r
octβ1R
octβ2r; octβ1r
D
type II boutons
0
50
100
150
200
***
**
wild type
octβ2r
octβ1r
octβ2r; octβ1r

Figure 3-4

Figure 3-4. Suppression of the overgrowth phenotype of *octß1r* by reducing cAMP. (**A,B**) *rut* mutation or overexpression of Dnc fully suppress the synaptic overgrowth phenotype, supporting the hypothesis that Rut and Dnc are downstream components of the Octß1R signaling pathway. (**C,D**) Octß1R and Octß2R appear to function partially independently. (**A,C**) Quantification of synaptopods. (**B,D**) Quantification of type II boutons. (N=175,21,24,13,14,13 in **A**. N=22,15,18,18,12,12 in **B**. N=175,13,21,12 in **C**. N=22,17,15,16 in **D**.)

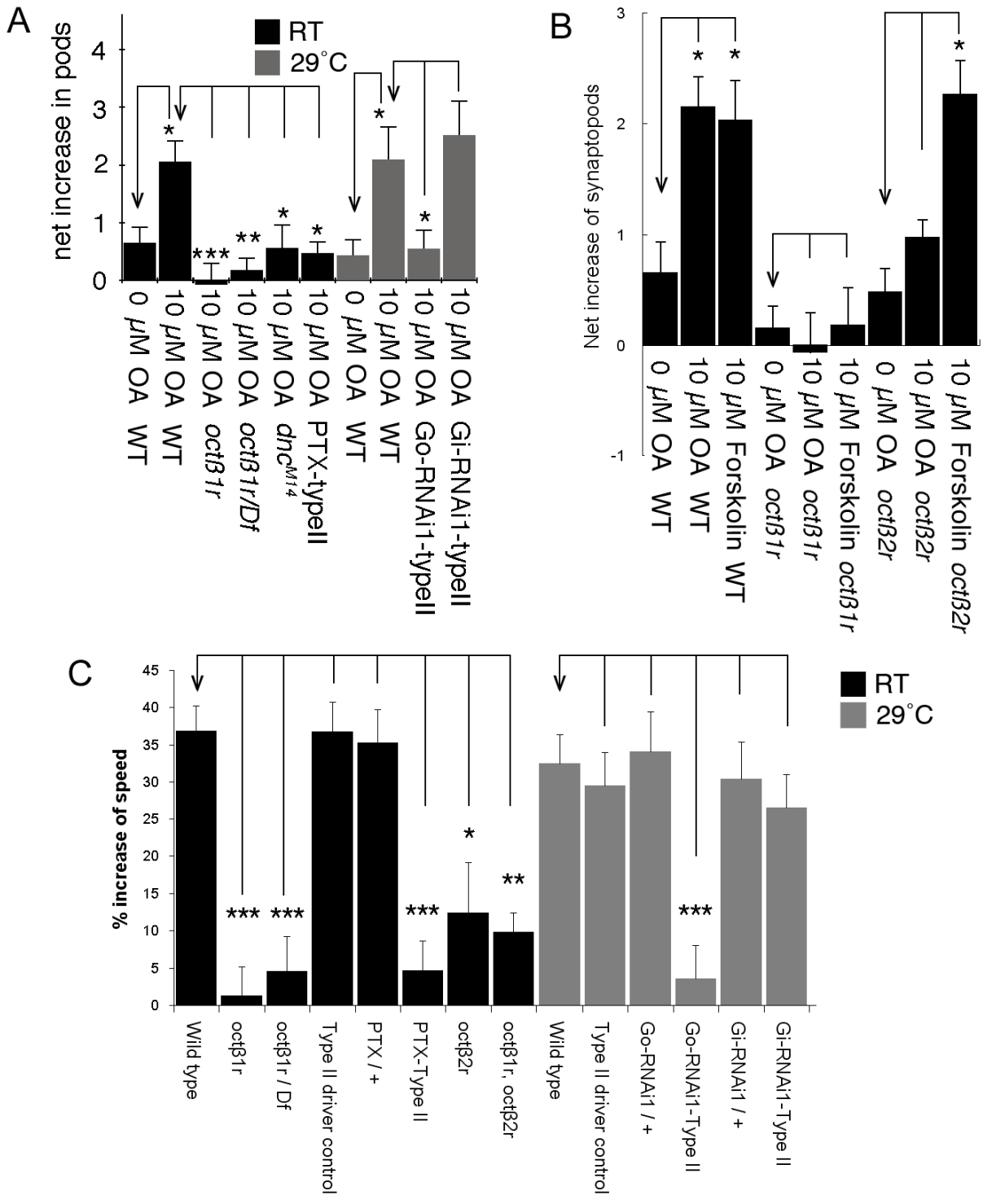

A
net increase in pods
RT
29˚C
0 μM OA WT
10 μM OA WT
10 μM OA octβ1r
10 μM OA octβ1r/Df
10 μM OA dncM14
10 μM OA PTX-typeII
0 μM OA WT
10 μM OA WT
10 μM OA Go-RNAi1-typeII
10 μM OA Gi-RNAi1-typeII
B
Net increase of synaptopods
0 μM OA WT
10 μM OA WT
10 μM Forskolin WT
0 μM OA octβ1r
10 μM OA octβ1r
10 μM Forskolin octβ1r
0 μM OA octβ2r
10 μM OA octβ2r
10 μM Forskolin octβ2r
C
% increase of speed
RT
29˚C
Wild type
octβ1r
octβ1r / Df
Type II driver control
PTX / +
PTX-Type II
octβ2r
octβ1r, octβ2r
Wild type
Type II driver control
Go-RNAi1 / +
Go-RNAi1-Type II
Gi-RNAi1 / +
Gi-RNAi1-Type II

