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INSULIN SIGNALING REGULATES NEURITE GROWTH DURING ECDYSONE-DEPENDENT NEURONAL REMODELING

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INSULIN SIGNALING REGULATES NEURITE GROWTH DURING ECDYSONE-DEPENDENT NEURONAL REMODELING

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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Abstract

The morphological rearrangement of neurons to accommodate new functions or activities is called “neuronal remodeling”. Although neuronal remodeling is an important feature of nervous systems, the mechanisms governing the transition of neurons, from relatively stable states to more dynamic and differentiative remodeling states, are largely unknown. In holometabolous insects, there is a major transition from maintenance growth to organizational growth near the onset of metamorphosis, and these changes provide an unparalleled opportunity to explore the underlying mechanisms of neuronal remodeling. Many differentiated larval neurons are maintained throughout metamorphosis and undergo extensive remodeling, which involves the elimination of larval dendrites and axons (neurites) and the outgrowth and elaboration of adult-specific projections (Levine and Truman 1982; Brown, Cherbas et al. 2006). Here, I show that a metamorphosis-specific increase in insulin and insulin-like-growth factor signaling (IIS) promotes neuronal growth and axon branching after a long period of morphological stability during the larval stages. In a previous gain-of-function genetic screen, we found that overexpression of a negative effector in the IIS pathway, Forkhead box, sub-group O (FOXO), blocked the metamorphic growth of peptidergic neurons that secrete crustacean cardioactive peptide (CCAP) and bursicon. RNA interference (RNAi) and CCAP/bursicon cell-targeted expression of dominant negative constructs for other components of the IIS pathway [Insulin-like receptor (InR), Pi3K92E, Akt1, and S6K] also partially suppressed the growth of the CCAP/bursicon neuron somata and neurite arborization. In contrast, expression of wild-type or constitutively active forms of InR, Pi3K92E, Akt1, Rheb, and Target of rapamycin (TOR), as well as RNAi for

negative regulators of the IIS pathway (PTEN and FOXO), stimulated overgrowth. Interestingly, InR displayed little effect on larval growth of the CCAP/bursicon neurons, but strong effects on the metamorphic outgrowth of these neurons. In addition, manipulations of IIS in a pan-peptidergic neuronal pattern revealed a general role in promoting organizational outgrowth of many neurons during metamorphosis. These results reveal that specific activation of IIS during metamorphosis facilitates renewed organizational growth in mature neurons. In order to further elucidate the molecular and cellular mechanisms governing IIS regulation of the metamorphic remodeling, I performed a genetic modifier screen to detect IIS-interacting genes. I screened 492 deficiency lines for modifiers of a *foxo* overexpression phenotype (wing expansion defects). A total of 14 deficiencies were confirmed as suppressors of *foxo*, and 19 were confirmed as enhancers. Two selected suppressors, *Df(1)Exel6221* and *Df(1)Exel6002*, strongly reversed the effects of *foxo* on neuronal outgrowth. *Df(1)Exel6221* also significantly rescued the phenotypes produced by expression of InR^{DN}, suggesting that the gene(s) within *Df(1)ExEL6221* might be involved in IIS-mediated growth during the neuronal remodeling process. The source of suppression in *Df(1)Exel6002* was mapped to an individual locus, *Su(z)2*. Reduced expression of *Su(z)2* by RNAi suppressed the effects of FOXO on neuronal outgrowth. Su(z)2 is a Zinc finger protein that belongs to the *Drosophila* Polycomb Group (PcG) protein family, the members of which function as negative regulators of transcription and chromatin modification (Brunk, Martin et al. 1991). This indicates that transcriptional regulation through chromatin modification by *Su(z)2* may play an important role in reprogramming neuronal entry into the organizational growth phase, or in the execution of that growth program.

CHAPTER 1

The roles of steroids and insulin/insulin-like-growth-factor signaling in insect metamorphic neuronal remodeling

Neuronal remodeling

The ability of mature neurons to reorganize their structures is essential for the nervous system to adapt to developmental transitions, changes in the environment, or nervous system damage. This morphological rearrangement, or plasticity, of neurons to accommodate new functions or activities is called "neuronal remodeling". The adult brain was once viewed as a hardwired system, with neuronal differentiation and outgrowth seen as possible only during early development. However, neuroscientists began to recognize greater neuronal plasticity in the 1970s, when several important investigations were conducted on patients with brain injuries (Stenevi, Bjorklund et al. 1973; Kaas, Merzenich et al. 1983; Dombovy and Bach-y-Rita 1988). The functional recovery of stroke patients also suggested the rewiring of the nervous system after damage to brain tissue (Dombovy and Bach-y-Rita 1988). Since that time, accumulating evidence has revealed the importance of neuronal remodeling in many situations, such as during puberty, seasonal changes in bird song control centers, and learning and memory, and in response to brain injury or stroke, chronic stress, or neurodegenerative disease (Finger and Almli 1985; Arendt, Schindler et al. 1997; Brenowitz 2004; Blakemore and Choudhury 2006; Zehr 2006; Knobloch and Mansuy 2008; Sousa, Cerqueira et al. 2008; Dadon-Nachum, Melamed et al. 2011; Dijkhuizen, van der Marel et al. 2012). Given this developmental potential in neurons, an

understanding of the genetic processes that control neuronal plasticity may help researchers to develop strategies to restore functional connections in damaged neural circuits.

Hormonal regulation of neuronal remodeling

A variety of internal and external factors contribute to neuronal remodeling during aging, sleep, learning and memory, and following injury (Walker 2008; Warraich and Kleim 2010; May 2011; Kolb and Teskey 2012). Among the known internal factors, hormonal regulation plays a vital role in neuronal remodeling. Besides the well-established functions of hormones in controlling the plasticity of peripheral tissue, emerging evidence has helped to unravel their significance in the central nervous system (Moult and Harvey 2008). For instance, *in vivo* and *in vitro* research has revealed an important role of estrogen in regulation of dendritic morphology in the hippocampus (Moult and Harvey 2008). During the natural estrous cycle, dendritic spines in rat hippocampal CA1 neurons undergo morphological transformation, from predominantly mushroom-shaped spines in the proestrus stage to abundant, thin spines in the estrus stage (Gonzalez-Burgos, Alejandre-Gomez et al. 2005). In addition, removal of circulating ovarian steroids through ovariectomy in rats significantly reduced dendritic spine density in hippocampal CA1 neurons, and the effect could be prevented by estradiol replacement (Gould, Woolley et al. 1990). This estrogen-dependent reorganization of dendritic spines in the hippocampus could contribute to differential information processing related to hippocampal activity during the estrous cycle.

Steroid-induced neuronal remodeling is present in many model animals, particularly in the context of behavioral activation. For example, in the Japanese quail, sex steroids induce seasonal volume changes in specific brain nuclei related to male sexual behavior though an increase in cell size or spacing and dendritic branching (Balthazart, Charlier et al. 2010). In the female rat, ovarian steroid hormones induce synaptic reorganization within the ventromedial nucleus of the hypothalamus to control sexual behavior (Griffin and Flanagan-Cato 2011). These various studies illustrate how steroid hormones play conserved roles in regulating neuronal morphology and function (Moult and Harvey 2008; Fernandez and Torres-Aleman 2012; Srivastava 2012).

In addition to steroid hormones, researchers have discovered a growing number of peptide hormones, including insulin, that contribute to the modulation of brain structure and function (Moult and Harvey 2008). In addition to the important peripheral effects of insulin on metabolism, there is mounting evidence implicating insulin in plasticity and growth-regulation in the central nervous system (CNS). For example, insulin has been shown in the hippocampus to regulate synaptic plasticity and promote neuronal survival (Schulingkamp, Pagano et al. 2000; van der Heide, Ramakers et al. 2006; Moult and Harvey 2008), and disruption of insulin signaling has been implicated in a variety of CNS disorders, such as Alzheimer's disease (Schulingkamp, Pagano et al. 2000; van der Heide, Ramakers et al. 2006). Interestingly, both insulin and estrogen can activate two major signaling pathways, the PI3K/Akt and the Ras/Raf/MEK pathways, through their respective receptor complexes (Moult and Harvey 2008). This suggests the potential interaction of these two hormonal systems in the regulation of

brain structure and function. However, the molecular and cellular mechanisms that govern hormonal regulation of neuronal remodeling in the CNS remain largely unknown.

Insect neuronal remodeling during metamorphosis

Insect metamorphic neuronal remodeling is one of the most remarkable and thoroughly studied examples of hormone regulation of neuronal plasticity in animals. In holometabolous insects, the nervous system undergoes extensive developmental reorganization during metamorphosis to support a complete transformation of the animal, from a crawling larva into a highly mobile and reproductively active adult. During this transition, three types of changes occur in the nervous system: programmed cell death of some larval neurons through autophagy or apoptosis, formation of new adult-specific neurons, and morphological remodeling of existing, fully differentiated larval neurons for adult purposes (Truman 1992; Weeks 2003; Choi, Lee et al. 2006). The remodeling of neurons in the latter group involves the elimination of larval dendrites and axons (neurites), followed by outgrowth and elaboration of adult-specific projections (Levine and Truman 1982; Brown, Cherbas et al. 2006).

Ecdysone signaling regulates metamorphic neuronal remodeling

Insect ecdysone signaling pathway

The major developmental transitions in insects are triggered and controlled by circulating steroid hormones, the ecdysteroids, of which the principal active form is 20-hydroxyecdysone (hereafter referred to as ecdysone) (Thummel 1996). A second hormone, juvenile hormone (JH), acts in the presence of ecdysone to perform a ‘status

quo' action in preventing precocious metamorphosis and allowing growth of larvae through multiple molts to reach the proper size for metamorphosis (Riddiford 1996; Truman and Riddiford 1999). The most dramatic response to ecdysone occurs at the end of the last larval stage, when a high pulse of ecdysone triggers the onset of metamorphosis. One of the earlier external manifestations of this transition is puparium formation. About 12 hours later, another ecdysone pulse initiates the prepupal to pupal transition.

A large body of work has revealed the essential role of ecdysone signaling in regulating metamorphic neuronal remodeling in two model organisms: the tobacco hornworm, *Manduca sexta*, and the fruit fly, *Drosophila melanogaster*. In *Drosophila*, ecdysone is produced in the prothoracic gland and in the ovary (Gilbert, Rybczynski et al. 2002). Ecdysone exerts its function by binding to and activating a nuclear receptor, the ecdysone receptor (EcR), which forms a heterodimer with its partner, ultraspiracle (USP), a homolog of the vertebrate RXR receptor (Oro, McKeown et al. 1990; Koelle, Talbot et al. 1991). Activation of the EcR/USP complex regulates a wide range of developmental processes and physiological responses, including embryogenesis, larval molting, metamorphosis, oogenesis, and reproduction (Clever and Karlson 1960; Ashburner 1990; Kozlova and Thummel 2000; Li and Bender 2000). The ligand-binding receptor complex triggers a genetic hierarchy by directly activating a small number of ecdysone early-response genes, including *E74, E75*, and *Broad-complex (BR-C)*. These ecdysone early-response genes encode transcription factors that in turn regulate the transcription of a large set of late-response regulatory genes (Thummel 1996; Wang, Miura et al. 1998).

The molecular mechanisms of ecdysone action are complex. For example, the EcR/USP heterodimer can function as both a transcriptional activator and repressor. Without ligand binding, EcR/USP often represses target gene transcription through interaction with co-repressors such as SMRTER (silencing mediator for RXR and TR-related ecdysteroid receptor interacting factor) (Tsai, Kao et al. 1999). However, EcR/USP can also serve as a repressor via ligand binding, when it binds to another co-repressor of EcR, *Drosophila* arginine methyltransferase 1 (DART1) (Kimura, Sawatsubashi et al. 2008). When ecdysone is present, the binding of the ligand to the EcR/USP complex causes a conformational change that promotes the release of co-repressors and the recruitment of co-activators (Bai, Uehara et al. 2000; Francis, Zorzano et al. 2010). The resulting activated receptor complex initiates the expression of a genetic hierarchy of early-response and late-response genes to regulate various biological events.

Isoform-specific regulation of spatial and temporary responses to ecdysone

Studies from *Drosophila* demonstrate the presence of three EcR isoforms, EcR-A, EcR-B1, and EcR-B2, which transfer the systemic hormonal signal into stage- and tissue-specific developmental responses (Talbot, Swyryd et al. 1993). The three EcR isoforms are produced through alternative splicing, with a common C-terminal region that includes the DNA binding domain and the ligand-binding domain. They differ only in the N-terminal region, which determines the transactivation specificity of each isoform (Talbot, Swyryd et al. 1993; Mouillet, Henrich et al. 2001; Gauthier, VanHaaften et al. 2012). It has been shown that isoform-specific spatial and temporal

expression patterns contribute to distinct cellular responses to ecdysone (Robinow, Talbot et al. 1993; Talbot, Swyryd et al. 1993; Truman, Talbot et al. 1994). In addition, isoform-specific mutations reveal distinct functionalities for the EcR isoforms. *EcR-B1* mutations disrupt early events of metamorphosis, while EcR mutations that map to regions of the protein common to all these isoforms cause embryonic lethality (Bender, Imam et al. 1997).

Variable expression of the three EcR isoforms in the CNS correlates with neuronal remodeling during *Drosophila* metamorphosis (Truman, Talbot et al. 1994). Antibodies specific to EcR-A and EcR-B1 (an EcR-B2-specific antibody is not available) have been used by Truman and others (Truman, Talbot et al. 1994) to examine the expression patterns of these isoforms and to correlate these patterns with the types of cellular responses to ecdysone. Most larval neurons express high levels of EcR-B1 at the onset of metamorphosis, during pruning of larval neurites. These neurons shift to prominent EcR-A expression during later metamorphosis, when adult neurite sprouting, neurite outgrowth, and synaptogenesis occurs (Truman, Talbot et al. 1994). In contrast to remodeling neurons, imaginal neurons, which are born but then arrest development during the larval stages, express only EcR-A at the onset of metamorphosis when they begin their adult outgrowth (Truman, Talbot et al. 1994).

To investigate the specific functions of each EcR isoform in metamorphic neuronal remodeling, genetic EcR mutations have been used to examine the pruning and outgrowth phenotypes of many neuronal cell types, including sensory neurons, mushroom body (MB) neurons, motoneurons, and peptidergic neurons (Schubiger,

Wade et al. 1998; Lee, Marticke et al. 2000; Kuo, Jan et al. 2005; Williams and Truman 2005). EcR-B mutants that lack both EcR-B1 and EcR-B2 expression fail to prune back larval dendrites in the thoracic ventral Tv neurons, a group of neuropeptide-producing cells (Schubiger, Wade et al. 1998). However, an EcR-B1-specific mutation does not block Tv neuron pruning. This indicates the presence of functional redundancy between EcR-B1 and EcR-B2 to support pruning, or a requirement for EcR-B2 alone. A similar block to pruning was observed in mushroom body (MB) γ neurons with EcR-B mutations (Lee, Marticke et al. 2000). In both cell types, these pruning defects were rescued by expression of either the EcR-B1 or EcR-B2 isoform, but not the EcR-A isoform (Lee, Marticke et al. 2000; Schubiger, Tomita et al. 2003). Thus, the EcR-B isoforms are associated with pruning of larval-specific projections during early metamorphosis, while the shift to EcR-A expression after pruning (Truman, Talbot et al. 1994) suggests that EcR-A may be responsible for regulating outgrowth of the adult arbor in mid- to late-metamorphosis.

To further dissect the specific roles of each EcR isoform in directing precise cellular responses during metamorphic neuronal remodeling, EcR dominant negative (EcR^{DN}) constructs have been used to study the remodeling of Tv neurons, sensory neurons, and serotonergic neurons in the *Drosophila* olfactory system (Kuo, Jan et al. 2005; Brown, Cherbas et al. 2006; Roy, Singh et al. 2007). The Cherbas lab (Cherbas, Hu et al. 2003) created two EcR^{DN} constructs, EcR^{F645A} and EcR^{W650A}. Both EcR^{DN} constructs contain a point mutation at highly conserved residues in helix 12 in the ligand binding domain that abolishes ligand-dependent transcriptional activation. Both bind to ecdysone-response elements in DNA, and the main difference between the two

EcR^{DN} constructs is in their ligand binding ability. EcR^{F645A} can bind to ecdysone, but it fails to mediate transcription activation, while EcR^{W650A} cannot bind to ecdysone. Since ligand binding can release co-repressors from the EcR/USP complex to terminate transcriptional repression, these differences in ligand binding ability may allow EcR^{F645A} to function as a conditional repressor, whereas EcR^{W650A} may be a constitutive repressor (Hu, Cherbas et al. 2003; Brown, Cherbas et al. 2006). When these two EcR^{DN} proteins were used to disrupt ecdysone signaling in peripheral sensory neurons, the class IV da (C4da) neurons (Kuo, Jan et al. 2005), and in neuroendocrine cells, the Tv neurons (Brown, Cherbas et al. 2006), both resulted in defective pruning of the larval arbor. This suggests that ecdysone-dependent transcriptional activation is required for the pruning back of larval neurites during metamorphosis.

The manipulation of EcR has more varied effects on neuronal outgrowth. With time-lapse microscope imaging of live Tv cell axons, Brown and colleagues (Brown, Cherbas et al. 2006) were able to observe differential effects of isoform-specific EcR^{DN} and EcR core region RNA interference [$EcR(core)^{RNAi}$] constructs on axonal outgrowth. Although the morphology of newly formed axonal branches was somewhat abnormal (Brown, Cherbas et al. 2006), outgrowth occurred in cells expressing EcR-$B1^{F645A}$, EcR-$B2^{W650A}$, or $EcR(core)^{RNAi}$, and it was associated with moderate filopodia activity, which is important in neurite outgrowth initiation (Mattila and Lappalainen 2008). Cells expressing EcR-$B1^{W650A}$ or EcR-A^{W650A} showed qualitatively different outgrowth defects, with very few filopodia formed during outgrowth, and retention of a more larval-like arbor into the pharate adult stage (Brown, Cherbas et al. 2006). The selective block of outgrowth with EcR-$B1^{W650A}$ but not with EcR-$B1^{F645A}$ or RNAi to

the common region of EcR suggests that the relief of transcriptional repression caused by binding of ecdysone to its receptor is essential for outgrowth during metamorphic remodeling.

Regulation of EcR expression facilitates neuronal remodeling during metamorphosis

Studies on the control of EcR expression shed further light on the genetic processes governing neuronal remodeling. Through a forward genetic mosaic screen, Zheng and colleagures (Zheng, Wang et al. 2003) discovered a crucial role of transforming growth factor β (TGF-β) signaling in controlling EcR-B1 expression to regulate remodeling of MB γ neurons. Two mutations, one affecting Baboon, a *Drosophila* TGF-β type I receptor, and the other affecting dSmad2, a downstream transcriptional effector for Baboon, block the pruning of larval axons by reducing EcR-B1 expression before the onset of metamorphosis. This remodeling defect can be rescued by restoration of EcR-B1 expression (Zheng, Wang et al. 2003). Later, the same lab discovered a ligand of *Drosophila* TGF-β signaling, Myoglianin (MYO), which enables the neuronal remodeling of MB γ cells through its binding to the Baboon receptor. Intriguingly, MYO must be secreted by glial cells to induce EcR-B1 expression (Awasaki, Huang et al. 2011). These results further illuminate the importance of glial cells, in addition to their engulfing action during pruning (Awasaki and Ito 2004; Watts, Schuldiner et al. 2004; Awasaki, Tatsumi et al. 2006), in directing neuronal remodeling.

In addition to TGF-β signaling, several other parallel molecular pathways regulate EcR-B1 expression to facilitate neurite remodeling of MB neurons, including

the orphan nuclear receptors, FTZ-F1 and HR39, and the cohesin protein complex (Dorsett 2008; Schuldiner, Berdnik et al. 2008; Awasaki and Lee 2011; Boulanger, Clouet-Redt et al. 2011). During MB neuron pruning, FTZ-F1 is required for repression of HR39 and activation of EcR-B1 expression. The repression of HR39 is important, since HR39 can compete with FTZ-F1 to inhibit EcR-B1 transcription. Boulanger's group also found that the role of FTZ-F1 and HR39 on neuronal remodeling is independent of TGF-β signaling (Boulanger, Clouet-Redt et al. 2011). Through a similar forward genetic mosaic screen, Schuldiner and colleagues identified the requirement of the cohesin complex for remodeling of MB neurons (Schuldiner, Berdnik et al. 2008). Mutations in *SMC1* and *SA*, two subunits of the cohesin complex (Losada and Hirano 2005; Nasmyth and Haering 2005), disrupted axon pruning by the MB neurons. This defect was associated with a significant reduction in EcR-B1 protein level and could be partially rescued by excess expression of EcR-B1. In addition, Smc1 has been shown to bind to the active EcR gene in cultured cells, which indicates a general transcription role of cohesin (Misulovin, Schwartz et al. 2008). These results reveal a novel function of the cohesin complex in neuronal remodeling, potentially through stimulation of *EcR-B1* transcription.

Signaling by ecdysone to regulate neurite pruning

A study of ecdysone-dependent gene expression revealed a role for the conserved RNA-binding protein, Boule in neuronal remodeling. Down-regulation of *boule* by ecdysone in MB γ neurons at the onset of metamorphosis is required for proper axon pruning, whereas forced mis-expression of *boule* in MB γ neurons is sufficient to block axon pruning (Hoopfer, Penton et al. 2008). Thus, Boule acts as a

negative regulator of axon pruning during metamorphic neuronal remodeling. In addition, an ecdysone early-response gene, *Sox14*, has been shown to be critical in triggering the initiation of dendritic pruning of dendritic arborization sensory neurons (ddaCs) by directly regulating the expression of its target gene *mical*, which encodes a cytoskeletal regulator (Kirilly, Gu et al. 2009). Later the same lab revealed that *sox14* is activated by the cooperation of ecdysone signaling and two epigenetic factors, a Brahma (BRM)-containing remodeling complex and the histone acetyltrasferase, CREB-binding protein (CBP) (Kirilly, Wong et al. 2011). In the presence of ecdysone, CBP associates with EcR-B1, with the facilitation of BRM, to affect *Sox14* transcription. These results indicate the importance of interactions between intrinsic epigenetic machinery and systemic ecdysone signaling to control neurite pruning during metamorphosis.

Other intracellular and extracellular mechanisms are also involved in ecdysone-induced neurite pruning during metamorphic neuronal remodeling. The intracellular signals include the ubiquitin-proteasome system, which participates in initiation of dendrite breakage of dendritic arborization C4da neurons (Kuo, Jan et al. 2005) and prior axon pruning of MB neurons (Hoopfer, Penton et al. 2008). Extracellular matrix metalloprotease activity is required for degradation of severed larval dendrites of C4da neurons (Kuo, Jan et al. 2005), and glial engulfment is required for removal of many neuronal processes (Awasaki and Ito 2004; Watts, Schuldiner et al. 2004; Awasaki, Tatsumi et al. 2006).

Ecdysone control of neurite outgrowth during metamorphosis

Compared to the extensive research on the pruning process, the mechanisms governing outgrowth of adult-specific neurites have received far less attention. A handful of studies have revealed a role for the zinc finger transcription factor, Krüppel-homolog 1 (KR-H1), in the outgrowth of remodeling neurons (Pecasse, Beck et al. 2000; Hewes 2008). Pecasse and colleagues recovered a P-element-induced prepupal mutant of *Drosophila* that displays normal embryonic and larval development and pupariation, but dies at the prepupal-pupal transition with head eversion defects (Pecasse, Beck et al. 2000). This mutation disrupts the *Kr-h1* gene, which was originally discovered during a screen for homologues of the segmentation gene, *Krüppel* (Schuh, Aicher et al. 1986). One clue to the remodeling function of *Kr-h1* then emerged from work in honeybees: increased KR-H1 expression is associated with the natural transition in workers to foraging behavior, a stage when extensive neurite outgrowth, branching, and synapse formation occur in the brain . In *Drosophila*, KR-H1 modulates ecdysone signaling to govern axon morphogenesis of MB neurons during metamorphosis (Shi, Lin et al. 2007). KR-H1 is normally expressed in MB neurons, with the expression level dropping precipitously during early metamorphosis, when the outgrowth of adult-specific neurites takes place (Shi, Lin et al. 2007). Targeted overexpression of *Kr-h1* in MB neurons inhibits re-elaboration of adult-specific λ axons during early metamorphosis, while loss of KR-H1 did not affect the neurite remodeling of MB neurons. However, removal of endogenous KR-H1 rescued the delayed morphogenesis phenotype caused by a *baboon* mutation in the dorsal cluster of Atonal-

positive (DC) neurons (Shi, Lin et al. 2007). This indicates that KR-H1 is involved in the negative regulation of neurite morphogenesis during neuronal remodeling.

Further studies link KR-H1 to ecdysone signaling. In response to ecdysone during late larval development, KR-H1 expression in salivary gland cells increased fivefold (Pecasse, Beck et al. 2000; Beck, Dauer et al. 2005). In addition, KR-H1 expression requires USP, as loss of *usp* in MB clones abolished *Kr-h1* expression. KR-H1 also regulates patterning of EcR-B1 in the late larval CNS (Shi, Lin et al. 2007). Therefore, KR-H1 appears to be an important regulator of metamorphosis in the larval CNS: it inhibits neurite morphogenesis, and that inhibition is released by ecdysone.

There are only a few other studies that provide insights into the control of ecdysone-dependent neuronal outgrowth during metamorphosis. In *Manduca sexta*, Broad Complex (BRC), a primary ecdysone response gene, is required in a non-cell-autonomous manner for dendritic outgrowth of motoneurons MN1-MN4 during metamorphosis (Consoulas, Levine et al. 2005). Multiple reports also show the importance of the pruning process and neuronal electrical activity in establishing the adult neuronal pattern during metamorphic remodeling (Hebbar and Fernandes 2004; Williams and Truman 2004). The above studies have started to reveal the control mechanisms for growth of adult-specific arbors during metamorphosis, but many more questions need to be addressed. For instance, how does the ecdysone signaling pathway interact with other signals to orchestrate the transition from pruning of larval-specific neurites to outgrowth of adult-specific arbors? What other signals regulate metamorphic neuronal outgrowth? To what extent are these signals context-dependent?

Interaction between JH and ecdysone regulate insect metamorphosis

In order to reveal the roles of endogenous JH, Riddiford and colleagues blocked the biosynthesis of JH in *Drosophila* through ablation of the JH producing cells in the corpora allata. This resulted in smaller larvae at pupariation, higher lethality at the head eversion stage, and precocious expression of EcR-B1 in photoreceptors and optical lobe neurons, which led to defects in the outgrowth of photoreceptors (Riddiford, Truman et al. 2010).

The molecular genetic mechanism by which JH interacts with ecdysone has been the subject of intense study. Progress has been realized through research on the metamorphosis of holometabolous insects, including *D. melanogaster*, *M. sexta,* the silkmoth *Bombyx mori,* and the beetle *Tribolium castaneum*. JH signals through two putative receptors, methoprene-tolerant (Met) and germ cell-expressed (Gce) (Wilson and Ashok 1998; Baumann, Barry et al. 2010; Abdou, He et al. 2011). Met and Gce both belong to the bHLH-PAS protein family and form heterodimers to regulate gene expression (Godlewski, Wang et al. 2006; Liu, Sheng et al. 2009; Abdou, He et al. 2011).

Details of the downstream JH signaling pathway are beginning to emerge. Wnt signaling has been shown to inhibit JH action by negatively regulating *met* and *gce* (Abdou, Peng et al. 2011). Cumulative evidence suggests that Kr-h1 also mediates JH action as an early JH-response gene downstream of Met (Minakuchi, Namiki et al. 2008; Minakuchi, Namiki et al. 2009). In *Drosophila* and *Tribolium*, Kr-h1 is

expressed during embryonic and larval stages, but it largely disappears during the pupal stage (Minakuchi, Namiki et al. 2009). In *Tribolium*, inhibition of JH biosynthesis induced a precocious larval-pupal transition and reduced *Kr-h1* transcription (Minakuchi, Namiki et al. 2009). Conversely, RNAi-mediated knockdown of *Kr-h1* expression during the larval stage of *Tribolium* caused premature metamorphosis and precocious expression of *broad* (*br*) (Minakuchi, Namiki et al. 2009). *br* is one of the ecdysone early-response genes (Crossgrove, Bayer et al. 1996) and has been implicated as a key mediator of the cross-talk between ecdysone and JH signaling (Zhou and Riddiford 2002; Dubrovsky 2005).

Indeed, the responses of BR to JH and ecdysone signaling play a role in regulating developmental transitions. During larval development, the presence of both JH and ecdysone suppresses *br* expression to ensure status quo (larval-to-larval) molts (Zhou, Hiruma et al. 1998; Abdou, Peng et al. 2011). At the onset of metamorphosis, JH levels decline, and a surge of ecdysone directly stimulates *br* expression. This *br* expression can be prevented by application of exogenous JH (Zhou, Hiruma et al. 1998; Dubrovsky 2005). The predominant expression of *br* during the larval-pupal transition, in turn, directly regulates the transcription of late ecdysone-response genes and specifies pupal development (Zhou and Riddiford 2002). The final adult molt occurs in the presence of a high titer of ecdysone and in the absence of JH and BR activity. JH mimic (JHM) application at the onset of the adult molt induces *Kr-h1* expression and *br* re-expression in *Manduca* and *Tribolium*, but only in the abdomen of *Drosophila*. Consequently, a second pupal molt is seen in both *Manduca* and *Tribolium*, and a second pupal cuticle is formed in the abdomen of *Drosophila* (Minakuchi, Zhou et al.

2008; Minakuchi, Namiki et al. 2009). These results are consistent with a model in which JH exerts its status quo function by regulating the ecdysone-dependent switch of *br* expression.

JH inhibition of neuronal remodeling

Several studies on *Manduca sexta* motoneurons (MNs) have revealed the anti-metamorphic functions of JH on neuronal remodeling. Ecdysone stimulates outgrowth of adult-specific neurites by MN, but this requires the absence of JH (Truman and Reiss 1988; Truman and Reiss 1995; Knittel and Kent 2005). Local or systemic application of JHMs has been shown to interfere with the effects of ecdysone action on MN remodeling (Truman and Reiss 1988; Knittel and Kent 2005). Systemic application of JHM prior to the pupal peak of ecdysone, when the outgrowth program initiates, prevents adult MN differentiation (Truman and Reiss 1988). However, local JHM application to target muscles at pupal stage specifically blocks the formation of adult motor terminals, but not the outgrowth of adult-specific MN dendritic arbors (Truman and Reiss 1995). Local injection of JHM into the CNS before the pupal peak of ecdysone disturbed the growth of adult-specific dendrites, but not the elongation and differentiation of adult motor terminals (Knittel and Kent 2005). These results demonstrate that JH regulates distinct mechanisms to control MN dendrite outgrowth and adult axon terminal formation.

Roles of insulin/insulin-like-peptide signaling in neuronal plasticity

In addition to steroid hormones and JH, recent studies have revealed important roles of insulin and related peptides in the development of neuronal circuits and in the

regulation of neuronal plasticity in adult brains in response to internal and external cues (Fernandez and Torres-Aleman 2012). These peptides include insulin and insulin-like growth factors (IGFs), hereafter collectively referred to as insulin-like-peptides (ILPs) (Fernandez and Torres-Aleman 2012). From cnidarians, nematodes, and flies to mammals, ILPs are evolutionarily highly conserved peptide hormones that regulate metabolism, growth, and neuronal survival (Nakae, Kitamura et al. 2001; Siddle 2011).

Insulin-like-peptide signaling

In vertebrates, ILPs function through several receptors, whereas in invertebrates, they act through a single receptor. In mammals, insulin, insulin-like growth factor 1 (IGF-1), and insulin-like growth factor 2 (IGF-2) all bind to their own receptors (although each ILP can bind to other receptors, often with lower affinity) to control various functions (Nakae, Kitamura et al. 2001; Siddle 2011). IGF-2 has also been shown to bind the IGF-1 receptor and insulin receptor isoform A with high affinity to regulate brain development (Alvino, Ong et al. 2011). In *Drosophila*, there are eight *Drosophila* insulin-like-peptides (DILPs) that are all thought to act through a single receptor (Brogiolo, Stocker et al. 2001). Despite the presence of various ligands, activation of these pathways occurs through recruitment of common downstream kinase-phosphatase cascades (Fernandez and Torres-Aleman 2012), including the RAS-mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K)-Akt-forkhead box protein O (FOXO) signaling cascade (Fernandez and Torres-Aleman 2012).

Insulin and IGF-1 receptor are members of the tyrosine kinase receptor family. Ligand binding causes the receptors to undergo a conformational change and become autophosphorylated. The autophosphorylation enables phosphorylation of a number of substrate proteins and activation of a series of kinases to engender metabolic, growth, and cell survival responses (Siddle 2011).

Functions of IIS in the vertebrate brain

Although both ILPs and their receptors were found in the brain in the early to mid-1980s (Havrankova, Brownstein et al. 1981; Laron and Galatzer 1985), their specific functions in developing and adult brain have been revealed only recently. ILPs and ILP receptors are expressed at high levels in many regions of the developing brain and then display reduced expression in the adult brain (Sandberg, Engberg et al. 1988; Adamo, Raizada et al. 1989; Valentino, Ocrant et al. 1990; Ayer-le Lievre, Stahlbom et al. 1991; Baron-Van Evercooren, Olichon-Berthe et al. 1991; Bondy 1991; Aguado, Sanchez-Franco et al. 1994; Devaskar, Giddings et al. 1994; Ghasemi, Haeri et al. 2012). In addition to local production of ILPs in different brain regions, peripheral ILPs can enter the brain, as evidenced by the insulin uptake from plasma into cerebrospinal fluid (CSF) and the binding of insulin to brain microvessels (Frank, Pardridge et al. 1986; Baura, Foster et al. 1993).

IIS in developing brain: In vertebrates, ILPs are important regulators of nervous system growth and maturation. ILPs and their receptors are highly abundant in the developing rat brain, with peaks of expression coinciding with periods of active cell proliferation and neurite outgrowth (Hynes, Brooks et al. 1988; Bondy and Lee 1993; Devaskar,

Giddings et al. 1994; D'Ercole, Ye et al. 1996). Transgenic mice with overexpression of IGF-1 exhibit postnatal brain overgrowth, while IGF-1, IGF-2, or IGF-R knockout mice display brain growth retardation and a smaller final brain size (Baker, Liu et al. 1993). In humans, patients with an IGF-1 gene deletion experience a severe growth defect with microcephaly (Camacho-Hubner, Woods et al. 2002). Based on these and many similar reports, the effects of insulin-like growth factors on the developing vertebrate brain are well documented.

More recently, insulin – which has primarily be known as critical regulator of nutrient homeostasis – has been implicated in the morphogenesis, functioning, and development of the central nervous system (Chiu and Cline 2010; Huang, Lee et al. 2010). For example, several neuronal cell culture studies have revealed a role of insulin receptor signaling in regulating neurite growth (Govind, Kozma et al. 2001; Choi, Ko et al. 2005), and *in vivo* studies in retinotectal circuits of *Xenopus laevis* have shown that insulin receptor signaling is required for dendritic arborization (Chiu, Chen et al. 2008). Thus, the entire set of vertebrate ILPs plays important roles in promoting neuron proliferation, differentiation, and survival as well as in building circuits in the developing brain.

Functions of IIS in the adult brain: Accumulating recent evidence has expanded our understanding of IIS functions in the brain to include many regulatory activities: IIS influences food intake and body weight, reproduction, learning and memory, and neurodegeneration. Examples of insulin regulation of energy homeostasis include the finding that an intracerebroventricular infusion of insulin inhibits caloric intake and

body weight by decreasing neuropeptide Y gene expression in the hypothalamus (Schwartz, Figlewicz et al. 1992). Likewise, neuron-specific disruption of the insulin receptor (IR) gene in mice induced diet-sensitive obesity and impaired spermatogenesis and ovarian follicle maturation (Bruning, Gautam et al. 2000). A link to reproduction was also revealed by Della Torre and colleagues, who demonstrated that amino acid-dependent activation of liver estrogen receptor alpha integrated energetic and reproductive responses by regulating hepatic IGF-1 levels (Della Torre, Rando et al. 2011). These results indicate the important roles of IIS in central regulation of energy homeostasis and reproduction as a result of food availability.

Compelling evidence also indicates that IIS has direct effects on learning and memory. In mammals, insulin receptor is highly expressed in the olfactory bulb and modulates memory, anxiety, and olfactory behaviors (Marks, Tucker et al. 2009), and IGF-1 regulates sensory map formation and axon guidance in the olfactory system (Scolnick, Cui et al. 2008). In addition, brain ILPs have been shown to associate with spatial learning and cognition. After spatial training, insulin receptors were up-regulated in the hippocampus of trained rats (Zhao, Chen et al. 1999). Training also increased the levels of downstream molecules in the insulin signaling pathway, such as insulin receptor substrate (IRS)-1 and Akt (Dou, Chen et al. 2005). Administration of brain insulin improved spatial learning and memory in rats and cognition in humans (Dhamoon, Noble et al. 2009; Haj-ali, Mohaddes et al. 2009).

ILP dysfunction has also been described in many neurodegenerative diseases, including Huntington's disease, Parkinson's disease, and Alzheimer's disease (AD)

(Cohen and Dillin 2008). For example, the strong association of AD with type-2 diabetes suggests a direct role of IIS in this neurodegenerative disease, and this has led to the proposal that AD is often a brain manifestation of diabetes (Freude, Plum et al. 2005). Although plasma insulin levels remain high in patients with AD, cerebrospinal fluid insulin levels are largely reduced (Craft, Peskind et al. 1998), and expression of IR, IGF-1 receptor (IGF-1R), IRS-1, and IRS-2 is strongly down-regulated (Freude, Schilbach et al. 2009). The severity of these changes progresses with the extent of neurodegeneration (Freude, Schilbach et al. 2009). It was also found that insulin can abolish the phosphorylation of a microtubule-associated protein tau, a hallmark indicator of the neurofibrillary tangles seen in AD, that is induced by depletion of insulin through intracerebroventricular administration of streptozotocin (Clodfelder-Miller, Zmijewska et al. 2006). A pair of recent studies showed that intranasal administration of insulin improved hippocampus-dependent memory in both healthy adults and also patients with AD (Benedict, Hallschmid et al. 2007; Dhamoon, Noble et al. 2009). These tantalizing findings draw considerable interest for the future clinical application of ILPs or ILP analogs as a treatment for memory and neurodegenerative disorders.

IIS regulation of neuronal plasticity

The previously discussed effects of IIS on learning and memory are via regulation of neuronal plasticity. In the brain, insulin receptors are highly concentrated in synaptic areas (Zhao and Alkon 2001), and a number of neuronal culture studies have revealed an important role of IIS in regulation of long-term depression (LTD) or long-term potentiation (LTP), two opposite forms of activity-dependent synaptic

modification (Bear and Malenka 1994). ILPs perform this modification by regulating intracellular trafficking, membrane expression, and activity of ion channels and neurotransmitter receptors (Davila, Piriz et al. 2007; Huang, Lee et al. 2010). For example, insulin or IGF-1 treatment induced LTD in the cerebellum by stimulating clathrin-dependent endocytosis of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Wang and Linden 2000; Ahmadian, Ju et al. 2004). In the hippocampus, insulin has been shown to promote rapid delivery of N-methyl-D-aspartate (NMDA) receptors to the cell surface to increase LTP (Skeberdis, Lan et al. 2001; Chen and Roche 2009). Similarly, activation of NMDA receptors and PI3K signaling are required in insulin-induced LTD or LTP in hippocampal neurons (van der Heide, Kamal et al. 2005).

In addition to regulating synaptic function, IIS also contributes to the structural remodeling of the synapse. For instance, insulin promotes dendritic spine formation in rat hippocampal neurons through activation of the PI3K/Akt/mTOR and Rac1 signaling pathway (Lee, Huang et al. 2011). IIS also regulates synaptic remodeling by activating the PKC signaling pathway (Nelson, Sun et al. 2008), which has been shown to produce dramatic memory-specific changes in the morphology of dendritic spines after spatial training (Hongpaisan and Alkon 2007).

There are also many examples in the literature of the important roles of IIS in promoting regeneration and neuronal survival following various brain injuries. IGFs are well-known neuroprotective agents that induce neurite outgrowth and promote neuronal survival after injury (Ishii, Glazner et al. 1994). The levels of IGF expression

and IGF signaling significantly increase after brain insult (Walter, Berry et al. 1997; Beilharz, Russo et al. 1998; Walter, Berry et al. 1999). IGF-1 knockout mice have been shown to display defects in neurological development and impaired recovery from neuronal injuries (Liu, Baker et al. 1993; D'Ercole, Ye et al. 2002). In rats, exogenous administration of IGF-1 or IGF-2 significantly improved nerve regeneration after nerve crush or transection (Glazner, Lupien et al. 1993; Apel, Ma et al. 2010). In addition to IGFs, insulin itself also functions as a potential neuronal growth factor to support nerve regeneration. *In vitro* research has revealed a direct neurite outgrowth effect of insulin, through its own receptor or through cross–activation of IGF-1 receptors (Recio-Pinto, Rechler et al. 1986; Recio-Pinto and Ishii 1988; Edbladh, Fex-Svenningsen et al. 1994). More direct evidence came from an *in vivo* study on axon regeneration after injury. Intrathecal insulin treatment promoted regeneration of sensory sural nerve axons and functional recovery after nerve crush injuries in rats. This action is associated with the up-regulation of the insulin receptor (Toth, Brussee et al. 2006).