Figure 3-5

Figure 3-5. *octß1r* mutation or disruption of Goα results in near-saturation levels of cAMP and defective starvation-induced behavior. (**A**) Bath application of octopamine increases synaptopods in control animals, but not in *octß1r*, *dnc* and animals expressing PTX or Go-RNAi. Net increase of synaptopods is quantified per 100um arbor (N=14,13,12,10,10,11,12,11,19,10). (**B**) Bath application of octopamine fail to increase synaptopods in both *octß1r* and *octß2r*, whereas bath application of forskolin increases synaptopods in *octß2r* but not in *octß1r* (N=14,13,11,10,12,13,11,13,10). (**C**) Percentage increase of larval crawling speed in response to 2 hr starvation in wild type, *octß1r*, *octß2r*, *octß1r;octß2r* double mutant, and animals expressing PTX, Go-RNAi or Gi-RNAi in octopaminergic neurons (N=26,16,16,15,16,16,16,16,17,13,19,16,16,17). RT: Reared at 25^{o}C. 29^{o}C: Reared at 29^{o}C. Animals used in RNAi experiments were reared at 29^{o}C to increase knockdown efficiency.

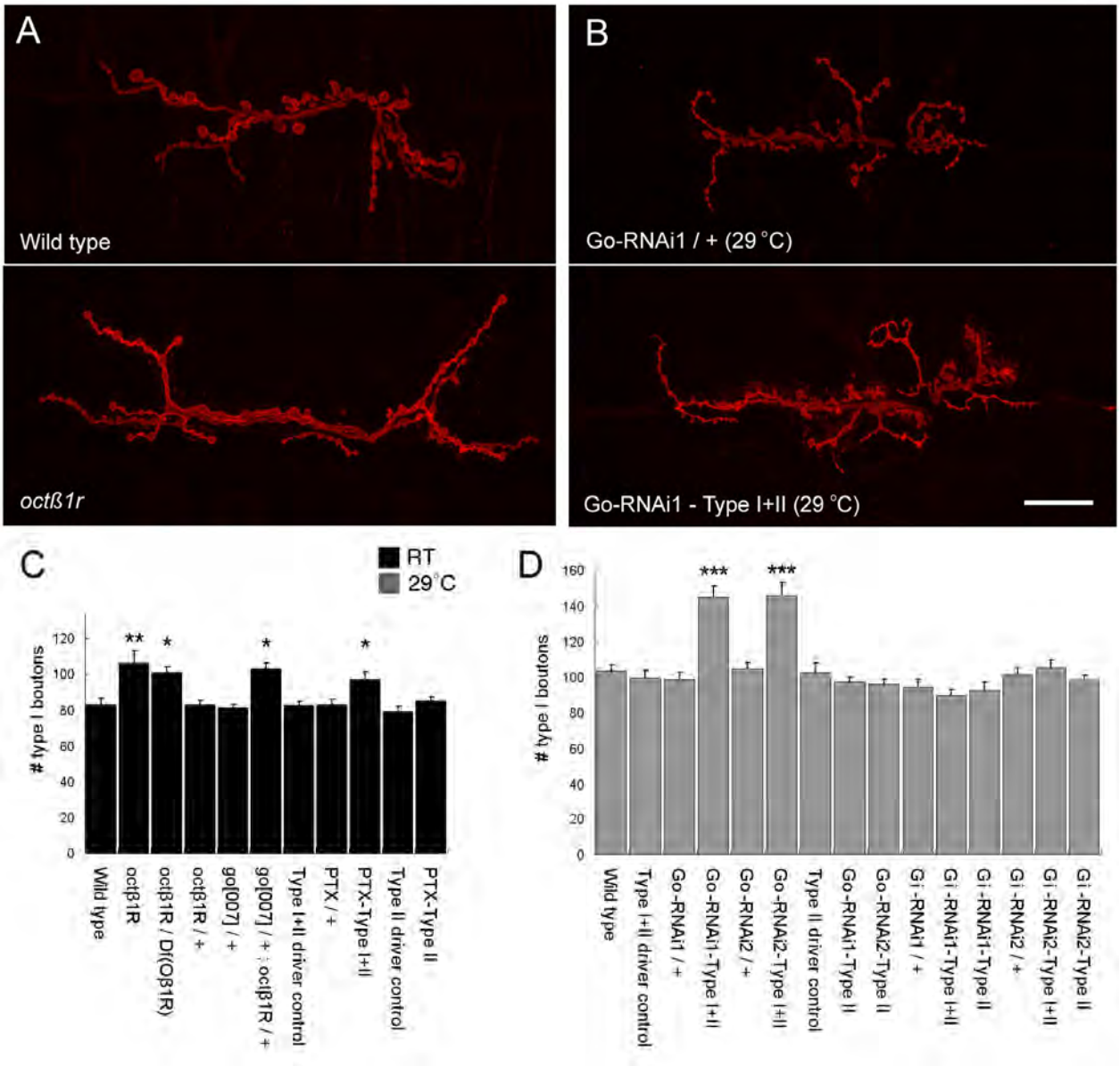
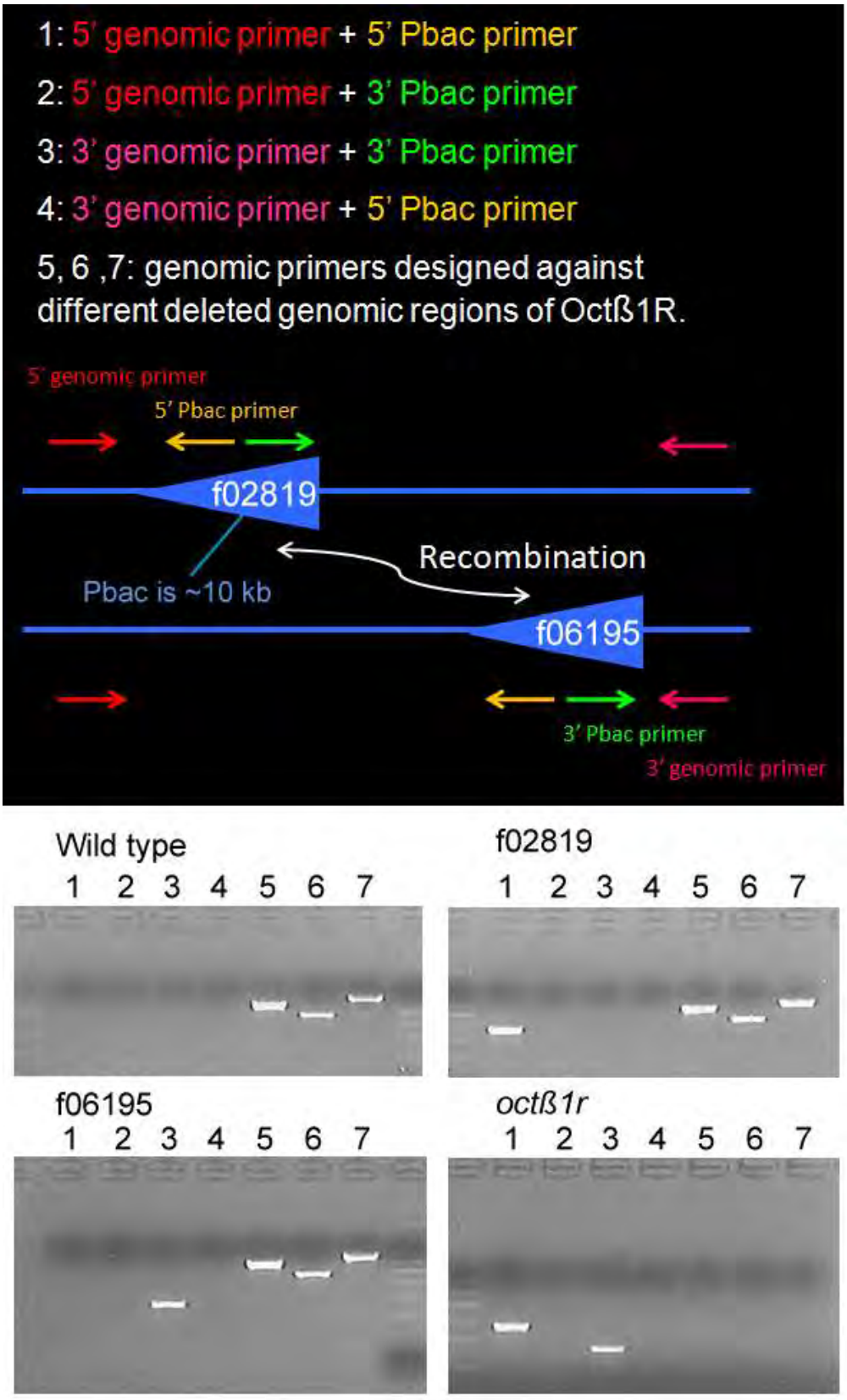