It is clear that IIS plays important roles in regulating neuronal plasticity through modulation of synaptic activity, structural remodeling, and neurite outgrowth after brain injury. However, the investigation of the roles of IIS in the vertebrate brain can be challenging, due to the profound effects of ILPs on metabolic processes in peripheral tissues and the relatively low amount of ILPs in the brain (Banks 2004). These problems may be overcome more readily in model systems. For example, through specific manipulation of the IIS pathway in *Drosophila* neurons, the effects on the brain can be separated from the effects on peripheral tissues, and thus the roles of IIS in this species have recently received substantial attention.

IIS in Drosophila

In *Drosophila*, a highly conserved insulin signaling pathway displays multifaceted functions in metabolism, growth, reproduction, and aging (Wu and Brown 2006). The *Drosophila* genome contains eight insulin-like genes (*dilps*) that display tissue- and stage-specific expression (Brogiolo, Stocker et al. 2001). DILP1, 2, 3, and 5 are highly expressed in and are secreted by seven pairs of neurosecretory cells in the brain (Brogiolo, Stocker et al. 2001; Rulifson, Kim et al. 2002). Genetic ablation of these insulin-producing neurons results in altered metabolism, growth retardation, developmental delay, reduced fecundity, increased resistance to oxidative stress and starvation, and extended lifespan (Rulifson, Kim et al. 2002; Broughton, Piper et al. 2005). DILP6 is produced by the fat body to mediate growth during pupal development, a non-feeding stage (Okamoto, Yamanaka et al. 2009; Slaidina, Delanoue et al. 2009). Expression of DILP6 is also induced by starvation (Slaidina, Delanoue et al. 2009). These results indicate that DILPs act as part of a nutrient sensor system that mediates systemic growth during non-feeding stages. DILP7, which is expressed in a small set of posterior ventral nervous system (VNS) neurons that innervate the female reproductive tract, has no discernible effect on development (Yang, Belawat et al. 2008). Instead, silencing of the DILP7-producing neurons interfered with egg-laying site decision-making in adult females and disrupted ovipositor motor programs (Yang, Belawat et al. 2008). However, the sterility of these female flies is independent of DILP7, as *dilp7* mutants are viable and fertile (Gronke, Clarke et al. 2010). Finally, two recent, independent studies identified a new *dilp* gene, *dilp8*, which is expressed and

secreted from imaginal discs under growth disturbance conditions to coordinate tissue growth and maturation throughout the animal (Colombani, Andersen et al. 2012; Garelli, Gontijo et al. 2012). Together, the ILPs act through regulation of energy homeostasis and growth to mediate divergent responses to developmental and environmental cues.

IIS in neuronal plasticity in Drosophila

Drosophila insulin-like peptide receptor (InR) transcripts are ubiquitously expressed throughout embryogenesis and are then concentrated in the developing nervous system after mid-embryogenesis where they remain at high levels through the adult stage (Garofalo and Rosen 1988). This suggests important roles of IIS in neuronal development and function. However, in contrast to the extensive research on effects of IIS in the vertebrate nervous system (Chiu and Cline 2010), very few studies have been done in invertebrates. Neuronal culture studies on isolated locust CNS revealed a neurotrophic role of insulin in promoting neurite outgrowth (Vanhems, Delbos et al. 1990). In addition, the effect of insulin was enhanced through synergistic interactions with ecdysone. Inhibition of IIS in the mushroom body reduced neuronal proliferation and impaired food intake in *Drosophila* larvae (Zhao and Campos 2012). In *Drosophila* motor neurons, overexpression of components in the IIS pathway, PI3K or Rheb, produced synapse overgrowth and enhanced synapse function (Knox, Ge et al. 2007; Howlett, Lin et al. 2008). These studies suggest a growth regulatory function of IIS in *Drosophila* neurons.

Interaction between IIS and other hormonal signaling pathways in Drosophila

IIS interacts with JH signaling: In recent years, extensive studies on insect development, especially *Drosophila,* have demonstrated cross talk between IIS and ecdysone and JH signaling pathways to regulate tissue growth and developmental timing. Brain insulin-producing cells project their axon terminals to the ring gland, the endocrine organ that produces ecdysone and JH (Ikeya, Galic et al. 2002). This indicates a potential interaction between insulin, ecdysone, and JH signaling. In InR mutant flies, JH levels were reduced and accounted for an extension of lifespan, as JH analog treatment restored normal lifespan (Tatar, Kopelman et al. 2001). This JH level reduction was not due to the inhibition of corpus allatum growth but rather due to effects on central neurons (Tu, Yin et al. 2005). In InR mutant animals, the neurons producing allatropins, a neuropeptide regulator of JH synthesis (Weaver and Audsley 2009), displayed reduced neuropeptide in the axon projections to the ring gland (Tu, Yin et al. 2005). These results suggest that IIS may affect JH production by influencing the neuropeptide regulation of JH synthesis. In addition, a link between IIS and JH signaling has been observed in mosquitos (Sim and Denlinger 2008). Ovarian development was arrested in InR knock down mosquitoes, but it could be rescued by JH application (Sim and Denlinger 2008). Thus, IIS may regulate JH production to influence insect development.

IIS interacts with ecdysone in Drosophila: During larval development, IIS is part of a nutrition sensor system that regulates larval growth until the attainment of critical weight, which is the minimal weight for larvae to enter metamorphosis (Mirth, Truman

et al. 2005). Starvation or a reduction in IIS before the critical weight significantly delays the onset of metamorphosis, whereas no delay occurs if larvae are starved after the critical weight is achieved (Shingleton, Das et al. 2005). After a larva reaches its critical weight, the JH titer declines, causing the release of PTTH, which in turn promotes ecdysone synthesis by the prothoracic gland (PG) to trigger metamorphosis (Nijhout and Williams 1974; Warren, Yerushalmi et al. 2006; Rewitz, Yamanaka et al. 2009). Cell-targeted activation of IIS in the PG caused larvae to prematurely reach their critical size, leading to precocious pupariation and small pupae and adults. In contrast, repression of IIS in the PG delayed the onset of metamorphosis and generated oversized animals (Caldwell, Walkiewicz et al. 2005; Colombani, Bianchini et al. 2005; Mirth, Truman et al. 2005). Ecdysone feeding mimicked the effects of enhanced IIS in the PG and it rescued developmental timing and growth in animals with decreased IIS in their PGs (Colombani, Bianchini et al. 2005). In addition, precocious ecdysone release and up-regulation of the ecdysone biosynthetic genes *phm* and *dib* is seen in flies with elevated IIS in the PG (Colombani, Bianchini et al. 2005). These results suggest that IIS in the PG stimulates ecdysone production and release, which in turn influences the rate and duration of larval growth. In other tissues, ecdysone signaling also directly influences IIS to mediate larval growth and maturation. The feeding of ecdysone to larvae showed cell autonomous repression of IIS in the fat bodies, through reduced PI3K activity and increased FOXO activity (Colombani, Bianchini et al. 2005). Thus, in feeding larvae, interactions of IIS and ecdysone signaling coordinate growth and developmental timing.

Recent work has also revealed interactions between IIS and ecdysone signaling to control growth during non-feeding stages. At the end of larval development, the larva stops feeding and climbs out of the food to initiate wandering, which is followed shortly thereafter by pupal development. During pupal development, the remodeling of larval tissue and morphogenesis of adult tissue both involve substantial cell growth, which is controlled by ecdysone and DILP/PI3K signaling (Ninov, Manjon et al. 2009).

Two labs recently discovered a fat body-derived DILP6, which promotes growth during the non-feeding phase (Okamoto, Yamanaka et al. 2009; Slaidina, Delanoue et al. 2009). DILP6 expression starts at the onset of metamorphosis and persists throughout pupal development. DILP6 mutants display growth defects during the non-feeding stage resulting in smaller adult size, but no alteration of carbohydrate and lipid metabolism. These growth defects can be rescued by expression of DILP6 in the fat body (Okamoto, Yamanaka et al. 2009; Slaidina, Delanoue et al. 2009). In addition, the developmental expression of DILP6 is controlled by ecdysone. Slaidina and colleagues showed that the silencing of EcR in the fat body largely reduced DILP6 expression. In contrast, exogenously applied ecdysone significantly induced DILP6 expression in the dissected larval fat body (Slaidina, Delanoue et al. 2009). Similar findings have been obtained in the silkmoth, *Bombyx mori*. An ecdysone-induced DILP6 from the fat body promotes the growth of adult-specific tissues during pupal development (Okamoto, Yamanaka et al. 2009). This finding suggests that the interactions between IIS and ecdysone signaling in mediating non-feeding growth are conserved in insects.

IIS and ecdysone have also been shown to coordinate tissue growth with developmental timing under various stress conditions, such as altered imaginal tissue growth associated with tumors, x-ray irradiation, and transdetermination, and regeneration after damage or tumor growth in the imaginal discs postponed maturation (Vanhems, Delbos et al. 1990; Garelli, Gontijo et al. 2012). By combining genetic and genomic methods, both labs found a highly induced, new insulin-like peptide, named DILP8 (Vanhems, Delbos et al. 1990; Garelli, Gontijo et al. 2012). The induction of *dilp8* expression and secretion by imaginal discs in turn delays metamorphosis by blocking ecdysone biosynthesis. In addition, DILP8 modulates growth plasticity by controlling growth rates through FOXO to maintain overall proportionality and left-right symmetry (Garelli, Gontijo et al. 2012). Therefore, IIS and ecdysone signaling cooperate closely to regulate growth and maturation during normal feeding (larval) and non-feeding (pupal) development and under abnormal conditions where growth rates are globally regulated.

Evidence of the direct interplay between these two hormonal signaling pathways also comes from a new coactivator of the ecdysone receptor, dDOR (Francis, Zorzano et al. 2010). DOR was first discovered in muscle tissue of diabetic rats (Baumgartner, Orpinell et al. 2007). In *Drosophila* dDOR mutants, the expression of two ecdysone signaling reporter genes, *E75* and *BR-C,* was inhibited in the Kenyon cells of the mushroom bodies. A combination of *in vitro* and *in vivo* studies has suggested that dDOR functions as a coactivator of EcR that is required for maximal transcriptional activity (Francis, Zorzano et al. 2010). Francis and colleagues also identified dDOR as a direct target of FOXO, a key negative regulator in the insulin signaling pathway. It

was known that in the fat body, activation of ecdysone signaling inhibits insulin signaling through reducing PI3K activity and increasing FOXO activity (Rusten, Lindmo et al. 2004; Colombani, Bianchini et al. 2005), and Francis and colleagues also showed that treatment with ecdysone in the fat body increased the expression of *dDOR* in a FOXO-dependent manner (Francis, Zorzano et al. 2010). Since dDOR is also required for maximal activation of ecdysone target genes, dDOR links these important hormonal signaling pathways through a antagonistic relationship.

The cross-talk between IIS and ecdysone signaling may also play a role in metamorphic neuronal remodeling. An early culture study on isolated locust CNS neurons revealed a synergistic relationship between IIS and ecdysone in promoting neurite outgrowth (Vanhems, Delbos et al. 1990). Mutants for *chico*, which encodes a *Drosophila* insulin receptor substrate, displayed significantly impaired olfactory associative learning (Naganos, Horiuchi et al. 2012). These memory formation defects, along with the structural changes in mushroom bodies, were restored by expressing *chico* in the mushroom bodies during development, but not during the adult stage (Naganos, Horiuchi et al. 2012). *Drosophila* mushroom body λ neurons undergo substantial reorganization during metamorphosis, and this neuronal remodeling is required for short-term courtship memory (Redt-Clouet, Trannoy et al. 2012). Therefore, the learning and memory defects of *chico* mutants may be related to the disruption of mushroom body neuron remodeling.

Despite these tantalizing results, there is still very little known about the molecular and cellular mechanisms governing the effects of IIS in metamorphic

neuronal remodeling, and many critical questions remain. How do different external signals coordinate, integrate, and regulate the remodeling process? How do neurons execute their responses to the hormonal regulation? Recent advances in the elucidation of the conserved IIS pathway in *Drosophila* and in understanding the roles of ecdysone in metamorphic neuronal remodeling hold the promise that studies in this genetic model organism will further our general understanding of neuronal plasticity.

The CCAP/bursicon neurons are an excellent model for studies of metamorphic neuronal remodeling

Larval morphology of the CCAP/bursicon neurons

In previous work, we demonstrated that a group of *Drosophila* neuroendocrine cells, the crustacean cardioactive peptide (CCAP)/bursicon producing neurons, are a powerful model system for examining metamorphic neuronal remodeling (Zhao, Gu et al. 2008). Anti-CCAP immunostaining and GFP expression driven by *CCAP-Gal4* in the third-instar larvae have been used to examine the morphology of the CCAP/bursicon neurons (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Zhao, Gu et al. 2008). There are at least two pairs of neurons in the brain, three pairs of neurons in the subesophageal ganglia, and at least 21 pairs of neurons in the ventral nerve cord (VNC) (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Zhao, Gu et al. 2008).

Bursicon is a heterodimeric neuropeptide hormone consisting of proteins encoded by the *burs* and *pburs* (*partner of burs*) genes (Luo, Dewey et al. 2005). Anti-BURS and anti-PBURS immunostaining and EGFP expression driven by *burs-Gal4* in third-instar larvae have been used to reveal the expression patterns of these bursicon

subunits. The α-subunit (BURS) is expressed in most of the CCAP neurons, except the pair in the brain, while the β-subunit (PBURS) displays a more restricted expression pattern consisting of two pairs of neurons in each of the A1-A4 neuromeres (Luo, Dewey et al. 2005; Peabody, Diao et al. 2008). However, the anti-PBURS antibody is of poorer quality than the anti-BURS antibody, and it may underreport the PBURS pattern. Most of the CCAP/bursicon neurons project within the VNC, but five pairs of these neurons (T3 and A1-A4) send efferent projections, via the segmental nerves, to the periphery and terminate on larval body wall muscles 12 or 13, where they form neuroendocrine endings (Hodge, Choi et al. 2005; Vomel and Wegener 2007; Zhao, Gu et al. 2008). Additional abdominal neurons send projections out posterior abdominal nerves, where they terminated blindly before contacting specific peripheral targets (Fig. 2-2 and 2-7).

Remodeling of the CCAP/bursicon neurons during metamorphosis

Although the CCAP/bursicon neurons grow in proportion to overall larval growth, their gross morphology remains relatively constant (see Chapter 2). We call this type of growth "maintenance growth". During metamorphosis, neurons undergo substantial remodeling. First, they prune back the larval dendrites and axons. This process is initiated 3 hr after puparium formation (APF) and completed by around 30 hr APF. The pruning includes both central and peripheral larval axons and dendrites, which are pruned back to the initial process. Then, outgrowth of new adult-specific projections takes place at 15-60 hr APF (Zhao, Gu et al. 2008). The new adult neurites form a peripheral, branch-like axonal arbor with neuroendocrine boutons along nearly

the entire length of the processes, thus differing from the thin, unadorned larval peripheral axons with neuromuscular junction-like endings (Zhao, Gu et al. 2008). During this period, the CCAP/bursicon neuron somata migrate within VNC, more than double in size, and adopt a multiangular shape (Zhao, Gu et al. 2008). We call this metamorphic neuronal outgrowth "organizational growth," as it involves substantial structural changes associated with axonal and dendritic pathfinding and elaboration of new neuronal arbors. At the pharate adult stage, there are typically 14 dorsal neurons in the abdominal ganglion (B_{AG} neurons) and 2 ventrally located neurons in the subesophageal ganglion (B_{SEG} neurons) that express both the α- and β-bursicon subunits at high levels (Luan, Lemon et al. 2006; Peabody, Diao et al. 2008; Zhao, Gu et al. 2008).

The CCAP/bursicon neurons control pupal ecdysis and wing expansion behavior

Cell ablation and cell silencing experiments have shown that the CCAP/bursicon neurons are required at two times during the life cycle, to regulate pupal ecdysis at the onset of metamorphosis and to regulate wing expansion after metamorphosis is completed (McNabb, Baker et al. 1997; Park, Schroeder et al. 2003). Prior to pupal ecdysis, CCAP is released into the hemolymph in response to ecdysone triggering hormone (ETH) to activate the ecdysis motor program (Park, Schroeder et al. 2003). Disruption of the CCAP/bursicon neurons has little effect on larval development, but it produces severe head eversion defects at pupal ecdysis. These animals fail to evert the adult head from the thorax and to fully extend the developing legs and wings, and they typically die at later pupal stages (Park, Schroeder et al. 2003; Dewey, McNabb et al.

2004; Zhao, Gu et al. 2008). Disruption of these neurons during metamorphosis leads to defects in post-adult eclosion events, including cuticular sclerotization and wing expansion behavior (Park, Schroeder et al. 2003; Dewey, McNabb et al. 2004; Zhao, Gu et al. 2008). Although the pupal ecdysis and wing expansion behaviors each last only a few minutes, head eversion and wing expansion defects persist for days and are easy to score (Zhao, Gu et al. 2008). This has allowed us to perform genetic screens to identify factors specifically contributing to metamorphic remodeling through selection of factors that preferentially disrupt wing expansion (and not head eversion).

Insulin signaling regulates neurite growth during metamorphic neuronal remodeling

We previously carried out a large-scale, gain-of-function genetic screen for genes that when overexpressed or mis-expressed in the CCAP/bursicon neurons could disrupt the metamorphic neuronal remodeling of these neurons and induce wing expansion defects (Zhao, Gu et al. 2008). From this screen, we found that overexpression of *foxo* blocked the metamorphic growth of the CCAP/bursicon neurons, resulting in reduced soma sizes, shorter neurites, and failed wing expansion. In contrast, transgenic *foxo* RNAi directed to the CCAP/bursicon neurons significantly increased soma size. Since the function of FOXO is negatively regulated by the IIS pathway, we then examined the role of IIS in metamorphic remodeling of the CCAP/bursicon neurons (Chapter 2).

In *Drosophila*, the DILPs bind to a single InR to activate an insulin receptor substrate (IRS), encoded by *chico*, which together with the PI3K adaptor protein, P60,

recruits PI3K to the cell membrane (Bohni, Riesgo-Escovar et al. 1999; Weinkove, Neufeld et al. 1999; Brogiolo, Stocker et al. 2001). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Engelman, Luo et al. 2006). This process can be reversed by Phosphatase and Tensin Homolog (PTEN), which thereby inhibits IIS (Goberdhan, Paricio et al. 1999; Huang, Potter et al. 1999; Gao, Neufeld et al. 2000). PIP3 recruits phosphoinositol-dependent kinase (PDK) to the cell membrane to activate Akt (protein kinase B) (Verdu, Buratovich et al. 1999). Activation of Akt inhibits the function of FOXO through phosphorylation, which blocks its nuclear translocation (Puig, Marr et al. 2003). When unphosphorylated, nuclear FOXO functions as a growth suppressor by promoting expression of the translational repressor, 4E-Binding Protein (4E-BP) (Junger, Rintelen et al. 2003; Puig, Marr et al. 2003). Activation of Akt also inhibits Tuberous Sclerosis Complex 1 and 2 (TSC1/TSC2), which form a heterodimer that negatively regulates Ras homolog enhanced in brain (Rheb), an activator of the Target of Rapamycin (TOR) complex (Saucedo, Gao et al. 2003; Wullschleger, Loewith et al. 2006). The activated TOR complex promotes growth through either phosphorylation of ribosomal protein kinase p-70-S6 (S6K) to increase protein synthesis or inhibition of 4EBP to enhance translation (Jaeschke, Hartkamp et al. 2002; Garami, Zwartkruis et al. 2003; Stocker, Radimerski et al. 2003). Therefore, to examine whether an increase in IIS can counteract the effects of *foxo* overexpression, we co-overexpressed *InR* or *PI3K* or *Akt* together with *foxo* in the CCAP/bursicon neurons (Chapter 2). The phenotypes of *foxo* overexpression were completely rescued by any of these three components of the IIS pathway. We then demonstrated the role of IIS in metamorphic outgrowth of the

CCAP/bursicon neurons through manipulation of the levels of both positive and negative components of the IIS pathway. CCAP/bursicon cell-targeted expression of dominant negative or RNAi constructs for InR, PI3K, and Akt suppressed neurite outgrowth and reduced soma size. In contrast, expression of wild-type or constitutively active form of InR, PI3K, Akt, Rheb, and Target of rapamycin (TOR), as well as RNAi for negative regulators of the IIS pathway (PTEN, FOXO), stimulated neurite overgrowth (Chapter 2).

Although our results displayed a profound effect of IIS in regulating the organizational growth of the CCAP/bursicon neuron somata and neurite arbors during metamorphosis, we observed little if any requirement for IIS for the normal maintenance growth of these neurons in larvae. We further examined the role of IIS in a pan-peptidergic neuronal pattern, which indicated the general role of IIS in controlling the metamorphic (organizational) growth of many neurons, with most neurons relatively refractory to IIS in larvae. Taken together, these findings reveal a fundamental shift in growth control mechanisms as neurons are remodeled, and they highlight an important role of IIS in this process. These findings are described in Chapter 2.

A *Drosophila* deficiency screen for modifiers of *foxo* and IIS during metamorphic neuronal remodeling

How does IIS interact with other biological factors/pathways, such as the ecdysone signaling pathway, to facilitate organizational growth during metamorphic neuronal remodeling? To address this general question, we carried out a genetic modifier screen for IIS-interacting genes (Chapter 3). Since the wing expansion

phenotype produced by *foxo* overexpression in the CCAP/bursicon neurons is easy to score and is sensitive to genetic interactions with InR, PI3K, and Akt, we conducted a deficiency-based screen for modifiers of the *foxo* overexpression phenotype. We screened 492 Exelixis, DrosDel, and Bloomington Stock Center (BSC) deficiencies with isogenic backgrounds and molecularly defined endpoints on the 1st, 2nd, and 3rd chromosomes, and together these deficiencies covered about 56% of the genome (Parks, Cook et al. 2004). A total of 14 deficiencies were confirmed as suppressors of *foxo* and 19 were confirmed as enhancers. One selected suppressor was mapped to a single gene, *Su(z)2*. Reduced expression of *Su(z)2* suppressed the effects of FOXO on neuronal outgrowth. Su(z)2 is a zinc finger protein in the *Drosophila* Polycomb Group (PcG) protein family, the members of which function as negative regulators of transcription through inhibiton of chromatin modifiers (Brunk, Martin et al. 1991). Here, our results reveal the function of *Su(z)2* in regulating neuronal remodeling through modification of the effects of FOXO during metamorphosis. Thus, the regulation of chromatin modification by PcG may play an important role in reprogramming neurons to re-enter a organizational growth phase. These findings are described in Chapter 3.

CHAPTER 2

Insulin signaling regulates neurite growth during ecdysone-dependent neuronal remodeling

Abstract

As neurons mature, their capacity for growth is often greatly reduced. However, under some circumstances such as regeneration following injury, they can return to a more embryonic state to undergo organizational growth. The mechanisms governing the transitions of neurons from the relatively stable maintenance state to an organizational growth state are largely unknown. In holometabolous insects, there is a major transition from maintenance growth to organizational growth near the onset of metamorphosis. Many differentiated larval neurons are maintained through metamorphosis and undergo extensive remodeling, involving the elimination of larval dendrites and axons (neurites) and the outgrowth and elaboration of adult-specific projections (Levine and Truman 1982; Brown, Cherbas et al. 2006). Here, we show that a metamorphosis-specific increase in insulin/insulin-like-growth factor signaling (IIS) promotes neuronal growth and axon branching after a long period of stability during the larval stages. In a previous gain-of-function genetic screen (Zhao, Gu et al. 2008), we found that overexpression of a negative effector in the IIS pathway, Forkhead box, sub-group O (FOXO), blocked metamorphic growth of peptidergic neurons that secrete crustacean cardioactive peptide (CCAP) and bursicon. RNA interference (RNAi) and CCAP/bursicon cell-targeted expression of dominant negative constructs for other components of the IIS pathway [Insulin-like receptor (InR), Pi3K92E, Akt1, and S6K] also partially suppressed the

growth of the CCAP/bursicon neuron somata and neurite arbor. In contrast, expression of wild-type or constitutively active forms of InR, Pi3K92E, Akt1, Rheb, and Target of rapamycin (TOR), as well as RNAi for negative regulators of the IIS pathway (PTEN and FOXO), stimulated overgrowth. Interestingly, InR displayed little effect on larval growth of the CCAP/bursicon neurons, in contrast to the strong effects on the metamorphic growth of these neurons. In addition, manipulations of IIS in a pan-peptidergic neuronal pattern revealed a general role in promoting the growth of many neurons during metamorphosis, but not during larval development. Taken together, these results reveal that specific activation of IIS during metamorphosis supports renewed organizational growth in mature neurons.

Introduction

Although fully differentiated neurons have relatively stable morphologies, they nevertheless undergo dynamic structural changes in order to sustain their functions. These maintenance growth processes include the recycling of membrane and other components of the cell (Kelly 1993; Zimmermann, Volknandt et al. 1993), the expansion or retraction of synaptic contacts (Zito, Parnas et al. 1999; Eaton, Fetter et al. 2002), and growth in proportion to changes in tissue size (Bentley and Toroian-Raymond 1981; Loesch, Mayhew et al. 2010). For example, mouse lumbar spinal motoneurons significantly elongate and thicken their dendritic branches, while maintaining their dendritic topology, in concert with the overall growth of surrounding tissue during the first two weeks of postnatal development, (Li, Brewer et al. 2005). Similarly, the *Manduca sexta* larval motoneurons MN-1 and MN-3 display proportional

growth in relation to overall body size – a process called allometric growth (Truman and Reiss 1988). In larval *Drosophila melanogaster*, sensory neurons also increase the sizes of their dendritic arbors in proportion to larval growth while maintaining their overall morphology and functional properties (Parrish, Xu et al. 2009).

Neurons display another, organizational form of growth that is associated with axonal and dendritic pathfinding and elaboration of new neuronal arbors. Organizational growth is normally restricted to the initial differentiation of neurons, but it also occurs in fully differentiated neurons under certain situations, such as during puberty, insect metamorphosis, and seasonal changes in bird song control centers, and in response to injury, stroke, or neurological disease (Levine and Truman 1982; Finger and Almli 1985; Brenowitz 2004; Blakemore and Choudhury 2006; Benowitz and Carmichael 2010). Mature neurons vary widely in their capacities to undergo organizational growth (Holm and Isacson 1999; Goldberg and Barres 2000), and the factors contributing to these differences are poorly understood. Regulators of neuronal organizational growth, such as neurotrophic factors, cell adhesion molecules, and modulators of cytoskeletal reorganization, are associated with neurodegenerative diseases (Mattson 1990; Cotman, Hailer et al. 1998; Kao, Davis et al. 2010). Thus, there is intense interest in finding ways to stimulate organizational growth in neurons to counter nervous system damage (Maier and Schwab 2006; Mattson 2008; Zhang, Yeh et al. 2008).

Insect neurons are a powerful model for examining transitions between maintenance and organizational growth and for studying differences in the control of

these distinct growth processes. In holometabolous insects, many fully differentiated larval neurons exhibit maintenance growth during the larval stages and a second, post-embryonic phase of organizational growth during metamorphosis. During this latter process many larval neurons are retained and undergo significant structural remodeling; larval axons and dendrites (neurites) are pruned back, and this is followed by the outgrowth of adult projections (Witten and Truman 1996).

The *Drosophila melanogaster* CCAP/bursicon neurons provide an excellent genetic model to examine post-embryonic organizational growth (Zhao, Gu et al. 2008). These neurons secrete multiple neuropeptides, including bursicon and CCAP, to regulate molting behaviors (Park, Schroeder et al. 2003; Dewey, McNabb et al. 2004). In larvae, the CCAP/bursicon neurons consist of at least 3 pairs of neurons in the brain. Two of them express the CCAP neuropeptide but not bursicon, while the other produces bursicon neuropeptide but not CCAP (Dewey, McNabb et al. 2004; Zhao, Gu et al. 2008). There are 3-4 pairs of CCAP/bursicon neurons in the lateral subesophageal ganglia, and at least 21 pairs of neurons in the ventral nerve cord (VNC) (Hodge, Choi et al. 2005; Vomel and Wegener 2007; Zhao, Gu et al. 2008). Most of these neurons project within the VNC, but several abdominal pairs send efferent projections via segmental nerves to the periphery to terminate on larval body wall muscles 12 and 13, where they form neuroendocrine endings (Hodge, Choi et al. 2005; Vomel and Wegener 2007; Zhao, Gu et al. 2008) and additional efferents terminate in the more posterior abdominal nerves (this study). The morphology of the CCAP/bursicon neurons is maintained throughout larval development, but they grow more than two-fold in size in proportion to the overall larval growth (Fig. 2-1). During metamorphosis, the larval

axons and dendrites are pruned back almost to the cell bodies, followed by outgrowth of adult-specific neurites, which include a peripheral tree-like axonal arbor with neuroendocrine boutons along nearly the entire length of the processes (Zhao, Gu et al. 2008).

Cell ablation and cell silencing experiments have shown that the CCAP/bursicon neurons are essential for completion of two events in the life cycle, pupal ecdysis at the onset of metamorphosis and wing expansion, which occurs after metamorphosis is completed and the adult has eclosed (McNabb, Baker et al. 1997; Park, Schroeder et al. 2003). Disruption of the CCAP/bursicon neurons prior to pupal ecdysis produces animals that fail to evert the adult head from the thorax and to fully elongate the developing adult legs and wings. Later perturbation of the CCAP/bursicon neurons during metamorphosis leads to viable and fertile adults with permanently unexpanded wings (Park, Schroeder et al. 2003; Dewey, McNabb et al. 2004; Zhao, Gu et al. 2008). Although the pupal ecdysis and wing expansion behaviors each last only a few minutes, the resulting head eversion and wing expansion defects persist for days and are easy to score, even by the unaided eye (Park, Schroeder et al. 2003; Dewey, McNabb et al. 2004; Zhao, Gu et al. 2008). Therefore, we can conduct large-scale genetic screens for factors that contribute selectively to organizational growth by selecting for genetic alterations in the CCAP/bursicon neurons that preferentially disrupt wing expansion.

In vertebrates, insulin and insulin-like growth factor 1 (IGF-1) are both important regulators of nervous system growth and maturation. IGF-1 has well-established functions in controlling neuronal growth, survival, plasticity, and cognitive

function throughout the lifespan (Aleman and Torres-Aleman 2009). Insulin, a critical regulator of nutrient homeostasis, has also been implicated more recently in the morphogenesis, functioning, and development of the central nervous system (Chiu and Cline 2010; Huang, Lee et al. 2010). Several neuronal cell culture studies have revealed a role for insulin receptor signaling in regulating neurite growth (Govind, Kozma et al. 2001; Choi, Ko et al. 2005), and *in vivo* studies in retinotectal circuits of the frog *Xenopus laevis* have shown that insulin receptor signaling is required for dendritic arborization (Chiu, Chen et al. 2008).

The IIS pathway has been highly conserved throughout evolution. The structure of the mature peptide hormone, consisting of an A and B peptide connected by disulfide bonds, is shared by mollusks, nematodes, insects, and humans (Conlon 2001; Claeys, Simonet et al. 2002). These peptides act on a small family of closely related receptor tyrosine kinases that stimulate a canonical intracellular signaling pathway (Claeys, Simonet et al. 2002). In *Drosophila*, the insulin-like peptides are encoded by eight genes (*dilp1-8*) and are produced in the central nervous system (CNS), gut, imaginal disks, and fat body (Brogiolo, Stocker et al. 2001; Colombani, Andersen et al. 2012; Garelli, Gontijo et al. 2012). Once secreted, all of the DILPs are thought to bind and activate a single *Drosophila* insulin-like-receptor (InR) (Brogiolo, Stocker et al. 2001), which in turn activates insulin receptor substrate (IRS). IRS activates a series of kinases, including phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B, to regulate metabolism, cell and tissue growth, longevity, and neuronal events (Saltiel and Kahn 2001; Ikeya, Galic et al. 2002; Rulifson, Kim et al. 2002; Broughton and Partridge 2009; Naganos, Horiuchi et al. 2012). For instance, overexpression of components in

the IIS pathway, such as PI3K or Ras-homolog enriched in brain (Rheb), produced synapse overgrowth and enhanced synapse function in *Drosophila* larval motor neurons (Knox, Ge et al. 2007; Howlett, Lin et al. 2008). A recent study has shown that the TOR pathway, one of the major downstream arms of the IIS pathway, is required in axon outgrowth of Mushroom Body (MB) γ neurons (post-pruning) during metamorphosis (Yaniv, Issman-Zecharya et al. 2012).

Here, we examined the role of IIS in growth of the CCAP/bursicon neurons. Our results show that signaling through InR strongly regulates the organizational growth of the CCAP/bursicon neuron cell bodies and neurite arbor during metamorphosis, but IIS plays only a small role in maintenance growth of the larval CCAP/bursicon neurons. We tested whether IIS regulates the growth of other CNS neurons, and in most cases, the organizational growth seen during metamorphosis was substantially more sensitive to IIS than larval maintenance growth. However, there were strong cell type-specific differences in the extent to which IIS controlled the organizational growth of various neurons. These findings reveal a fundamental shift in growth control mechanisms as many neurons are remodeled, and they highlight an important role of IIS in this process.

Results

Overexpression of foxo *disrupts metamorphic growth of the CCAP/bursicon neurons*

In a previous gain-of-function genetic screen, we found that overexpression of

foxo (*forkhead box, sub-group O*; FlyBase ID FBgn0038197) in the CCAP/bursicon neurons disrupted normal wing expansion (Zhao, Gu et al. 2008). Since FOXO is a negative regulator of IIS (Puig, Marr et al. 2003), we examined this phenotype further to determine the timing and extent of IIS regulation of CCAP/bursicon neuron growth in larvae and during metamorphosis. We also examined the effects of FOXO loss-of-function manipulations (see below).

Following single crosses to bring the Gal4 and UAS elements together, all flies expressing *UAS-FOXO* under the control of a *CCAP-Gal4* driver had completely folded wings as adults at 25°C (n=122). Since the CCAP/bursicon neurons are essential for initiation of wing expansion behavior (Park, Schroeder et al. 2003; Dewey, McNabb et al. 2004; Zhao, Gu et al. 2008), this result suggested that *foxo* overexpression disrupts the development, function, or survival of these neurons. To test this hypothesis, we performed anti-bursicon immunostaining on stage P14 pharate adult CNS (Bainbridge and Bownes 1981) (Fig. 2-2). We observed a 65% reduction in the number of bursicon-immunopositive somata (Fig. 2-2A and D), and the remaining cells displayed abnormal morphology, with largely reduced soma size, reduced peptide expression, and a near complete loss of central and peripheral neurites (Fig. 2-2A). We also observed the same loss of cell bodies and neurites due to *foxo* overexpression and detected using membrane-associated mCD8::GFP expressed under the control of *CCAP-Gal4* (data not shown).

We observed less severe phenotypes produced by *foxo* overexpression in the same group of neurons under a different, but strong Gal4 driver, *bursicon-Gal4*,

although the level of GFP expression driven by *bursicon-Gal4* was significantly higher than the expression driven by *CCAP-Gal4* (Fig. 2-3B and C). At the P14 pharate adult stage, most of the CCAP/bursicon neurons with *foxo* overexpression driven by *bursicon-Gal4* remained (Fig. 2-3A middle panels and D), albeit with largely reduced somata sizes and reduced branching in the peripheral axon arbor. In addition, the pharate adult somata displayed a linear arrangement reminiscent of the larval stage (Fig. 2-3A middle panels). We speculate that the differences between the responses of these neurons to *foxo* overexpression are due either to cell-cell interactions (there are fewer cells in the *bursicon-Gal4* pattern), insertion position effects, or difference in the genetic backgrounds of these strains.

We then asked whether *foxo* overexpression disrupted the normal metamorphic remodeling of the CCAP/bursicon neurons, or their earlier development (Fig. 2-2 B-G). We conducted anti-BURS immunostaining on wandering 3rd instar stage larvae, which are entering the early stages of metamorphosis. All CCAP/bursicon cell somata were present. Moreover, there were no statistically significant changes in soma size (Fig. 2-2E), bouton number at the larval neuromuscular junctions (NMJ) (Fig. 2-2F), or area covered by the NMJ (Fig. 2-2G). This demonstrated that *foxo* overexpression specifically inhibited growth of the CCAP/bursicon neurons during metamorphic remodeling.

During metamorphic remodeling of the CCAP/bursicon neurons, the pruning of larval neurites peaks at approximately 12 hr after puparium formation (APF) and continues until ~30 hr APF. Peak outgrowth of adult neurites occurs at 36-54 hr APF,

and outgrowth is largely completed at ~60 hr APF (Zhao, Gu et al. 2008). To determine whether pruning and/or outgrowth was disrupted by *foxo* overexpression, we examined anti-bursicon immunostaining on the CCAP/bursicon neurons at key stages during metamorphic remodeling. There were no changes observed at 0 hr APF, near the onset of metamorphosis. However, at 24 hr APF, when pruning of larval neurites is largely complete (Zhao, Gu et al. 2008), we observed an equal extent of pruning in *foxo* overexpressing cells (*CCAP-Gal4>UAS-FOXO*) and controls (*CCAP-Gal4>+*) (Fig. 2-4). At 48 hr APF, when pruning in control cells is complete and adult neurite outgrowth is well underway (Zhao, Gu et al. 2008), the *foxo* overexpressing cells displayed reduced soma sizes (Fig. 2-4) and a much smaller and less branched peripheral axon arbor (data not shown). Therefore, *foxo* overexpression spared neurite pruning, but the growth (or maintenance) of adult-specific neurites was largely blocked, and many neurons disappeared altogether (or ceased to express bursicon and *CCAP-Gal4*). Because *foxo* overexpression under the *bursicon-Gal4* driver, and other IIS manipulations with the *CCAP-Gal4* driver, did not result in substantial cell loss, we did not determine whether the loss in *CCAP>UAS-FOXO* animals was due to cell death, although FOXO has known neurotoxic functions in other systems (Kanao, Venderova et al. 2010; Siegrist, Haque et al. 2010).

InR regulates metamorphic growth of the CCAP/bursicon neurons

The IIS pathway negatively regulates FOXO. Specifically, Akt, a key downstream component of the pathway, phosphorylates FOXO, thereby blocking its nuclear translocation and thus its transcriptional regulatory functions (Puig, Marr et al. 2003). Since the IIS pathway can inhibit the function of FOXO, we tested whether co-

overexpression of *foxo* and other genes that positively regulate the IIS pathway [*insulin receptor* (*InR*) and *PI3K*], would restore the normal development and function of the CCAP/bursicon neurons. When either *InR* or *PI3K* was overexpressed with *foxo*, we found that all of the adult progeny had fully expanded wings, and the morphology of the CCAP/bursicon neurons was restored to normal (Fig. 2-5 and data not shown). The above results implicated IIS in the regulation of CCAP/bursicon neuron remodeling. To test this directly, we changed the level of IIS through cell-targeted downregulation and upregulation of InR function and examined the effects of altered IIS on the CCAP/bursicon neurons at pharate adult stage (Fig. 2-6). Down-regulation of InR by expression of a dominant negative mutant of *InR* (InR^{K1409A}, hereafter referred to as InR^{DN}) or InR^{RNAi} in the CCAP/bursicon neurons reduced the soma area to 30% of normal (Fig. 2-6C-E) and the peripheral axon arbor area to 38% of normal (Fig. 2-6C′, D′ and F). In addition, the number of peripheral axon branches was reduced by 40% (Fig. 2-6C′, D′, and G). Overexpression of *InR* or expression of a constitutively active mutant of *InR* (InR^{R418P}, hereafter referred to as InR^{act}) (Parks, 2004; (Wu, Zhang et al. 2005) in the CCAP/bursicon neurons led to a 208% increase in soma area (Fig. 2-6B and E). In addition, the area covered by the peripheral axon arbor was increased to 189% of controls (Fig. 2-6B′ and F), and the number of branches in the peripheral axon arbor was increased to 140% of normal (Fig. 2-6B and G). These results showed that CCAP/bursicon neuron soma growth, peripheral axon arbor growth, and axon branching during metamorphosis are strongly dependent on the activity of InR.

Since the major impacts of *foxo* overexpression in the CCAP/bursicon neurons were observed during metamorphosis (and not in larvae), we wondered whether altered InR function would also affect metamorphic growth of these neurons in a stage-dependent manner. To test this hypothesis, we conducted anti-bursicon immunostaining on wandering 3rd instar stage larvae expressing InR^{DN} or InR^{act} in the CCAP/bursicon neurons (Fig. 2-7). In control larvae, five pairs of neurons have efferent axons that terminate on muscles 12 and 13 to form neuromuscular junctions (NMJ)-like endings in 5 central body segments (Fig. 2-7A). The gross morphology of the CCAP/bursicon neurons and the peripheral axon projections of the efferent neurons were essentially unchanged following InR^{DN} or InR^{act} expression (Fig. 2-7B and C).