A
Wild type
B
Go-RNAi1 / + (29 °C)
octß1r
Go-RNAi1 - Type I+II (29 °C)
C
RT
29°C
type I boutons
Wild type
octβ1R
octβ1R / Df(Oβ1R)
octβ1R / +
go[007] / +
go[007] / + ; octβ1R / +
Type I+II driver control
PTX / +
PTX-Type I+II
Type II driver control
PTX-Type II
D
type I boutons
Wild type
Type I+II driver control
Go-RNAi1 / +
Go-RNAi1-Type I+II
Go-RNAi2 / +
Go-RNAi2-Type I+II
Type II driver control
Go-RNAi1-Type II
Go-RNAi2-Type II
Gi -RNAi1 / +
Gi -RNAi1-Type I+II
Gi -RNAi1-Type II
Gi -RNAi2 / +
Gi -RNAi2-Type I+II
Gi -RNAi2-Type II

Figure 3-6

Figure 3-6. Octß1R regulates type I synaptic growth (**A-B**) NMJ at muscles 6/7 of segment A3 of (**A**) wild type and *octß1r* mutant, and (**B**) Go-RNAi/+ control and Go-RNAi-Type I+II. *octß1r* and Go-RNAi-Type I+II have increased type I boutons comparing to their corresponding controls. Preparations labeled with anti-HRP. (**C-D**) Quantification of total type I bouton numbers from muscle 6/7 of segment A3. (**C**) Total bouton numbers of wild type, combinations of *oct1ßr* mutants, *goα* mutants and larvae with neuronal-specific expression of Pertussis toxin (PTX). *octß1r* and *goα* showed genetic interaction in the increase of type I boutons (N=18,15,16,16,12,18,14,14,16,12,14). (**D**) Total bouton numbers from controls and Go- or Gi-RNAi lines (N=15,14,12,16,12,16, 11,16,16,12,14,10,12,16,16). RT: Reared at 25^{o}C. 29^{o}C: Reared at 29^{o}C. Animals used in RNAi experiments were reared at 29^{o}C to increase knockdown efficiency. Calibration bar is 10 μm in (**A-B**).