We also examined the effects of InR manipulations on larval CCAP/bursicon neuron soma size and NMJ. In the abdominal ganglia, there are 8 lateral pairs of abdominal CCAP/bursicon neurons on each side of the CNS. Within each 'a/b' neuron pair, the 'a' neuron has a higher level of bursicon expression then 'b' (Hodge, Choi et al. 2005; Zhao, Gu et al. 2008). We measured the soma size of A1a (the a cell in abdominal ganglion 1), A4a, and A7a, and the NMJ bouton number in segment 4. Interestingly, InR^{DN} had no statistically significant effect on soma size (Fig. 2-7B′ and D), the area covered by the larval NMJ (Fig. 2-7B″), and the larval NMJ bouton number (Fig. 2-7E). Similarly, cell-targeted expression of InR^{RNAi} with *dicer2* in the *CCAP/bursicon* neurons had no significant effect on these cellular parameters (Fig. 2-8). These results indicate that IIS plays little if any role in normal soma and synapse growth of the CCAP/bursicon neurons during larval development. However, we did observe a significant increase in soma size with InR^{act} expression in the cells in more posterior segments (Fig. 2-7C′ and D),

which suggests that the IIS pathway components are present and functional to some degree in larval CCAP/bursicon neurons, even if they are not normally active. In conclusion, IIS strongly promotes metamorphic outgrowth of the CCAP/bursicon neurons, but the effects of IIS on larval growth of these neurons are very limited.

FOXO and TSC/TOR regulate metamorphic outgrowth by the CCAP/bursicon neurons

Given that InR regulates the growth of the CCAP/bursicon somata and peripheral axon arbor, we then asked whether other components of the canonical IIS pathway also promoted these aspects of metamorphic development. First, we looked at the effects of PI3K, Akt, and Phosphatase and tensin homolog (PTEN), all of which are key upstream components of the IIS pathway (Wu and Brown 2006). The kinases PI3K and Akt are two positive regulators of the IIS pathway, whereas PTEN functions as a negative regulator of this pathway by inhibiting PI3K signaling. Increased IIS in the pharate adult stage, through cell-targeted expression of PI3K, $PI3K^{act}$, Akt, or $PTEN^{RNAi}$, strongly stimulated growth of both the cell bodies and peripheral axon arbor (Fig. 2-9). In contrast, decreases in IIS through RNAi to PI3K and Akt, as well as PTEN overexpression, suppressed neurite branching and growth of the CCAP/bursicon neuron somata (Fig. 2-9). These actions of PI3K, Akt, and PTEN in the CCAP/bursicon neurons further confirmed the role of insulin signaling in regulating the outgrowth of these neurons during metamorphosis.

The IIS pathway regulates cellular processes through at least three distinct downstream branches, the FOXO, Tuberous Sclerosis Complex (TSC)/Target of Rapamycin (TOR), and Shaggy (SGG)/Glycogen synthase kinase3 (GSK3) pathways

(Junger, Rintelen et al. 2003; Oldham and Hafen 2003; Papadopoulou, Bianchi et al. 2004; DiAngelo and Birnbaum 2009). In *Drosophila*, SGG has been shown to play an important role in the circadian clock (Martinek, Inonog et al. 2001), but it has little effect on IIS-stimulated cell growth (Papadopoulou, Bianchi et al. 2004; DiAngelo and Birnbaum 2009). Therefore, we did not examine the role of SGG in growth regulation of the CCAP/bursicon neurons, and we instead focused on FOXO and TSC/TOR. Given that *foxo* overexpression produced a strong phenotype (Fig. 2-2), we first examined the effects of *foxo* loss-of-function. Following CCAP/bursicon cell-targeted *foxo* RNAi, 98% of the adults displayed unexpanded wings (UEW), and the rest had partially expanded wings (PEW) (n=54). In addition, soma size was increased (Fig. 2-10G and I), although the size of the peripheral axon arbor was unchanged (Fig. 2-10L). These results suggest that the FOXO arm of the IIS pathway is involved in the regulation of CCAP/bursicon soma growth during metamorphic remodeling, whereas outgrowth and branching of the CCAP/bursicon peripheral axon arbor is regulated by other downstream targets of IIS.

We next examined whether TSC/TOR mediated the effects of IIS on axon growth and branching in the pharate adults. Activation of IIS inhibits TSC1/TSC2, a heterodimer that negatively regulates Rheb, an activator of the TOR complex (Saucedo, Gao et al. 2003; Wullschleger, Loewith et al. 2006). The TOR complex promotes growth through either phosphorylation of ribosomal protein kinase p-70-S6 (S6K) to increase protein synthesis, or inhibition of 4EBP to enhance translation (Jaeschke, Hartkamp et al. 2002; Garami, Zwartkruis et al. 2003; Stocker, Radimerski et al. 2003). Activation of the TSC/TOR arm of the IIS pathway through CCAP/bursicon-targeted expression of *UAS-Rheb* completely blocked wing expansion in adults (n=100), increased soma size, and produced an expanded

peripheral axon arbor (Fig. 2-10B and B′). Activation of the TSC/TOR arm of the IIS pathway by RNAi to TSC1 also resulted in flies with 36% UEW and 64% expanded wings (n=14) (the wing expansion phenotype for RNAi to TSC2 was not tested). Similarly, RNAi to S6K produced flies with 34% UEW, 11% PEW, and 55% expanded wings (n=44). We also observed a significant increase in soma size and peripheral axon arbor folowing RNAi to TSC1 and TSC2, and decrease in soma size and arborization produced by $S6K^{RNAi}$ (Fig. 2-10D-F and D′-F′). The fact that both over- or under-growth of the CCAP/bursicon neurons through cell-targeted stimulation or inhibition of IIS in these cells could lead to wing expansion defects suggests that the normal morphology of the CCAP/bursicon neurons is required for proper functioning of the cellular network. These results also reveal an important role of the TSC/TOR arm of the IIS pathway in regulating metamorphic outgrowth of the CCAP/bursicon neurons.

IIS regulates growth of the Tv neurons during metamorphosis

We next asked whether IIS play a universal role in regulating the metamorphic remodeling of all cell types or a cell-type specific role. To address this question, we first studied another class of neuroendocrine neurons, Tv neurons, for which there are excellent cell markers (e.g., anti-RFamide neuropeptide antibodies) and in which neuronal remodeling during metamorphosis has been well characterized (Brown, Cherbas et al. 2006). We targeted expression of *UAS-FOXO*, $UAS\text{-}InR^{act}$, and $UAS\text{-}InR^{DN}$ to the Tv neurons and conducted anti-RFamide immunostaining (Benveniste, Thor et al. 1998) on pharate adult animals. Similar to the effects in the CCAP/bursicon neurons (Fig. 2-6B), expression of InR^{act} led to a 28% increase in Tv neuron soma size and a 36% increase in the area covered

by the adult peripheral axon arbor (Fig. 2-11B and E-G). In contrast, cell-targeted expression of InR^{DN} in the Tv neurons significantly reduced soma size to 81% of normal (Fig. 2-11C and E), although there was no significant difference in the area covered by the axon arbor (Fig. 2-11F and H). The latter measurements may underreport the effects of IIS on Tv neuron axon branching and outgrowth, since the arbor has a highly variable and complex branching pattern. Nevertheless, it is clear that changes in IIS led to changes in soma growth and neurite outgrowth, although the effects were more modest in comparison to the changes seen in the CCAP/bursicon neurons (Fig. 2-6B-C and B′-C′).

IIS regulates the organizational growth, but not maintenance growth, of many peptidergic neurons

The effects of IIS on organizational growth of two groups of neurons, the CCAP/bursicon neurons and the Tv neurons, suggests that IIS may regulate the growth of many neurons during metamorphosis. To test this hypothesis, we manipulated InR activity under the control of *386-Gal4*, a pan-peptidergic driver (Taghert, Hewes et al. 2001). It is difficult to separate and quantify changes in the neurites of single neurons within such a broad neuronal pattern, and the effects of IIS on soma size generally paralleled the ones on neurites in the CCAP/bursicon and Tv neurons (e.g., Fig. 2-6 and Fig. 2-11). Therefore, we measured soma size as a way of characterizing the effects of InR on growth of diverse peptidergic neurons in the *386-Gal4* pattern. Based on soma morphologies and locations, we selected five different groups of neurons that were easily distinguished at the wandering 3rd instar larval stage (groups a to e) and five distinct groups of neurons that were identifiable at the pharate adult stage (groups f to j) (Fig. 2-12A and B). For example, the

larval group c neurons had large, round somata located along the central, dorsal midline in the larval ventral nerve cord (Fig. 2-12A and C), while the pharate adult group h neurons (the insulin-producing cells (IPCs)) had large, triangular somata located in the medial protocrebrum of the brain (Fig. 2-12B and C). For each group, we measured the cross-sectional area of cells labeled with the CD8::GFP marker. In larvae, four of the five cell types displayed no change in soma size in response to InR^{act} or InR^{DN} (Fig. 2-12C and D). In contrast, all five groups of pharate adult neurons displayed marked changes in soma size in response to the changes in InR (Fig. 2-12C and E). In general, the growth of most larval neurons appeared refractory to changes in IIS, whereas most neurons were highly responsive during metamorphosis. These results suggest that the stage-dependent regulation of CCAP/bursicon growth by IIS is representative of many neurons.

Heterogeneity in responses of the CCAP/bursicon neurons to IIS

Even within the CCAP/bursicon neuron group, not all cells responded equally to changes in IIS. At the pharate adult stage, busicon is highly expressed in 14 dorsal neurons in the abdominal ganglia (B_{AG} neurons) and 2 ventral neurons in the subesophageal ganglia (B_{SEG} neurons) (Luan, Lemon et al. 2006; Peabody, Diao et al. 2008; Zhao, Gu et al. 2008). Within this population, we observed a gradient of responses to changes in IIS. To quantify this effect, we labeled the B_{AG} neurons 1 to 14 based on their positions (posterior to anterior), and measured the soma size for cells in positions 3, 6, and 9. While soma size was significantly altered by InR^{act} and InR^{RNAi} in all three locations, these effects were substantially greater for the more anterior position (cell 9) (Fig. 2-13). In fact, almost all of the manipulations of IIS components that we

have tested have greater effects on the more anterior B_{AG} neurons (Table 1). In contrast, the B_{SEG} soma size was generally insensitive to IIS manipulations (data not shown). In addition, similar to the CCAP/bursicon neuron group, we measured the change in soma size of the Tv neurons in the three different thoracic ganglia in response to the expression of InR^{act} and InR^{DN}. The Tv neurons also displayed heterogeneous responses to changes in IIS, although in this case, the more posterior neurons exhibited the greatest changes in soma size (data not shown). Therefore, even among populations of neurons with similar morphologies and transmitters, IIS exerted differential effects based on segment identity.

Local source of IIS for regulation of metamorphic growth of the CCAP/bursicon neurons

In *Drosophila*, insulin-like peptides are encoded by eight genes, *dilp1-8* (Brogiolo, Stocker et al. 2001; Colombani, Andersen et al. 2012; Garelli, Gontijo et al. 2012), and which differ in their spatial and temporal expression patterns. To determine which DILP(s) regulate metamorphic growth of the CCAP/bursicon neurons, we tested three of the known sources of circulating DILP hormones: the brain IPCs, the fat body, and the VNC IPCs (Fig. 2-14). The brain IPCs are seven pairs of cells in each brain hemisphere that synthesize DILP 2, 3 and 5 and secrete these hormones into the hemolymph to regulate glucose homeostasis and growth (Brogiolo, Stocker et al. 2001; Ikeya, Galic et al. 2002). We ablated the brain IPCs by expressing the pro-apoptotic cell death genes, *reaper (rpr)* and *hid* (Schetelig, Nirmala et al. 2011) under the control of the *dilp2* promoter (Rulifson, Kim et al. 2002). In this cross, only the female

progeny contained the *UAS-rpr* and *UAS-hid* transgenes. The male progeny were normal, but in females, the developmental time was extended from 12 to 22 days at 25 ℃, and the body size was substantially reduced (data not shown), consistent with earlier findings (Rulifson, Kim et al. 2002). Nevertheless, in females we observed normal metamorphic growth of the CCAP/bursicon neurons (Fig. 2-14A and D), indicating that the brain IPCs were not necessary for this growth. We next tested DILP6, which is highly expressed in the fat body after the late 3rd instar and is secreted into hemolymph to regulate growth during metamorphosis (Okamoto, Yamanaka et al. 2009; Slaidina, Delanoue et al. 2009). We altered the level of DILP6 by expression of *UAS-dilp6* or of *UAS-dilp6*^{RNAi} with *UAS-dicer2* under the control of a fat body-specific driver, *cgg-Gal4*. Neither of these *dilp6* manipulations had any effect on the CCAP/bursicon neurons (Fig. 2-14C and F), even though both constructs have been reported to markedly alter post-feeding growth regulation when expressed in fat body tissue (Okamoto, Yamanaka et al. 2009). Finally, we examined the role of DILP7 in the dMP2 neurons, which are located in the posterior of the ventral nerve cord and innervate the female reproductive tract (Miguel-Aliaga, Thor et al. 2008; Yang, Belawat et al. 2008). Targeted expression of *dilp7*^{RNAi} with dicer2 in the dMP2 neurons had no effect on CCAP/bursicon somata or axon arbor (Fig. 2-14D and E). Thus, metamorphic growth of the CCAP/bursicon neurons did not require DILP2, 3, and 5 from the brain IPCs, DILP6 from the fat body, or DILP7 from the dMP2 neurons. While other interpretations are possible (e.g., compensatory changes in insulin signaling or a role of DILP8), these results provide indirect evidence to suggest that the sources of insulin for regulation of metamorphic CCAP/bursicon neuron growth may be local.

Discussion

Stage-dependent effects of IIS on neuronal development

It is well established that the IIS pathway is crucial for regulating cell growth and division in response to nutritional conditions in *Drosophila* (Hietakangas and Cohen 2009). However, most studies have focused on the systematic growth of the body or individual organs, and comparatively little is known about the roles of IIS during neuronal development, particularly in later developmental stages. *Drosophila* InR transcripts are ubiquitously expressed throughout embryogenesis, but then are concentrated in the nervous system after mid-embryogenesis and remain at high levels in nervous system through the adult stage (Garofalo and Rosen 1988). This suggests that IIS plays important roles in the postembryonic nervous system. Recently, several studies in different fly neurons, including motor neurons, mushroom body neurons, and IPCs, revealed important roles of PI3K and Rheb in synapse growth or axon branching (Knox, Ge et al. 2007; Howlett, Lin et al. 2008). One recent study on *Drosophila* mushroom body neurons revealed effects on IIS on larval neuron proliferation, but not cellular morphology (Zhao and Campos 2012), although a second group reported clear morphological defects in the mushroom body neurons in adult flies under the same genetic manipulations (Acebes, Martin-Pena et al. 2011). These studies reveal some growth regulatory functions of IIS in the CNS, but they have not explored whether the control of neuronal growth by IIS is temporally regulated.

Here, we have shown that IIS strongly stimulates organizational growth of neurons during metamorphosis, whereas the effects of IIS on larval neurons are comparatively modest (Fig. 2-12). Recently, another group reported similar results in mushroom body neurons, in which the TOR pathway strongly promoted axon outgrowth of γ-neurons after metamorphic pruning, but not during the initial growth of α/β-neurons (Yaniv, Issman-Zecharya et al. 2012). Expression of FOXO or dominant negative InR had no significant effect on larval growth of the CCAP/bursicon neurons (Fig.2-2, 2-7, and 2-8) or on the soma size of many other larval neurons (Fig. 2-12). Thus, while IIS has been shown to regulate motoneuron synapse expansion in larvae (Knox, Ge et al. 2007; Howlett, Lin et al. 2008), our findings indicate that IIS does not play a major role in regulating structural growth in many larval neurons. This is consistent with a recent report that concluded that the *Drosophila* larval CNS is insensitive to changes in IIS (Cheng, Bailey et al. 2011).

When we used InR^{act} to activate IIS without ligand, we saw a modest but significant increase in CCAP/bursicon neuron soma size during larval development (Fig. 2-7J). This result indicates that the IIS pathway is present and fully functional in these larval neurons, but it is normally unstimulated by ligand. During metamorphosis, unlike in larvae, down-regulation of IIS by altering the level of either InR or downstream components of the pathway significantly reduced the growth of the CCAP/bursicon neurons (Fig. 2-6 to 2-10). Thus, we conclude that IIS is strongly upregulated during metamorphosis to support postembryonic, organizational growth of CNS neurons, and this activation is at least in part due to the secretion of as yet unidentified InR ligands during metamorphosis.

We attempted to identify the ligand source for supporting metamorphic neuronal growth by eliminating, in turn, most of the known sources of systemic DILPs: the brain IPCs (DILPs 2, 3, and 5), DILP6 in the fat body, and DILP7 in the VNC IPCs. None of these manipulations had any effect on metamorphic growth of the CCAP/bursicon neurons. These results are consistent with three possible mechanisms. First, there may be a compensatory IIS response to loss of some *dilps*. For example, a compensatory increase in expression of peripheral DILPs has been observed in the fat body in response to ablation of brain *dilps* (Gronke, Clarke et al. 2010). Second, their growth may be regulated by another systemic hormone (e.g., DILP8) that was not tested. Third, a local insulin source may be responsible for stimulating metamorphic outgrowth of the CCAP/bursicon neurons. Consistent with this view, a recent report showed that DILPs secreted from glial cells were sufficient to reactivate neuroblasts during nutrient restriction without affecting body growth, while overexpression of seven *dilp* genes (dilp1-7) in the IPCs had no effect on neuroblast reactivation under the same conditions (Sousa-Nunes, Yee et al. 2011). It is likely that local sources of DILPs, possibly glia, may play an important role in regulating metamorphic growth of the CCAP/bursicon neurons and other neurons, but further experiments will be needed to test this model.

Embryonic or larval origin of CCAP/bursicion neurons in the posterior ventral nerve cord

We have used *UAS-CD8::GFP*, driven by *CCAP-Gal4*, and anti-CCAP and anti-BURS immunostaining to examine the morphologies of the CCAP neurons (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Zhao, Gu et al. 2008). In third-instar

larvae, both CCAP markers are expressed in two pairs of neurons in the brain, three pairs of neurons in the subesophageal region, one pair of neurons in each of the first two thoracic neuromeres (T1 and T2), two pairs of neurons in the third thoracic neuromere (T3), and two pairs of neurons in each of the first four abdominal neuromeres (A1-A4) (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Zhao, Gu et al. 2008). In the next three abdominal neuromeres (A5-A7), one pair of CCAP/bursicon neurons is strongly labeled with both the GFP marker and anti-CCAP immunostaining, while another pair of neurons is occasionally observed by anti-CCAP immunostaining (Vomel and Wegener 2007). There are three pairs of neurons located in the last two abdominal neuromeres (A8-A9), although GFP reporter expression in these neurons is sometimes weak or absent (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Zhao, Gu et al. 2008). Therefore, the *CCAP-Gal4* expression pattern may not be fully representative of all CCAP-positive neurons. This may explain the absence in late larval development of nuclear GFP in the second pair of CCAP neurons in each of the A5-A7 neuromeres and in the CCAP neurons in the A8/A9 abdominal neuromeres that was reported by Veverytsa and Allan (2012) to suggest the late differentiation of these neurons at 10-12 hr APF. In contrast to the Veverytsa and Allan report, our lab is one of several that have observed these neurons through anti-CCAP immunostaining or GFP expression driven by *CCAP-Gal4* in second (Gu, Zhao et al. *in preparation*) or third-instar larvae (Park, Schroeder et al. 2003; Vomel and Wegener 2007; Hari, Deshpande et al. 2008; Zhao, Gu et al. 2008).

Interactions between IIS and ecdysone

A handful of studies on the interaction between IIS and ecdysone, a well-known regulator of metamorphic neuronal remodeling (Truman 1996), suggest that interactions between these systems may be important for regulating the metamorphic growth of neurons. In cell cultures of isolated locust CNS neurons, IIS and ecdysone had synergistic, stimulatory effects on neurite outgrowth (Vanhems, Delbos et al. 1990). Evidence of the direct interplay between these two hormonal signaling pathways also comes from a new coactivator of the ecdysone receptor, dDOR (Francis, Zorzano et al. 2010). DOR was first discovered in muscle tissue of diabetic rats (Baumgartner, Orpinell et al. 2007). In *Drosophila* dDOR mutants, the expression of two ecdysone signaling reporter genes, *E75* and *BR-C*, was inhibited in the Kenyon cells of the mushroom bodies. A combination of *in vitro* and *in vivo* studies has shown that dDOR functions as a coactivator of EcR that is required in some circumstances for maximal transcriptional activity (Francis, Zorzano et al. 2010). Francis and colleagues also identified dDOR as a direct target of FOXO, a key negative regulator in the insulin signaling pathway. In the fat body, activation of ecdysone signaling inhibits insulin signaling by reducing PI3K activity and increasing FOXO activity (Rusten, Lindmo et al. 2004; Colombani, Bianchini et al. 2005), and ecdysone treatment increased the fat body expression of *dDOR* in a FOXO-dependent manner (Francis, Zorzano et al. 2010). Since dDOR is also required for maximal activation of ecdysone target genes, this results a feed-forward regulatory loop in the fat body. Therefore, dDOR links these important hormonal signaling pathways through an antagonistic relationship.

The roles of IIS on age- and context-dependent neuronal regenerative ability

Our results indicate that IIS is critical for organizational growth, a type of growth that also occurs during neuronal regeneration. After injury, some neurons can initiate organizational growth to replace axons and dendrites. However, this regenerative ability of neurons is age-dependent and context-dependent (Selzer 2003; Park, Liu et al. 2010); immature neurons possess a more robust regenerative capacity, while the regenerative potential of many mature neurons is largely reduced. In particular, the adult vertebrate CNS displays a very limited regeneration capacity, and this is in marked contrast to the regeneration abilities displayed by the peripheral nervous system (PNS) (Ferguson and Son 2011). Recent studies on adult mouse corticospinal tract regeneration suggest that age-dependent inactivation of mTOR contributes to the reduced regenerative capacity of adult corticospinal neurons, and activation of mTOR activity through PTEN deletion promoted robust regenerative growth of corticospinal tract axons in injured adult mice (Liu, Lu et al. 2010). Our genetic experiments demonstrate a requirement for activity of mTOR, as well as several other IIS pathway components both upstream and downstream of mTOR, in controlling organizational growth of the CCAP/bursicon and many other peptidergic neurons. This suggests that under certain conditions, the activation of IIS may be a crucial component of the conversion of mature neurons to a more embryonic state, in which reorganizational growth after injury or as a function of developmental stage is possible. Given the strong evolutionary conservation of these systems and the powerful genetic tools available to identify novel regulatory interactions in *Drosophila*, studies on the

control of organizational growth in this species hold great promise for revealing factors that are crucial for CNS regeneration following injury.

Materials and Methods

Fly strains and genetic manipulations. Fly stocks were cultured on a standard cornmeal–yeast–agar medium at 22–25 ℃. Test crosses were performed at 25 ℃. The following strains were obtained from the Bloomington Drosophila Stock Center: *CCAP-Gal4* (*y* w*;P{ccap-GAL4.P}16*; FBti0037998); *UAS-InR* (*y^1 w^{1118}; P{UAS-InR.Exel}2*; FBst0008262); *UAS-InR^{act}* (*y^1 w^{1118}; P{UAS-InR.R418P}2*; FBst0008250); *UAS-InR^{DN}*; (*y^1 w^{1118}; P{UAS-InR.K1409A}2*; FBst0008259); *UAS-PI3K* (*y^1 w^{1118}; P{UAS-Pi3K92E.Exel}2*; FBst0008286); *UAS-$PI3K^{act}$* (*P{UAS-Pi3K92E.CAAX}1, y^1 w^{1118}*; FBst0008294); *UAS-$PI3K^{DN}$* (*P{UAS-Pi3K92E.A2860C}1, y^1 w^{1118}*; FBst0008288); *UAS-Akt* (*P{UAS-Akt1.Exel}1, y^1 w^{1118}*; FBst0008192); *UAS-$PTEN^{RNAi}$* (*w^{1118}; P{UAS-Pten.dsRNA.Exel}3*; FBst0008550); *UAS-Rheb* (*y^1 w^*; P{Mae-UAS.6.11}$Rheb^{LA01053}$/TM3, Sb^1 Ser^1*; FBst0022248); *UAS-S6K* (*w^{1118}; P{UAS-S6k.M}2/CyO*; FBst0006910); *CyO, tubPGal80* (*w^*; l(2)$DTS91^1$ noc^{Sco}/CyO, P{tubP-GAL80}OV2*; FBst0009491); and Oregon-R (wild type; FBst0004269). All RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC). *UAS-$FOXO^{w+[m3-1]}$* and *UAS-$FOXO^{TM[f3-9]}$* were kindly provided by Marc Tatar. *w; bursicon-Gal4[P12]* was made by Willi Honegger and provided Ben White. *w^*, UAS-dicer2* was made by Stephan Thor by mobilizing the *UAS-dicer2* insertion (FBti0101430) (Dietzl, Chen et al. 2007) to a new X chromosome location to enhance the effect of RNAi, and that was kindly provided by Paul Taghert.

Phenotype scoring. Defects in head eversion and wing expansion were scored as described (Luan, Lemon et al. 2006). The wing phenotypes were recorded as unexpanded wings (UEW), partially expanded wings (PEW), and expanded wings.

Immunostaining. Immunostaining was performed on isolated central nervous systems or on whole-animal fillets of wandering 3rd instar larvae or staged pupae (Bainbridge and Bownes 1981) according to previously described procedures (Hewes, Park et al. 2003; Hewes, Gu et al. 2006). Control and test groups of animals were dissected in parallel in calcium-free saline (182 mM KCl, 46 mM NaCl, 2.3 mM $MgCl_2.6H_2O$, 10 mM 2-Amino-2-(hydroxymethyl) propane-1,3-diol (Tris), pH=7.2). Tissues were fixed for 1 hr at room temperature (RT) in either 4% paraformaldehyde (PFA) or 4% paraformaldehyde with 7% picric acid (PFA/PA). Primary antisera were used overnight at 4℃ and were directed against the following proteins: CCAP (1:4000, PFA/PA) (Park, Schroeder et al. 2003), Bursicon α-subunit (1:5000, PFA/PA) (Luan, Peabody et al. 2006) , and FOXO (1:1000, PFA) (Puig, Marr et al. 2003). Tissues were mounted with Vectashield (Vector Labs, Burlingame, CA) for observation using an Olympus FluoView FV500 confocal microscope (Center Valley, PA).

Staining quantification. Confocal image quantification was performed as described (Hewes, Park et al. 2003; Hewes, Gu et al. 2006), and the images shown in the figures are representative of the means for cellular parameters that were quantified. The same confocal scanning settings, which were optimized to avoid image saturation, were used for all preparations within each experiment. For quantification of cell soma area, we manually circled the cell border and obtained a count for the bordered pixels in

Adobe Photoshop (Adobe Systems, San Jose, CA). The mean value of the histogram was the cell intensity. For quantification of arbor area, we used the inversion and threshold function in Adobe Photoshop (with the same threshold of 235 for all images) to convert the background to white and all remaining pixels (arbor and somata) to black. The somata and any obvious artifacts were manually cut from each image, and then we obtained a count of the black pixels. One-way ANOVAs and Tukey-kramer multiple comparison *post hoc* tests were performed using NCSS-2001 software (NCSS, Kaysville, UT).

Table 1. Differential effects of IIS on anterior versus posterior abdominal ganglion CCAP/bursicon cells

Genotype	Soma size ratio (cell9:cell3)
CCAP-Gal4/+, UAS-InR^{act}/+	1.42
CCAP-GAL4/+, UAS-InR/+	1.46
CCAP-GAL4/+, UAS-Akt1	1.32
CCAP-GAL4/+, UAS-PI3K/+	1.1
CCAP-GAL4/+, UAS-PI3K^{act}	1.12
CCAP-GAL4, UAS-Akt1	1.02
CCAP-GAL4, UAS-PI3K	1.32
CCAP-GAL4, UAS-PI3K^{act}	1.03
CCAP-GAL4, UAS-Rheb/+	1.34
UAS-dicer2/+; CCAP-GAL4/+;UAS-TSC1^{RNAi}/+	1.66
UAS-dicer2/+; CCAP-GAL4/+;UAS-TSC2^{RNAi}/+	2.2
UAS-dicer2/+; CCAP- GAL4/+, UAS-FOXO^{RNAi}/+	0.96
CCAP-GAL4/+, UAS-InR^{DN}/+	0.75
UAS-dicer2/+; CCAP-GAL4/+, UAS-InR^{RNAi}/+	0.64
UAS-dicer2/+, UAS-S6K^{RNAi}/+; CCAP-GAL4/+	0.63

The Cell9:cell3 ratio was calculated as:
$(Cell9_{experimental}/Cell9_{control})/(Cell3_{experimental}/Cell3_{control})$, n=5-11.
The control group is either CCAP-Gal4/+ or UAS-dicer2/+;CCAP-Gal4/+.

Figure 2-1. The CCAP/bursicon cells maintain overall morphology during larval development, but significantly increased in size

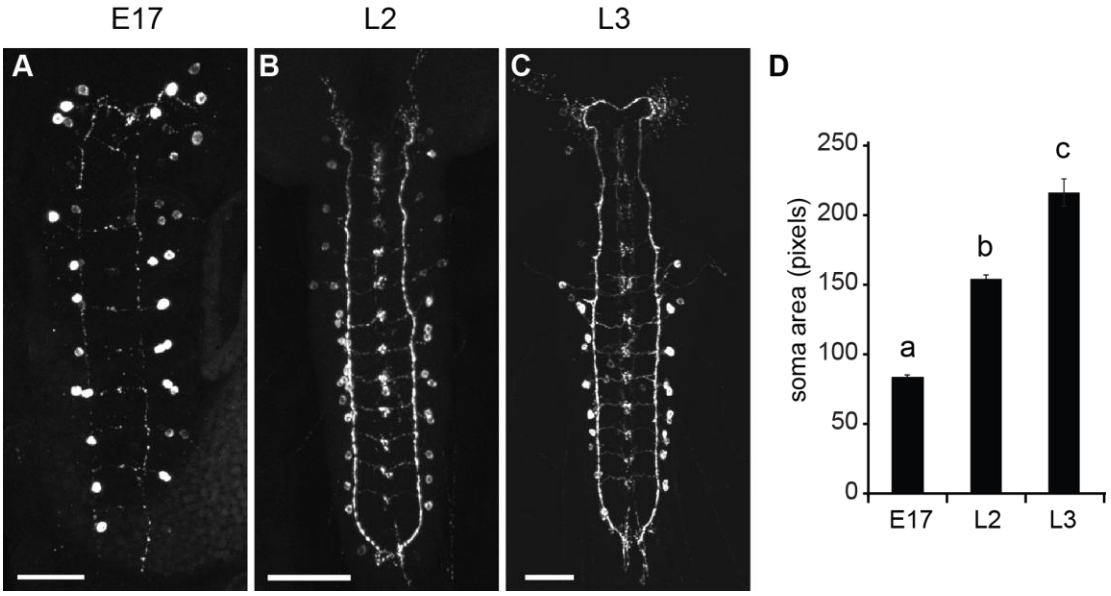

Figure 2-1. The CCAP/bursicon cells maintain overall morphology during larval development, but significantly increased in size

(A-C) CCAP/bursicon neurons from embryonic stage 17 (A), 2nd instar larvae (B), and wandering 3rd instar larvae (C). Scale bars: (A) 20µm, (B) 50µm, (C)100µm.

(D) Quantification of soma size for CCAP/busicon cells of the genotypes shown in (A-C). From embryonic stage 17 (A) to the wandering 3rd instar larval stage, the soma size of the CCAP/bursicon neurons was significantly increased. Data are presented as means ± SEM. Bars labeled with different letters are significantly different ($P<0.0001$, one way ANOVA; Tukey's HSD *post-hoc* test; n=6-8).

Figure 2-2. Overexpression of *foxo* led to loss of somata, neuritis in pharate adult, but not larvae

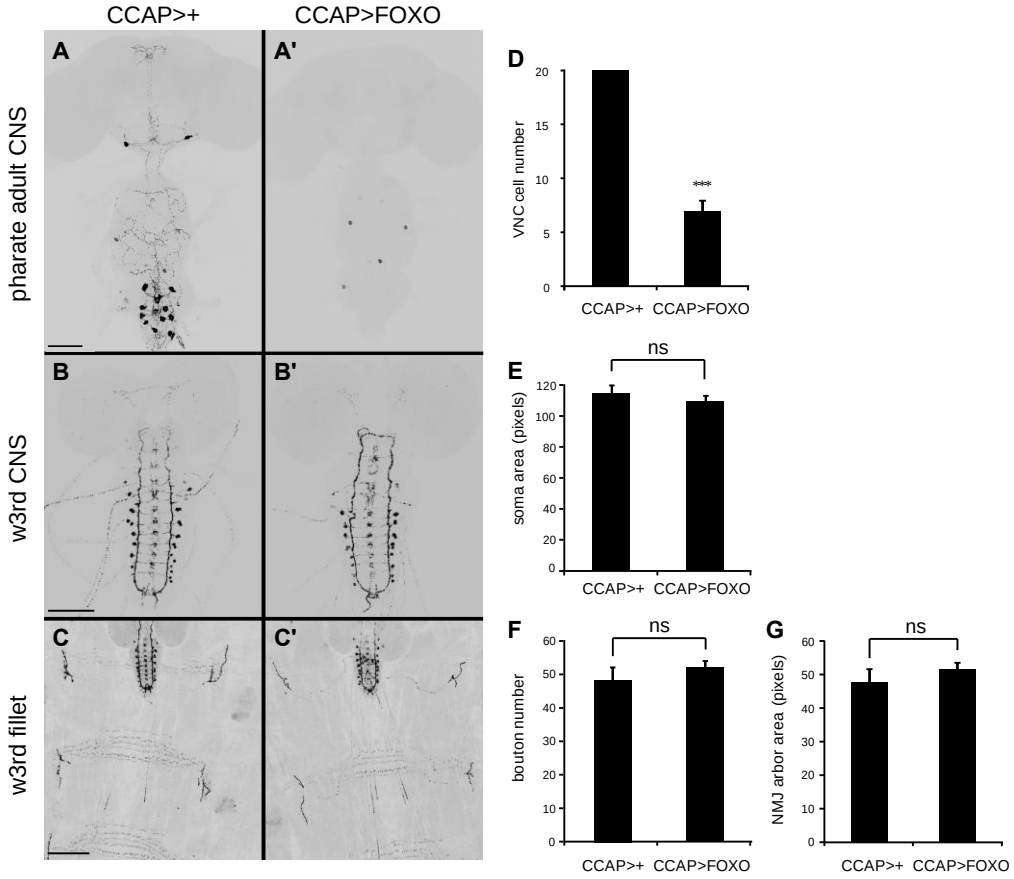

Figure 2-2. Overexpression of *foxo* in the CCAP/bursicon cells led to loss of somata, neurites in pharate adult, but not larvae

(A) Cell-targeted expression of *foxo* in the CCAP/bursicon neurons (A′) caused the near complete loss of adult-specific neurites and disappearance of the majority of the cell bodies at P14 pharate adult stage. Cells were labeled by anti-bursicon immunostaining, and the control genotype (*CCAP-Gal4/+*) is shown in (A). (B and C) In larvae, the CNS pattern and morphology (B′) and the peripheral axon arbor (C′) was largely normal. Scale bars: (A and A′, B and B′) 100μm, (C and C′) 50μm.

(D-G) Quantification of cellular parameters for the experiments shown in (A-C). Overexpression of *foxo* significantly reduced the CCAP/bursicon cell number at the P14 pharate adult stage (D) ***$P < 0.001$ ($P = 0.0000355$, student's t-test, n=3-5), but there was no change in larval soma size (E) ($P = 0.27$, student's t-test, n=5-6), bouton number at the larval neuromuscular junction (NMJ) (F) ($P = 0.35$, student's t-test, n=7), or size of the NMJ (G) ($P = 0.35$, student's t-test n=7). ns, non significant. Data are presented as means ± SEM.

Figure 2-3 Expression of *foxo* or *InR*^{act} driven by *burs-Gal4* significantly alters metamorphic growth of the CCAP/bursicon cells

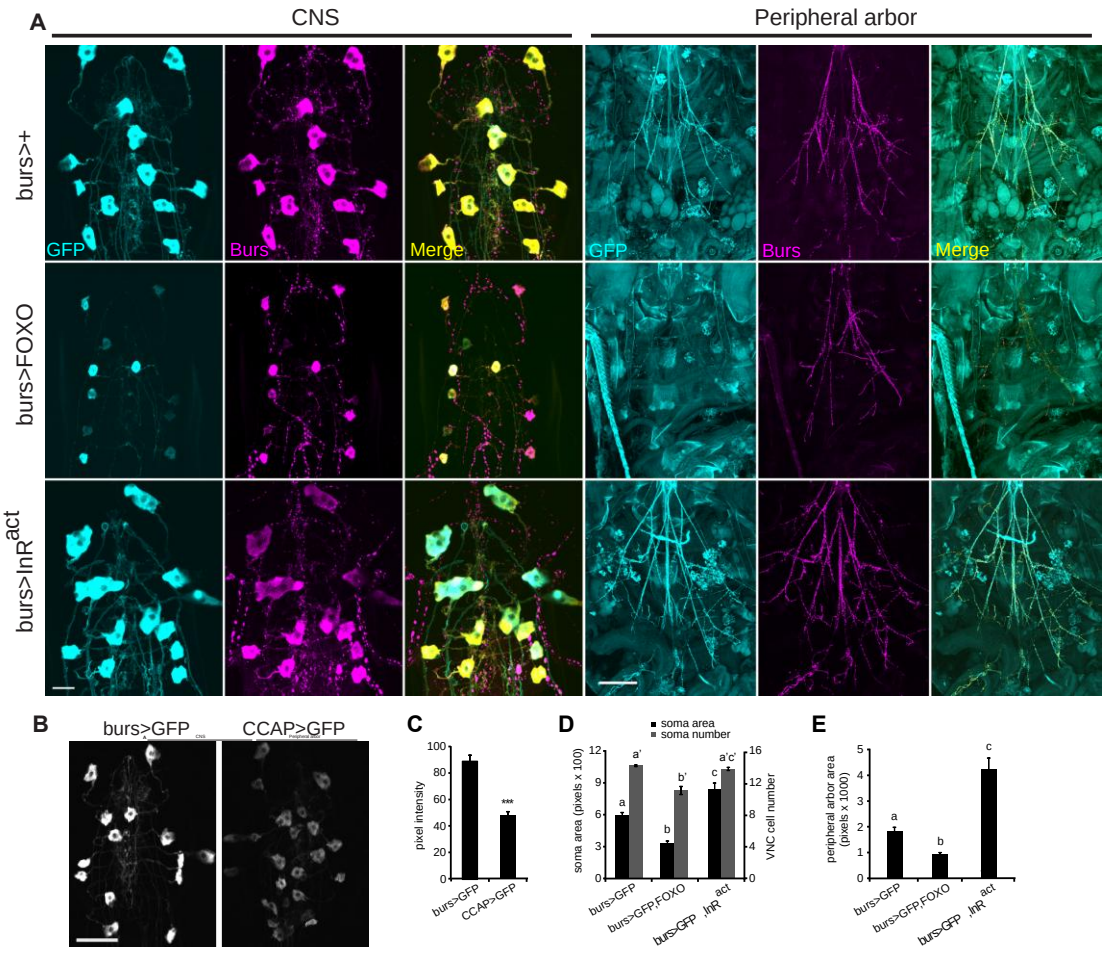

Figure 2-3. Expression of *foxo* or InR^{act} driven by *burs-Gal4* also significantly alters metamorphic growth of the CCAP/bursicon cells

(A) *bursicon-Gal4*-driven co-expression of *UAS-CD8::GFP* and *UAS-FOXO* caused a significant reduction in the growth of soma and peripheral arbor and a loss of a few cell bodies (middle panels) at pharate adult stage. In contrast, expression of InR^{act} significantly increased soma size and peripheral axon arbor (lower panels). Both GFP (cyan) and anti-busicon immunostaining (magenta) signals largely overlapped and GFP expression persisted in the soma, suggesting that the reduction of peripheral axon arbor with *foxo* overexpression was not due to the loss of cell markers.

(B) The expression level of GFP driven by *burs-Gal4* was significantly higher than the one driven by *CCAP-Gal4*. Scale bars: (A) CNS: 20 μm, (A) peripheral arbor: 200μm, and (B) 50μm.