Supplemental Figure 3-1

Supplemental Figure 3-1. Verification of *octß1r* mutant by genomic PCR Primer pair #1 verifies the presence of Pbac[f02819]. Primer pair #2 is negative control for primer pair #1. Primer pair #3 verifies the presence of Pbac[f06195]. Primer pair #4 is negative control for primer pair #3. Primer pair #5, 6 and 7 verifies the presence of Octß1R. *octß1r* is positive for both #1 and #3, indicating a successful recombination event. It is negative for #5-7, indicating that the deleted Octß1R fragment is not present in the genome.

CHAPTER 4

General Discussion

Aminergic modulation of synaptic and behavioral plasticity has been known for decades. However, the underlying mechanisms are still unclear. In fact, studies that successfully bridge across the role of aminergic regulation in synapses and behavior are sparse. A likely explanation is that the circuitry underlying behavioral modulation by aminergic signaling is still poorly defined. One might be able to identify a neural circuit responsible for a particular behavior and study how octopamine affects it, but the knowledge of this circuit might not have sufficient resolution or accessibility to investigate potential structural and functional changes elicited by aminergic modulation. On the other hand, one might be able to identify structural and functional changes elicited by biogenic amines at the cellular level in a preparation that allows detailed examination, but the isolation of this preparation might involve substantial damage to the animal, making it difficult to apply the knowledge obtained to the behaving animal. The research detailed in this thesis is a bold attempt to bridge across synaptic remodeling and behavioral changes elicited by aminergic modulation. The range and depth of this research is primarily due to the following advantages of our chosen preparation: (1) The accessibility and tractability of the *Drosophila* larval NMJ allowed us to perform studies with single synaptic bouton resolution in a defined population of synapses. Such resolution is not possible in other preparations involving fairly undefined population of synapses. (2) The simple layout of octopaminergic and glutamatergic neuronal terminals at specific muscles allowed us to carry studies of synaptic terminals with minimal interference from other synaptic terminals. (3) The transparency of the larval cuticle and muscles allowed us to trace and image the same NMJs of an intact animal during development from first to third instar larval stages. (4) The relatively simple behaviors of the *Drosophila* larva facilitated the establishment of clear relationships between

behavioral and synaptic changes in response to aminergic signaling. (5) The availability of powerful genetic tools and RNAi collections allowed us to conveniently manipulate and visualize neurons *in vivo*.

Dual modes of octopamine signaling broaden the level of control

At the *Drosophila* larval NMJ, three type II motorneurons per segment innervate most of the body wall muscles in that segment. This layout suggests that octopamine is likely to globally regulate plasticity, by tuning the excitability levels of multiple synapses in the entire body wall. Our observation that an excitatory and an inhibitory octopamine receptor are coexpressed in this system indicates that this global regulation can be tipped towards general excitation or inhibition. This dual mode of controlling excitability likely provides enhanced flexibility, allowing a broader level of control over synaptic functions. We unraveled the presence of an autoregulatory mechanism in octopaminergic neurons, which was dependent on both Octß1R and Octß2R receptors. Our data suggest that these receptors activate or inhibit cAMP production, regulating CREB-mediated protein synthesis and synaptic growth. This mechanism is necessary for normal locomotor increase during starvation. At the same time, octopamine also regulates glutamatergic neurons via the same receptors. This paracrine signaling is important for normal synaptic growth of glutamatergic neurons during development.

Demonstration of structural plasticity mediated by starvation and octopamine

While previous studies have demonstrated a relationship between octopamine signaling and starvation in the locust and in *C. elegans* (Davenport and Evans, 1981;

Suo et al., 2006), this relationship had not been well established in *Drosophila*. In addition, in the locust and *C. elegans*, the specific cellular mechanism mediating this regulation had not been elucidated. In *Drosophila*, food-deprivation is known to induce faster crawling and synaptic changes at the functional level (Steinert et al., 2006). Increased locomotor activity during food deprivation is sufficient to induce larger quantal sizes and enhanced evoked synaptic transmission at the larval NMJ. Yet, the involvement of octopamine was not examined. The work described in this thesis, for the first time, demonstrated real-time structural changes in neurons mediated by starvation and octopamine.

Unveiling the mechanisms of aminergic synaptic growth

To date, our understanding of the development and plasticity of aminergic terminals is meager – not only in *Drosophila*, but in other organisms as well. Given the vast literature on aminergic control of plasticity, it is hard to believe that few have investigated the development of the aminergic neurons themselves. In the second chapter of this dissertation, we have discovered an autoregulatory mechanism in octopaminergic neurons. The existence of octopamine autoreceptors was previously suggested in locusts. Yet, the receptor was not identified (Howell and Evans, 1998). This work has identified Octß1R and Octß2R as autoreceptors for octopamine, and both receptors are important for normal synaptic and behavioral plasticity.

We also made the surprising finding that type II motorneurons require electrical activity for establishing primary innervation. Such severe effect of activity deprivation on neuronal structure is unusual, as it was thought that neurons devoid of activity can still

form their initial terminals (Aamodt and Constantine-Paton, 1999). Our finding is in contrast to the widely held view that activity is only necessary for refinement of existing synapses but not required for synaptogenesis. Our study raises the possibility that the development of modulatory aminergic terminals could have major differences from arbors that mediate classical neurotransmission.

A possible explanation for an apparent controversy over the effect of octopamine at the *Drosophila* larval NMJ

The role of octopamine at the *Drosophila* larval NMJ has been investigated in three studies. Two previous studies from the same group found that perfusion of octopamine results in an increase of excitatory junctional potential (Kutsukake et al., 2000; Nagaya et al., 2002) while another study found the effect of octopamine to be inhibitory (Nishikawa and Kidokoro, 1999). In the second chapter of this dissertation, we showed that octopamine increases the amplitude of glutamatergic excitatory junctional potentials, supporting the results of the two former studies (**Fig. 2-1J**). We found this excitatory effect to be dependent on Octß2R, as bath application of octopamine to *octß2R* mutants did not change EJP amplitude as observed in wild type (**data shown in text, page 45 last paragraph – page 46 first paragraph**). In the third chapter, we identified an inhibitory octopamine receptor, Octß1R, that pairs with inhibitory G-protein Goα. This supports the latter study noted above of octopamine-induced inhibition. The work in this thesis suggests that the resulting excitatory or inhibitory effect of octopamine is likely dependent on the expression levels, affinity and availability of these receptors on the target cells. Since the conflicting studies mentioned above recorded from different

muscles at different larval stages, one possible explanation for the apparent discrepancy is the expression of different octopamine receptors in different muscles and/or neurons at different larval stages.

Possible ways of cAMP modulation using both excitatory and inhibitory octopamine receptors simultaneously

How could Octß1R and Octß2R control cAMP levels in opposite ways, given the assumption that they are expressed by the same cell and receive the same ligand? While currently we cannot fully resolve this question, a few different hypotheses based on the knowledge about G-protein-coupled receptors can be proposed. For example, if Octß1R and Octß2R have different affinities for octopamine binding, they could be activated by different concentrations of octopamine, resulting in differential cellular responses, depending on the strength of the stimulus. Indeed, it has been shown that in honeybees and olive fruit flies, low concentrations of octopamine are inhibitory while high concentrations are excitatory to cardiac contraction (Papaefthimiou et al., 2010). In another study, it was shown that 10 nM octopamine is sufficient to induce a significant Ca^{2+} increase mediated by OAMB, but it takes at least 50 nM octopamine to induce cAMP changes mediated by Octß1R (Balfanz et al., 2005). These studies strongly suggest that different octopamine receptors could have different affinities for octopamine.

An alternative possibility is based on the observation that internalization of GPCRs after ligand-binding is a common phenomenon (Calebiro et al., 2010). For example, it has been shown that ß2-adrenergic receptors can undergo clathrin-mediated

endocytosis upon activation by its ligands (Gagnon et al., 1998). It is very likely that this mechanism would play a role in maintaining an appropriate ratio of octopamine receptors at the cell surface, actively keeping or removing octopamine receptor mediated excitation or inhibition, depending of physiological states.

A third alternative is that receptors could be post-translationally modified upon ligand binding, which might also affect their downstream functions. For example, it has been demonstrated that dimerization of ß2-adrenergic receptors can inhibit its adenylate cyclase activating activity (Hebert et al., 1996). On the other hand, phosphorylation of ß2-adrenergic receptors by PKA reduces the affinity for Gs and increases the affinity for Gi/o (Martin et al., 2004).

Lastly, the localization of Octß1R and Octß2R could be physically separated in neurons. One receptor could be localized closer to the site of octopamine release. Therefore, even receptors of the same affinity may experience different concentrations of octopamine simultaneously. In any case, the presence of both excitatory and inhibitory octopamine receptors in *Drosophila* has opened up many different possibilities for octopamine to modulate cellular cAMP levels.

The coexpression of both excitatory and inhibitory GPCR is not a unique phenomenon in our system. For instance, mammalian dopamine receptors can also couple to both stimulatory and inhibitory G-proteins. The D1 receptor-like family is coupled to Gs, whereas the D2-like family is coupled to Gi/o (Beaulieu and Gainetdinov 2011). It is not uncommon for GPCRs receiving the same ligand to activate different signaling pathways. In fact, dopamine autoreceptors have also been identified (Bello et al., 2011). However, so far only the inhibitory D2 receptors have been found to be an

autoreceptor (Bello et al., 2011), whereas we have demonstrated the presence of both excitatory and inhibitory octopamine autoreceptors in our study.

Universality of CREB-mediated synaptic plasticity

CREB is a transcription factor involved in multiple forms of long-term synaptic plasticity, including late LTP and long-term memory. The macromolecular synthesis underlying long-term memory formation requires CREB-dependent transcription. Its function on mediating the transcription of synaptic effector proteins is conserved in both vertebrates and invertebrates (Benito and Barco, 2010). In adult *Drosophila*, long-term appetitive memory is CREB-dependent (Krashes et al., 2008). In *C. elegans*, octopamine receptors activate CREB signaling during food-deprivation (Suo et al., 2006). Now, at the *Drosophila* larval NMJ, we demonstrated that the structural changes mediated by the autoregulatory mechanism in type II motorneurons require CREB. Remarkably, there seems to be great universality of mechanisms utilized by the neurons of different organisms to increase long-term synaptic strength using biogenic amines. It is certainly in our interest to examine what genes are being actively regulated by CREB, and which of these genes contributes to synaptopod formation. From our work in chapter 2, we have found numerous synaptic proteins that are present in synaptopods and pods-related structures (**Fig. 2-2**). However, since CREB may not directly regulate the transcription of these synaptic proteins, experiments like chromatin immunoprecipitation may be difficult.