(C-E) Quantification of GFP fluorescence intensity (C), soma size (D), soma number (D), and peripheral arbor area (E) of the CCAP/busicon cells shown in genotypes in (A-B). Black bars and grey bars represent soma size and soma number, respectively. Bars labeled with different letters are significant different. Student's t-test was performed on GFP intensity ***$P < 0.001$ ($P = 0.00000019$, n=5-7). One way ANOVA, Tukey's HSD *post-hoc* tests were performed on soma size ($P < 0.001$, n=6-8), soma number ($P < 0.001$, n=6-8), and peripheral arbor area ($P < 0.001$, n=5-7). Data are presented as means ± SEM.

Figure 2-4. Overexpression of *foxo* disrupts metamorphic outgrowth of the CCAP/bursicon cells

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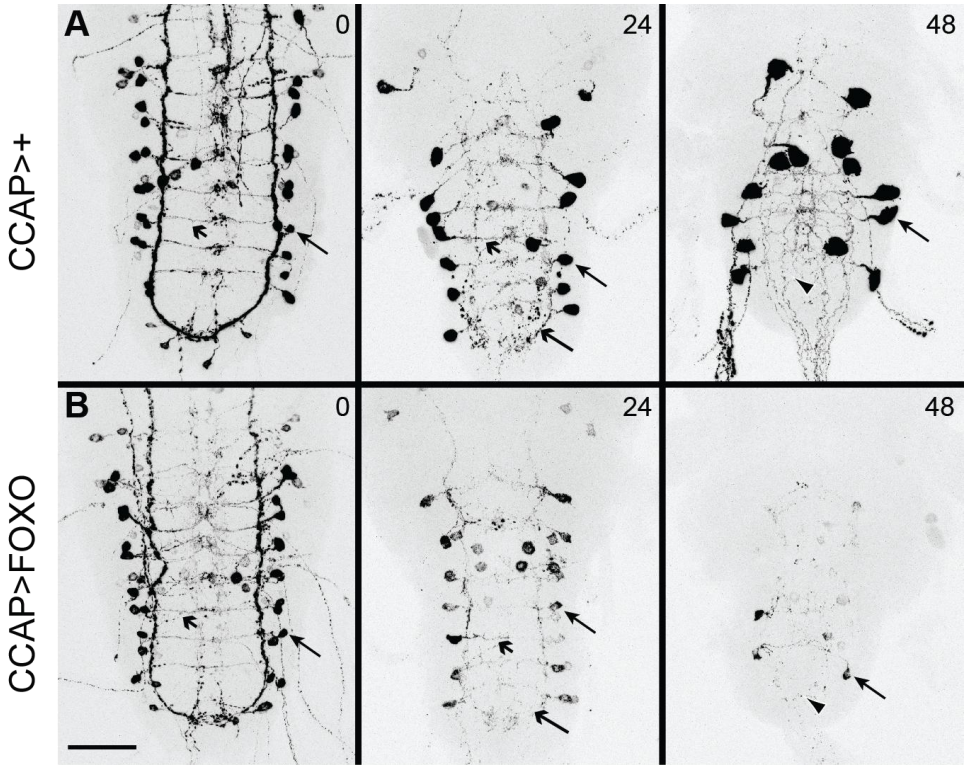

Figure 2-4. Overexpression of *foxo* disrupts metamorphic outgrowth of the CCAP/bursicon cells

Anti-bursicon immunostaining of the ventral nerve cord at 0, 24 and 48 hr after puparium formation (APF) (n=3-9). In contrast to the controls (top row, with only the *CCAP-Gal4* driver), CCAP/bursicon cell-targeted expression of *foxo* disrupted soma growth (solid arrows) and outgrowth of adult-specific neurites (solid arrowheads). A similar extent of pruning of the larval neurites was occurred at 24 hr in the two genotypes. Feathered arrows, lateral longitudinal tracks; feathered arrowheads, midline arbor. Scale bar: 100μm.

Figure 2-5. InR inhibits the effects of FOXO in the CCAP/bursicon cells.

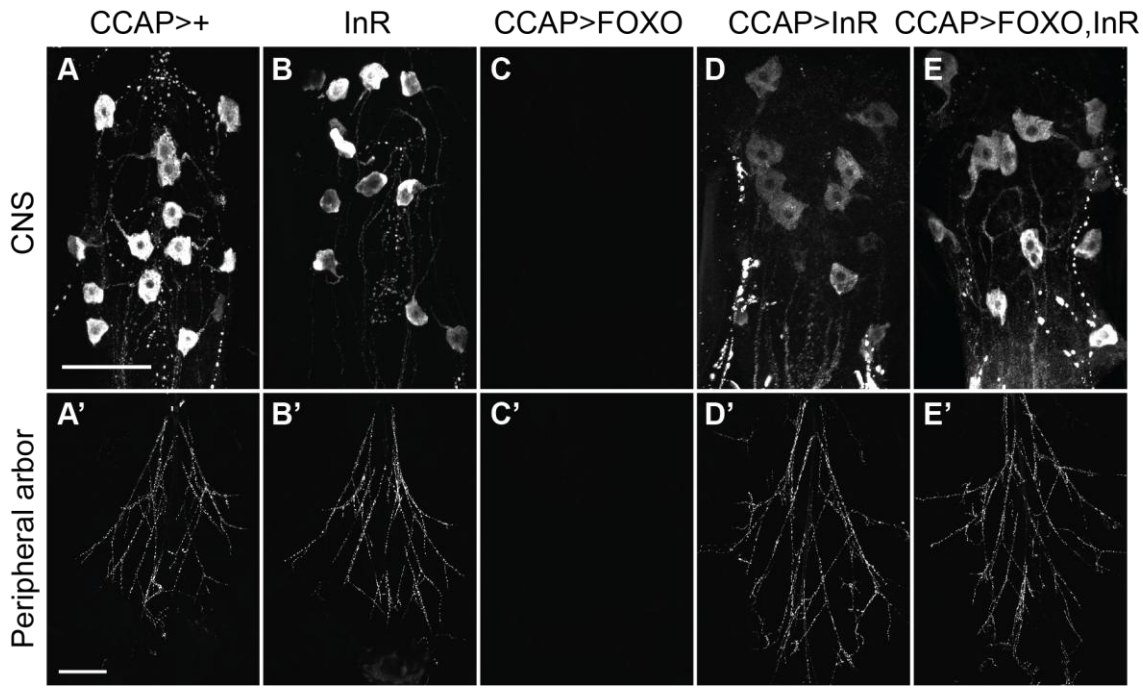

Figure 2-5. InR inhibits the effects of FOXO in the CCAP/bursicon cells

(A-E) Cell-targeted expression of InR in the CCAP/bursicon neurons completely rescued the cell loss phenotype induced by *foxo* overexpression (C and C′). (A and A′) *CCAP-Gal4* driver-only controls. (B and B′) *UAS-InR* controls. (C and C′) *CCAP-Gal4; UAS-FOXO.* (D and D′) *CCAP-Gal4; UAS-InR.* Scale bar: (A-E) 50μm, (A′-E′) 200μm.

Figure 2-6. InR regulates metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

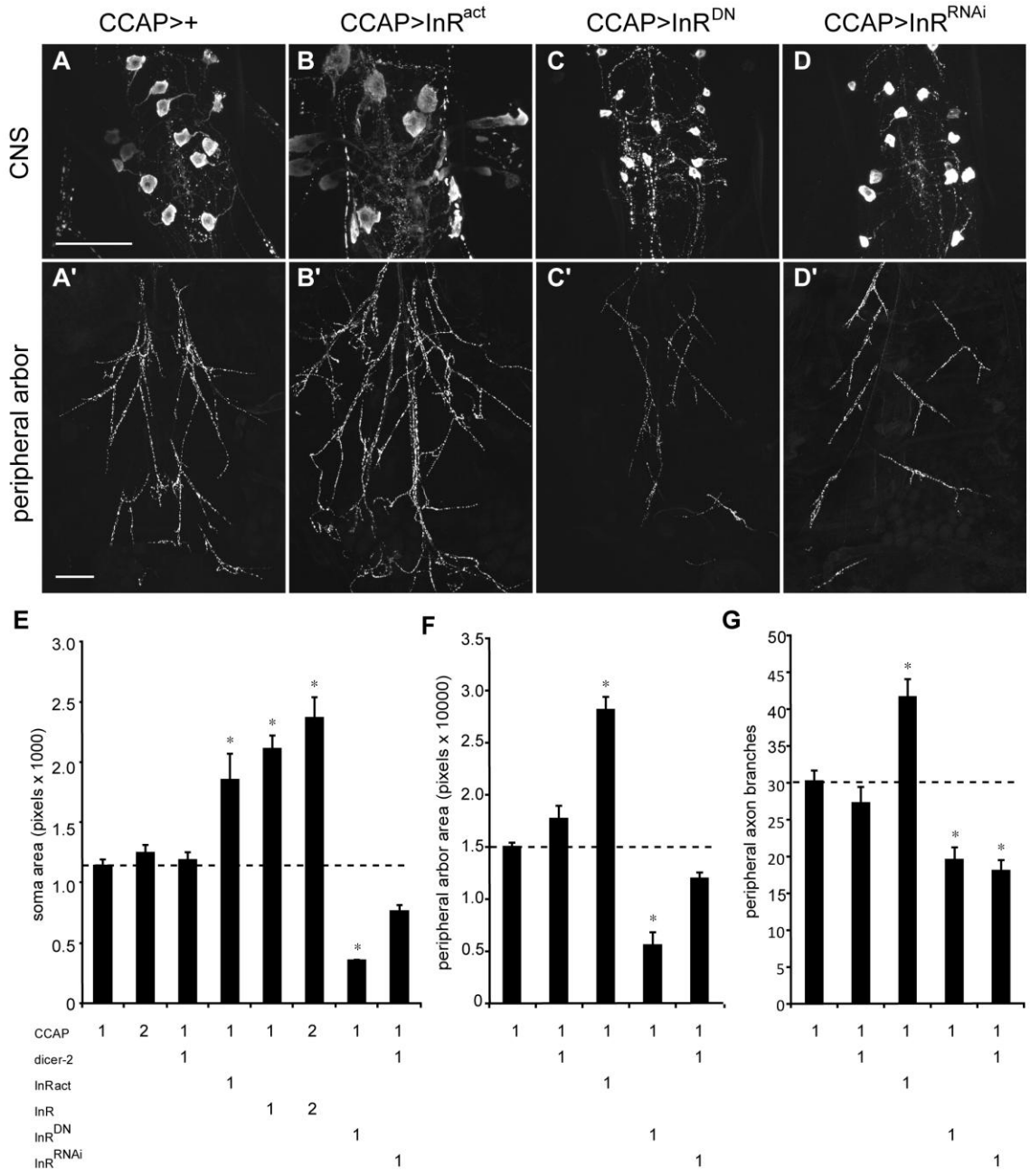

Figure 2-6. InR regulates metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

(A-D and A′-D′) Cell-targeted expression of InR^{act} in the CCAP/bursicon neurons increased the soma size (B) and extent of the peripheral axon arbor (B′) (anti-bursicon immunostaining, stage P14 pharate adults). In contrast, expression of InR^{DN} and InR^{RNAi} produced smaller somata (C and D) and reduced the peripheral arbor (C′ and D′). (A and A′) *CCAP-Gal4* driver-only controls. Scale bars: (A-D) 100µm, (A′-D′) 200µm.

(E-G) The CCAP/bursicon somata size (E), area covered by the peripheral axon arbor (F), and number of axonal branches (G) were dependent on InR activity. One or more copies of each transgene were present in each genotype as indicated below the histograms: CCAP = *CCAP-Gal4*; dicer-2 = *UAS-dicer-2*; InR^{act} = $UAS\text{-}InR^{act}$; InR^{DN} = $UAS\text{-}InR^{DN}$; InR^{RNAi} = $UAS\text{-}InR^{RNAi}$. One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size ($P < 0.001$, n=5-18), peripheral arbor area ($P < 0.001$, n=3-11), and peripheral arbor branches ($P < 0.001$, n=3-11). Data are presented as means ± SEM. *$P < 0.05$.

Figure 2-7. InR^{DN} has no effect on the larval soma and synapse growth of the CCAP/bursicon cells, but expression of *InR^{act}* significantly increased the CCAP/bursicon soma size

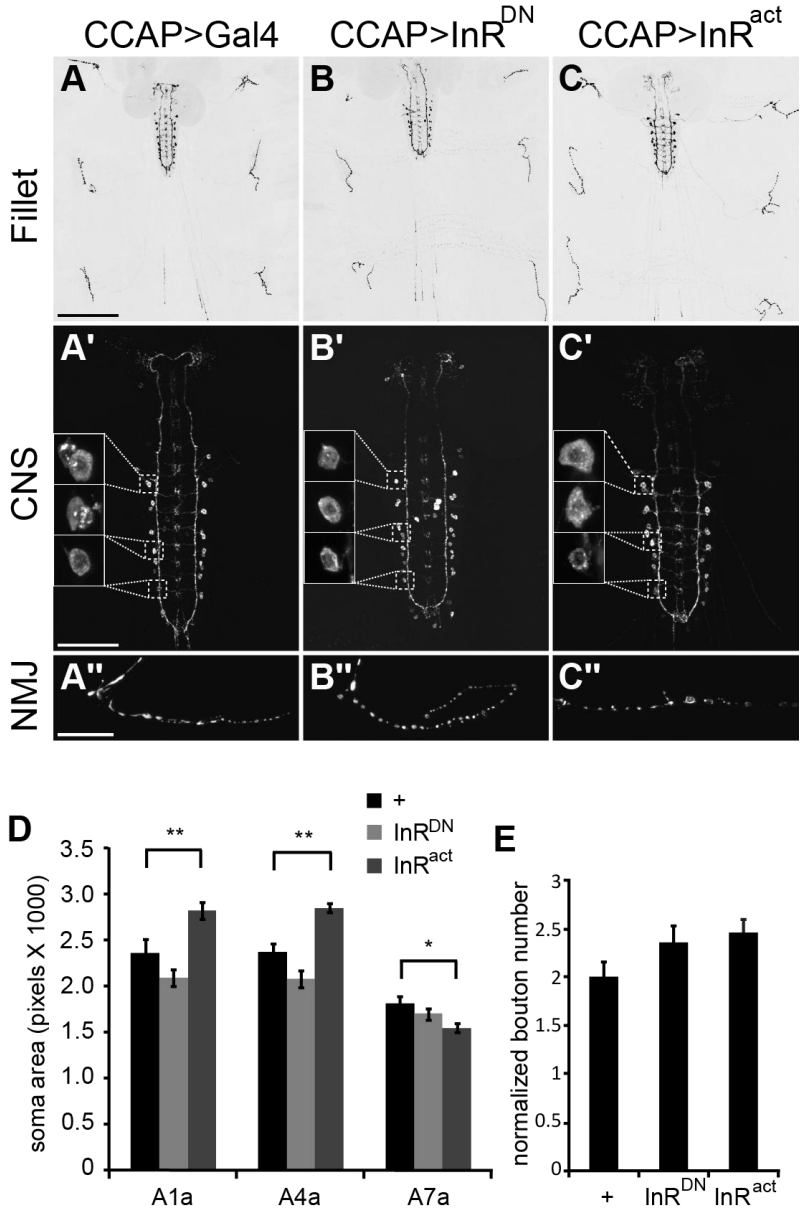

Figure 2-7. InR^{DN} has no effect on the larval soma and synapse growth of the CCAP/bursicon cells, but expression of InR^{act} significantly increased the CCAP/bursicon soma size

(A-C, A′-C′, and A″-C″) Cell-targeted expression of InR^{DN} and InR^{act} in the CCAP/bursicon neurons had little effect on the larval peripheral arbor of the CCAP/bursicon neurons (B and C), central pattern (B′-C′), or the nueromuscular junctions (NMJ) (B″-C″) (anti-bursicon immunostaining on wandering 3rd intar larvae). The insets in (A, A′, and A″) show the A1a, A4a, and A7a somata (from anterior to posterior). Scale bars: (A-C) 200µm, (A′-C′) 100µm, (A″-C″) 20µm.

(D-E) Quantification of soma size of A1a, A4a, and A7a (D) and normalized bouton number (E) of the CCAP/busicon cells shown in genotypes in (A-C). InR^{act} significantly affected the soma sizes of A1a, A4a, and A7a. In contrast, InR^{DN} had no effect on the soma size. Both InR^{DN} and InR^{act} had no effect on the normalized bouton number (E). One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size of A1a ($P < 0.0001$, n=5-10), A4a ($P < 0.0001$, n=6-10), and A7a ($P = 0.0185$, n=6-10), and normalized bouton number ($P = 0.1428$, n=6-10). Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 2-8. InR^{RNAi} has no effect on the larval soma and synapse growth of the CCAP/bursicon cells

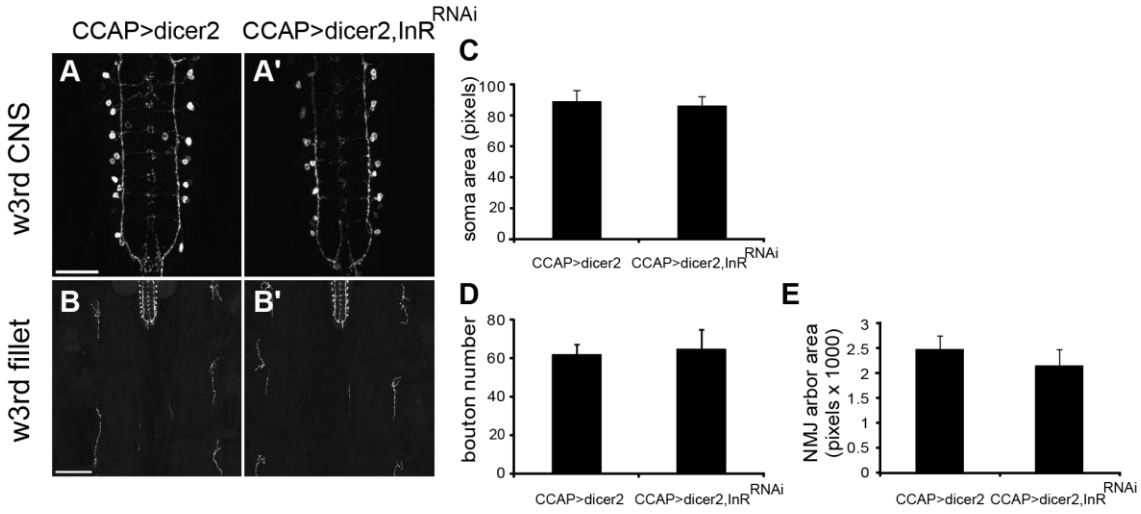

Figure 2-8. InR^{RNAi} has no effect on the larval soma and synapse growth of the CCAP/bursicon cells

(A-B and A′-B′) Cell-targeted expression of InR^{RNAi} with *dicer2* in the CCAP/bursicon neurons had no effect on the morphology of the central CCAP/bursicon neuron pattern in larvae (A-A′), peripheral arbor of the CCAP/bursicon neurons (B and B′), and their neuromuscular junction (NMJ) (anti-bursicon immunostaining on wandering 3rd intar larvae). (A and B) *UAS-dicer2*, *CCAP-Gal4* controls. (A′-B′) *UAS-dicer2*, *CCAP-Gal4/UAS-*InR^{RNAi}. Scale bars: (A-A) 100µm, (B′-B′) 200µm.

(C-E) InR^{RNAi} had no significant effects on soma area (C) (P = 0.74, student's t-test, n=6-8), bouton number (D) (P = 0.8, student's t-test, n=6), and NMJ bouton area (E) (P = 0.47, student's t-test, n=5-6). Data are presented as means ± SEM.