Octopamine's function may be more complex than simply signaling sugar reinforcement in classical conditioning of *Drosophila*

The aversive memory paradigm requires five sessions of training to induce long-term memory (Tully et al., 1994). In contrast, the appetitive memory paradigm induces long-term memory with a single session of training (Krashes et al., 2008). Remarkably, the appetitive paradigm requires octopamine and starvation of flies prior to the assay, and *tbh* mutants are defective in this type of conditioning (Schwaerzel et al., 2003). It has been proposed that octopamine functions to reinforce the sugar rewards in the appetitive memory paradigm (Hammer and Menzel, 1998; Schwaerzel et al., 2003). Our work demonstrated that the presence of octopamine reduced the requirement for activity to induce structural plasticity (**Fig. 2-3**), raising the possibility that octopamine may also function to modulate synaptic structures during appetitive conditioning.

Presynaptic synaptopod-like structures are present in the CNS and may contribute to synaptogenesis

One question is whether synaptopod-like structures are only present at the NMJ. To address this question, we searched for presynaptic filopodia-like structures in the CNS in animals expressing mCD8-GFP using Tdc2-Gal4. We observed en passant boutons in both the ventral ganglion and the brain lobes (**Fig. 4-1A,B**). These en passant boutons contain TBH at their terminals, indicating they are axonal. To our excitement, synaptopod-like structures were found near these en passant boutons (**Fig. 4-1C**). Furthermore, another Gal4 line, Ap-Gal4 (Calleja et al., 1996. Gift from Dr. John Thomas), which labels interneurons, also shows very dynamic filopodia-like structures in

the ventral ganglion. We were able to capture this kind of dynamic morphological plasticity in a living larval preparation without any kind of stimulation (**Fig. 4-2**). These data suggest to us that synaptopod-like filopodia could be very commonly found throughout the CNS, and that the effect of octopamine on synaptopods we examined in this study may not be limited to just the NMJ.

Unlike postsynaptic filopodia, which are widely considered the precursor of dendritic spines and contribute to activity-dependent synaptogenesis (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998), the role of presynaptic filopodia-like structures is relatively unclear. Very few studies have shown that presynaptic filopodia-like structures contribute to synaptogenesis (Nikonenko et al., 2003). Our work clearly indicates that synaptopods contribute to synapse formation at the NMJ, and we speculate that similar processes may also occur in the CNS.

A model for octopaminergic control of synaptic and behavioral plasticity: working hypotheses and speculations

Based on the work detailed in the previous chapters of this thesis and the currently available literature, I propose the following working model for octopaminergic signaling in regulating synaptic and behavioral plasticity in the *Drosophila* larva. Future experiments will be required, however, to support some of the speculations.

In the *Drosophila* embryo, synaptogenesis by glutamatergic type I motorneurons on the body wall muscles does not require activity (Aamodt and Constantine-Paton, 1999). In contrast, octopaminergic type II innervation of the body wall is completely dependent on activity (**Fig. 2-4**). TBH is highly abundant in type II boutons (**Fig. S2-3**), presumably

to synthesize octopamine at the sites of release. Once the initial type II connections are established at the body wall muscles, the basal level of activity in type II is likely to facilitate octopamine secretion from matured type II boutons. This basal level of octopamine is required during development for normal synaptic growth of both type I and type II (**Fig. 2-1, 2-6, 2-7**). This is mostly mediated by octopamine receptor Octß2R which is present in both type I and type II (**Fig. 2-6**), and presumably activates the cAMP pathway via Gs proteins. But when food is not available, starvation of the larva will induce more activity in octopamine neurons, which will release more octopamine. The autoregulatory control of synaptic growth by Octß2R leads to continuous amplification of synaptic growth and synaptic strength. This leads to an increase in locomotor activity in order to forage for food. However, once food is encountered, this positive feedback mechanism needs to be turned off. This could be accomplished via the inhibitory Go-coupled Octß1R receptors, which function as a brake for the continuous excitation.

Critique of the current model and future directions

Despite the fact that we have a fairly comprehensive model that fits well with our data, there are still numerous parts of the model that could be further tested or refined. For instance, all our manipulations of type II motorneurons are currently performed using Tdc2-Gal4. However, this Gal4 is not only expressed in type II motorneurons exclusively, but is also expressed widely in the brain lobes as well. The fact that we manipulated all octopaminergic neurons in the CNS in all our experiments is a concern. It raises the question whether our observations of neuronal morphological changes and larval behavioral changes were really due to type II terminals or some other

octopaminergic cells in the CNS. One may argue that our manipulation could affect appetitive responses that relate to food-seeking and thus locomotion. Fortunately, since all the morphological changes we observed are at the NMJ, and since the type II terminals are the closest terminals that release octopamine (whereas octopamine from the CNS will have to travel across the blood brain barrier), it is more likely that the NMJs are modulated by the type II octopaminergic terminals instead of the ones in the CNS. In addition, our electrophysiology data have demonstrated that even without input from the CNS, octopamine can still affect EJPs at the NMJ. Therefore, even though we cannot exclude the involvement of the CNS in regulating larval movement speed during starvation, our data indicate that the octopamine from type II motorneurons alone is sufficient to increase movement speed. Nevertheless, it would be ideal if another type II Gal4 line without CNS expression could be used to reproduce the data in this thesis. That would add an extra level of confidence to our claim of the functions of type II motorneurons.

In our studies, we noticed a very interesting correlation between the increase of synaptopods in response to octopamine and the percentage increase of larval locomotor speed during starvation. In all the genotypes that we have examined (including all the mutants and all the genetically rescued animals), all of them demonstrated this correlation. Animals that showed a slight defect in octopamine-induced synaptopod increase showed a slight defective in starvation-induced locomotor increase, whereas animals that showed a strong defect in one showed a strongly defect in the other. However, currently we are not able to determine its significance, nor do we know if there is a causal relationship between the two. One experiment that could be done is

expressing Kir2.1 in muscles to suppress muscle depolarization using the Gal4 system while expressing ChR2 in type II motorneurons to acutely increase activity using the LexA system. We could then access whether the increase of synaptopods could still occur without involvement of locomotor activity or muscle contraction.

Other experiments that can be performed in the future include the re-feeding of *Drosophila* larva after starvation to investigate if the increase of synaptopods is reversible when octopamine levels are lowered. However, we are aware that in other insects, like the locust, the effect of octopamine can persist for hours even after re-feeding (Davenport and Evans, 1984).

An important question that is currently unanswered is the localization of Octß1R and Octß2R in motorneurons. Since we had great difficulties raising antibodies against Octß1R and Octß2R and overexpression of these receptors appears to be a problematic method, we may attempt to tag these receptors using the recombineering technique in the future. This would allow us to analyze the localization of these tagged receptors expressed at endogenous level.

Another experiment of interest would be to investigate the affinity of Octß1R and Octß2R by expressing these receptors in *Drosophila* S2 cell culture and monitoring cAMP changes in response to octopamine. This experiment would tell us whether these receptors are activated by different concentrations of octopamine, and would also test their antagonistic actions on cAMP. However, this experiment does face the same problem of other octopamine receptor overexpression studies, which is potentially inducing unnatural coupling to Gsα. Nevertheless, it is possible that lower expression

levels of receptors may circumvent this problem. Also, prior to the experiment, it would be necessary to verify the expression level of both Gsα and Goα in S2 cells.

Last but not least, it would be very interesting to monitor real-time cAMP levels in live-preparations or intact animals using Epac-camps, a genetically encoded cAMP FRET sensor (Shafer et al., 2008). This would allow us to measure cAMP levels in our receptor mutants and allow us to examine cAMP changes in these animals in response to octopamine.

Conclusion

In conclusion, my thesis research has uncovered a number of mechanisms by which octopamine modulates synaptic and behavior plasticity. It is likely that the analysis of the other *Drosophila* octopamine receptors would further elucidate the complex control of plasticity by octopamine, and provide important implications to other biological processes, systems and organisms.

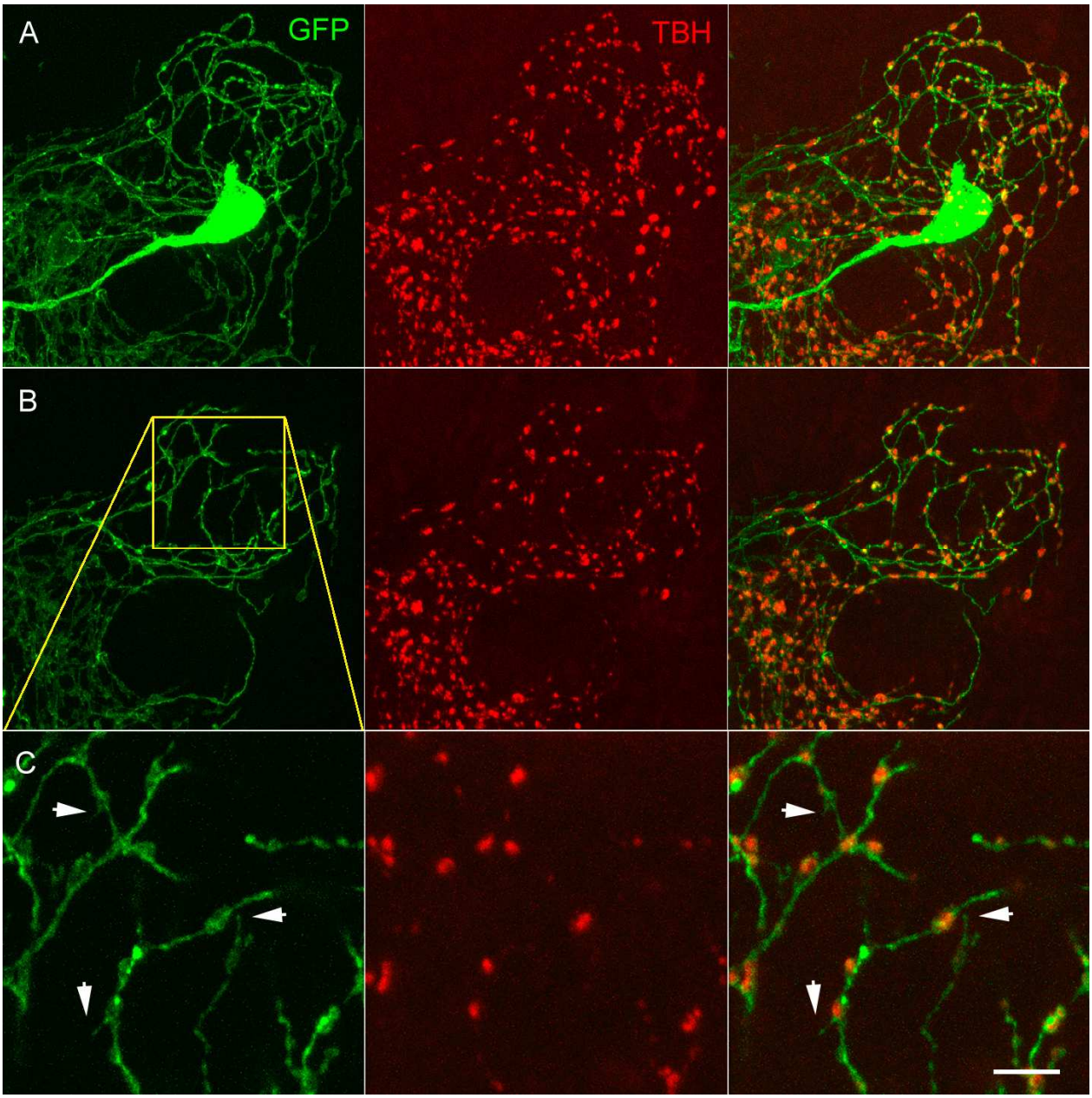


Figure 4-1

Figure 4-1. Synaptopod-like structures are not limited to the NMJ. Brain lobe of a fixed third instar larval preparation. mCD8-GFP is being expressed in octopaminergic neurons using Tdc2-Gal4. Labeled with anti-GFP and anti-HRP. (**A**) A section of approximately 15 μm of the brain lobe. Octopaminergic neurons form en passant boutons containing TBH. (**B**) A sub-section of (**A**) of approximately 6 μm. (**C**) High magnification view of (**B**) showing synaptopod-like structures near octopaminergic terminals in the CNS. Calibration bar is 4 μm for **A, B**, and 1.7 μm for **C**.

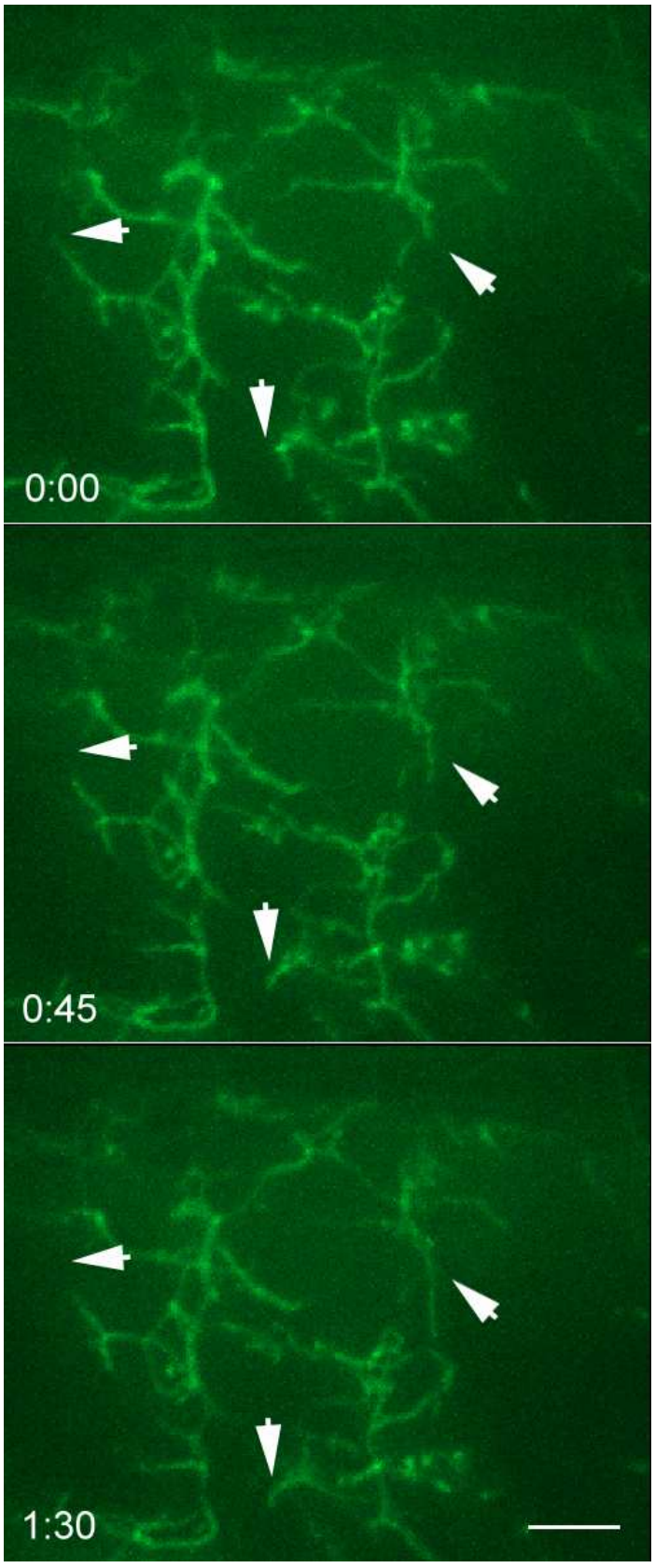


Figure 4-2

Figure 4-2. Dynamic filopodia-like structures are not limited to octopaminergic neurons. Ventral ganglion of an unstimulated third instar living larval preparation. mCD8-GFP is being expressed in interneurons using Ap-Gal4. The filopodia-like structure indicated by the white arrow on the right extended over 3 μm within 1.5 minutes. Movie length was 1 min 30 sec. Calibration bar is 3.5μm

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