Figure 2-9. Akt, PI3K, and PTEN regulate metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

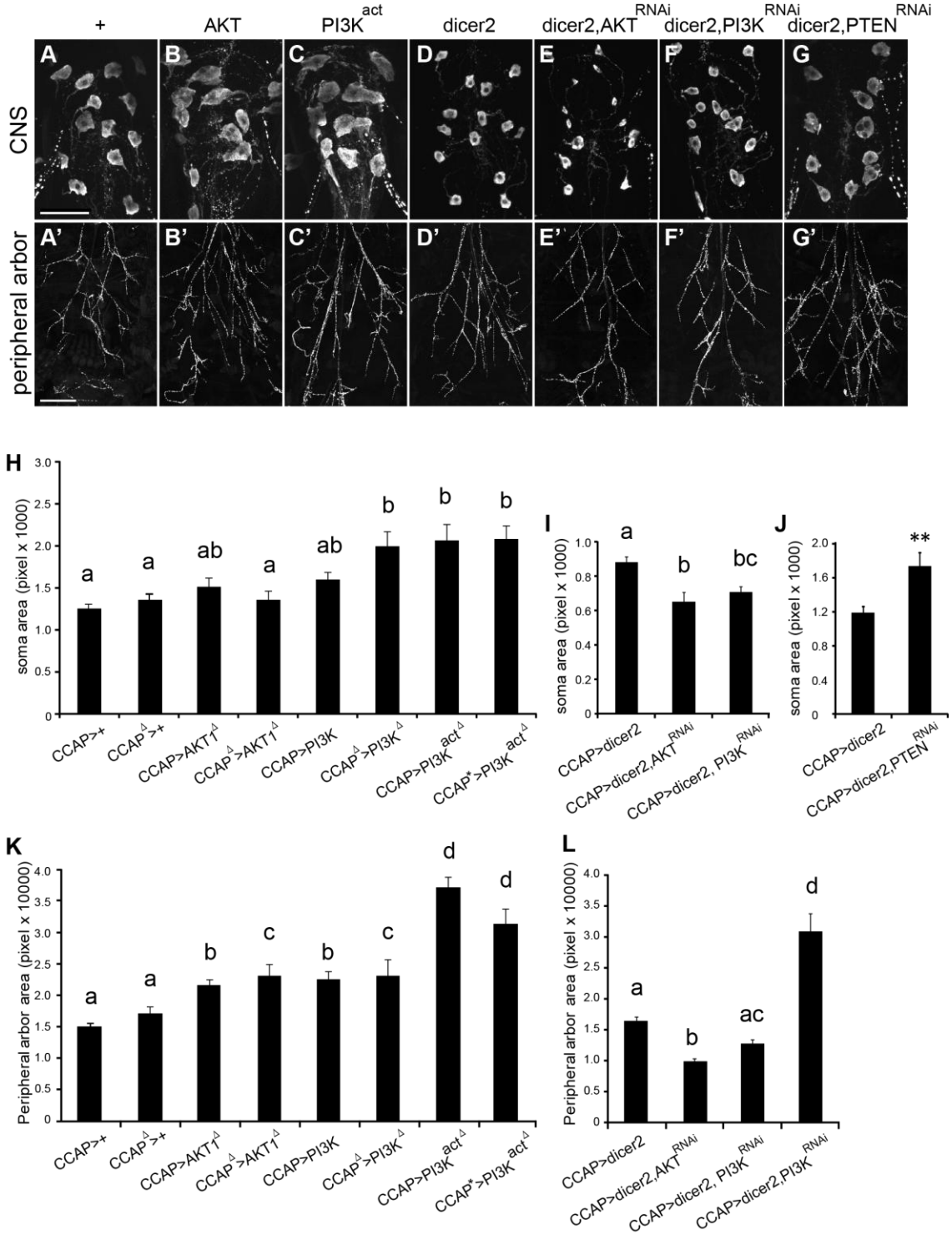

Figure 2-9. Akt, PI3K, and PTEN regulate metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

(A-G and A′-G′) Cell-targeted expression of *Akt*, $PI3K^{act}$, and $PTEN^{RNAi}$ with *dicer2* in the CCAP/bursicon neurons increased soma size (B, C, and G) and extent of the peripheral axon arbor (B′, C′, and G′) (anti-bursicon immunostaining, stage P14 pharate adults). In contrast, expression of Akt^{RNAi} and $PI3K^{RNAi}$ with *dicer2* produced smaller somata (E and F) and reduced the peripheral arbor (E′ and F′). (A and A′) show *CCAP-Gal4* driver-only controls. (D and D′) *UAS-dicer2, CCAP-Gal4* controls. Each element used here is heterozygous (A-G and A′-G′). Scale bars: (A-G) 50µm, (A′-G′) 200µm. (H-L) The CCAP/bursicon soma size (H and I) and peripheral axon arbor area (J-L) were dependent on the activity of Akt, PI3K and PTEN. Each element used here is heterozygous unless indicated by **Δ.** One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size (H) ($P < 0.0001$, n=5-13), (I) ($P = 0.000239$, n=6-10), and (J) ($P = 0.0019$, n=5). Kruskal-Wallis Multiple-Comparison Z-Value Tests with Bonferroni correction were performed on peripheral arbor area (K) (n=4-8) and (L) (n=5-7). Bars labeled with different letters are significantly different. Data are presented as means ± SEM. **$p < 0.01$.

Figure 2-10. Two major branches of IIS, FOXO and Tor, regulate metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

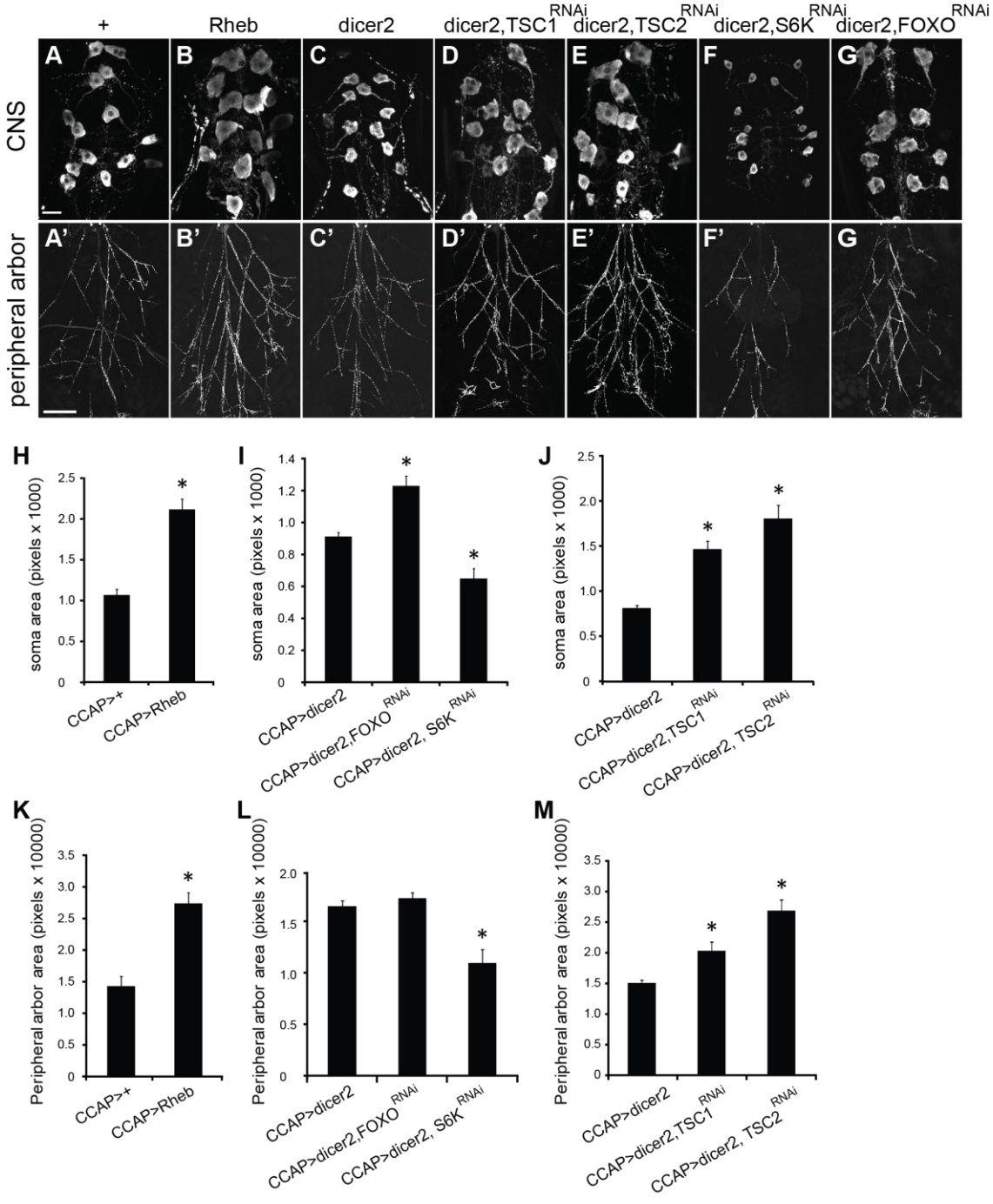

Figure 2-10. Two major branches of IIS, FOXO and Tor, regulate metamorphic growth of CCAP/bursicon cell somata and peripheral axon arbor

(A-B and A′-B′) *CCAP-Gal4* driven the expression of *Rheb*, and *dicer2* with $TSC1^{RNAi}$, and $TSC2^{RNAi}$ increased the soma size (B, D, and E) and the extent of the peripheral axon arbor (B′, D′, and E′). Expression of $FOXO^{RNAi}$ with *dicer2* increased the soma size (E), but not the peripheral axon arbor (G′). In contrast, expression of $S6K^{RNAi}$ with dicer2 decreased the soma size (F) and the peripheral axon arbor (F′). Cells were labeled by anti-bursicon immunostaining at P14 pharate adult stage. (A and A′) *CCAP-Gal4* driver-only controls. (C and C′) *UAS-dicer2, CCAP-Gal4* controls. Each element used in the experiments is heterozygous. Scale bars: (A-E) 100μm, (A′-D′) 200μm.
(H-M) Quantification of soma size (H-J) and peripheral axon arbor (K-M) for CCAP/bursicon cells of the phenotypes shown in (A-G and A′-G′). The soma size of the CCAP/bursicon cells was dependent on the activity of Rheb, TSC1, TSC2, and FOXO (H-J). The peripheral axon arbor was dependent on Rheb, TSC1 and TSC2, but not FOXO (K-M). Student's t tests were performed on soma size shown in (H) ($P < 0.0001$, n=5-6) and peripheral arbor area shown in (K) ($P = 0.0005$, n=5-6). One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size shown in (I) ($P < 0.0001$, n=5-25) and (J) ($P<0.001$, n=5-9) and peripheral axon arbor shown in (L) ($P = 0.000128$, n=5-25) and (M) ($P = 0.00218$, n=4-6). Bars labeled with different letters are significantly different. Data are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 2-11. Moderate effect of IIS on the metamorphic growth of Tv neurons

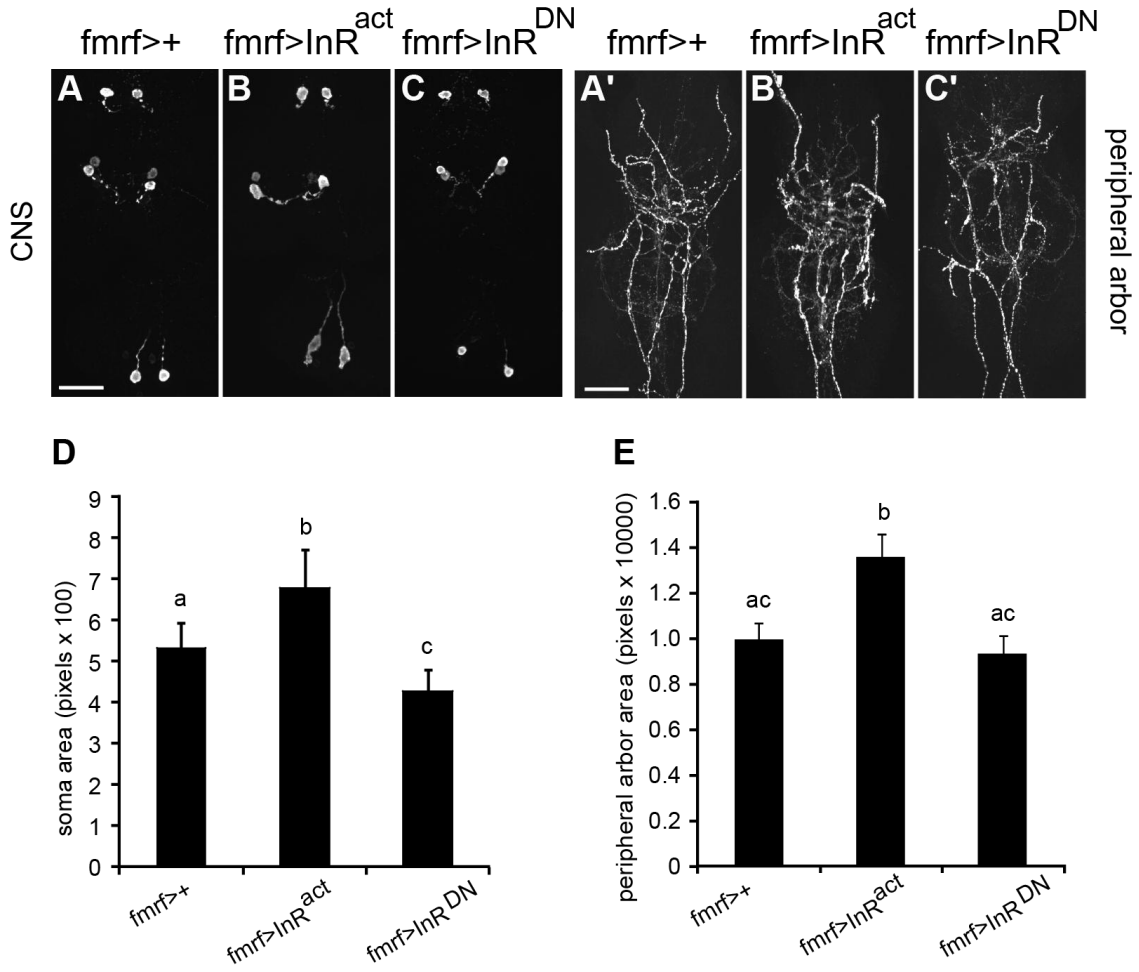

Figure 2-11. Moderate effect of IIS on the metamorphic growth of Tv neurons

(A-C and A'-C') Cell-targeted expression of InR^{act} or InR^{DN} in the Tv neurons changed the soma size (B and C), but not the peripheral axon arbor (B′ and C′) (anti-bursicon immunostaining, stage P14 pharate adults). (A and A′) *FMRF-Gal4* driver-only controls. Scale bar: (A-C) 100µm (A′-C′) 100µm

(D and E) Manipulate the activity of InR significantly influenced the soma size of Tv neurons (D), but not the peripheral axonal arbor (E). One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size ($P < 0.0001$, n=6) and peripheral axon arbor ($P < 0.0001$, n=6). Bars labeled with different letters are significantly different. Data are presented as means ± SEM.

Figure 2-12. IIS significantly affects the soma area of most pharate adult neurons but not larval neurons

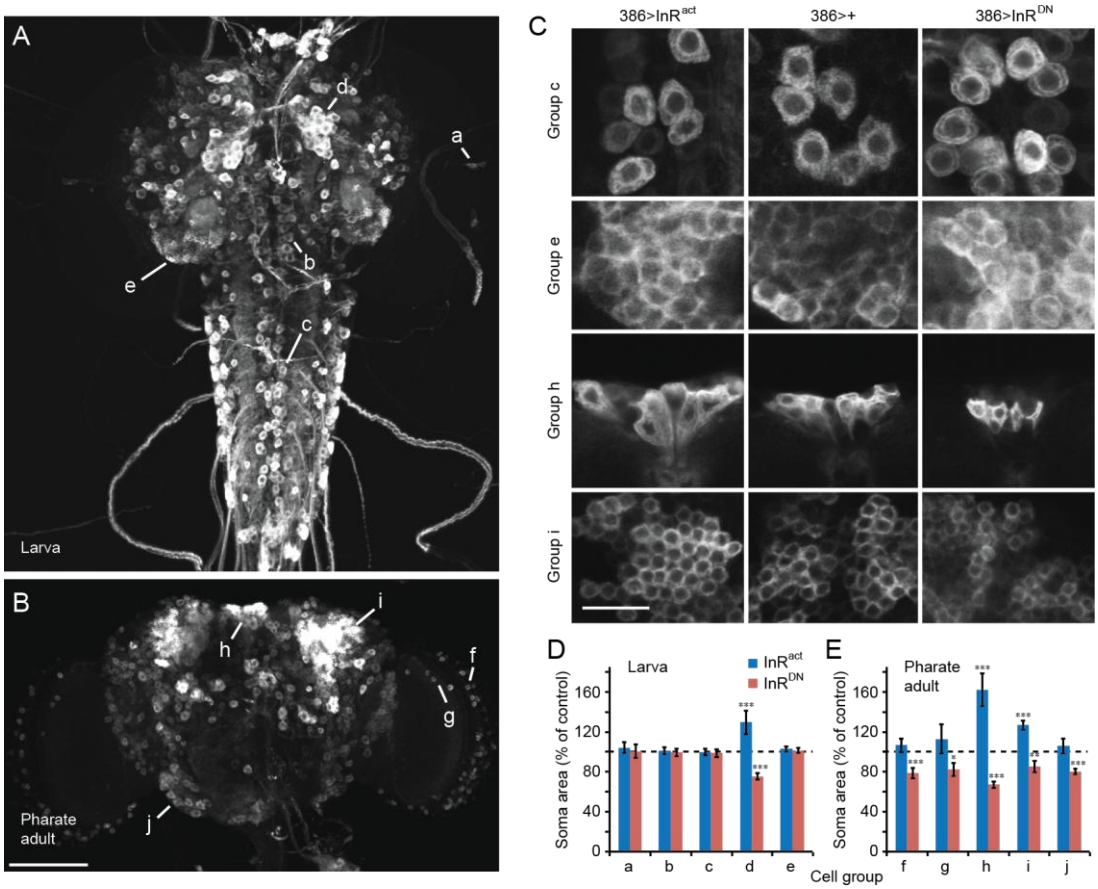

Figure 2-12. IIS significantly affects the soma area of most pharate adult neurons but not larval neurons

(A and B) Pan-peptidergic expression pattern *386-Gal4,UAS-CD8::GFP* at the wandering 3rd stage (A) and the pharate adult stage (B). Soma sizes for five larval groups of neurons labeled by a to e (A) and five pharate adult groups of neurons labeled by f to j (B) were analyzed. Scar bar: 100 µm.

(C) Higher magnification views of selected neurons groups (c, e, h, and i) expressing *InR*^{act}, *InR*^{DN} or just the *386-Gal4* driver. Groups e and i are the mushroom body Kenyon cells. Groups d and h are the brain insulin-producing cells. Scar bar: 100 µm.

(D and E) Soma sizes for the larval (D) and pharate adult (E) groups of neurons indicated in (A) and (B) following *InR*^{act} or *InR*^{DN} expression. One way ANOVA; Tukey's HSD *post-hoc* tests were performed on soma size ($P < 0.0001$, n=5) and peripheral axon arbor ($P = < 0.0001$, n=5). *$p < 0.05$ ** $p < 0.001$ *** $p < 0.001$ (n=6-11).

Figure 2-13. Heterogeneity in the responses of the CCAP/bursicon cells to insulin signaling

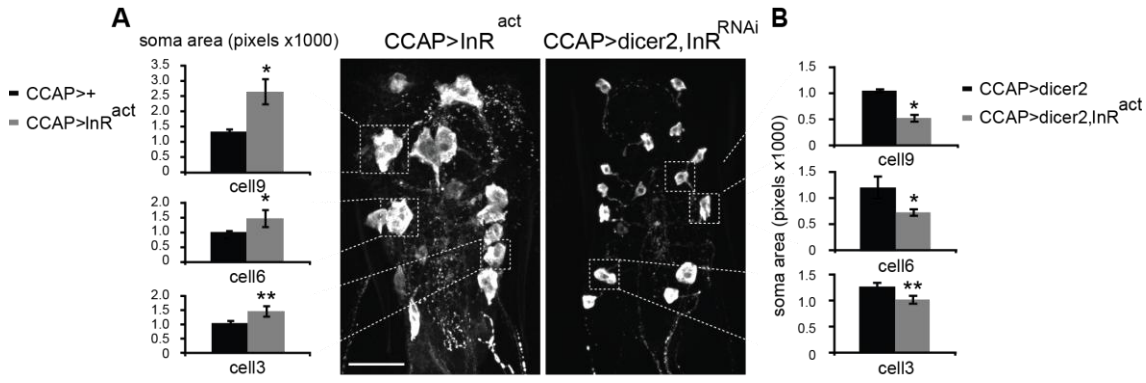

Figure 2-13. Heterogeneity in the responses of the CCAP/bursicon cells to insulin signaling

(A and B) Expression of InR^{act} in the CCAP/bursicon neurons increased the soma size of cell 9 more significantly than cell 6 and cell 3 (A) (anti-bursicon immunostaining, stage P14 pharate adults). In contrast, the soma size of cell 9 was the smallest among the three cells in animals with expression of InR^{RNAi} with *dicer2* (B). Scale bar: 100 μm. Black bars and grey bars indicate the controls groups and experimental groups in each tests, respectively. Students t-test was performed on the soma area (n=6-18). *$p < 0.05$ ** $p < 0.01$.

Figure 2-14. Circulating DILPs from the brain IPCs, fat body, and Dmp2 neurons are not required for metamorphic growth of the CCAP/bursicon neurons

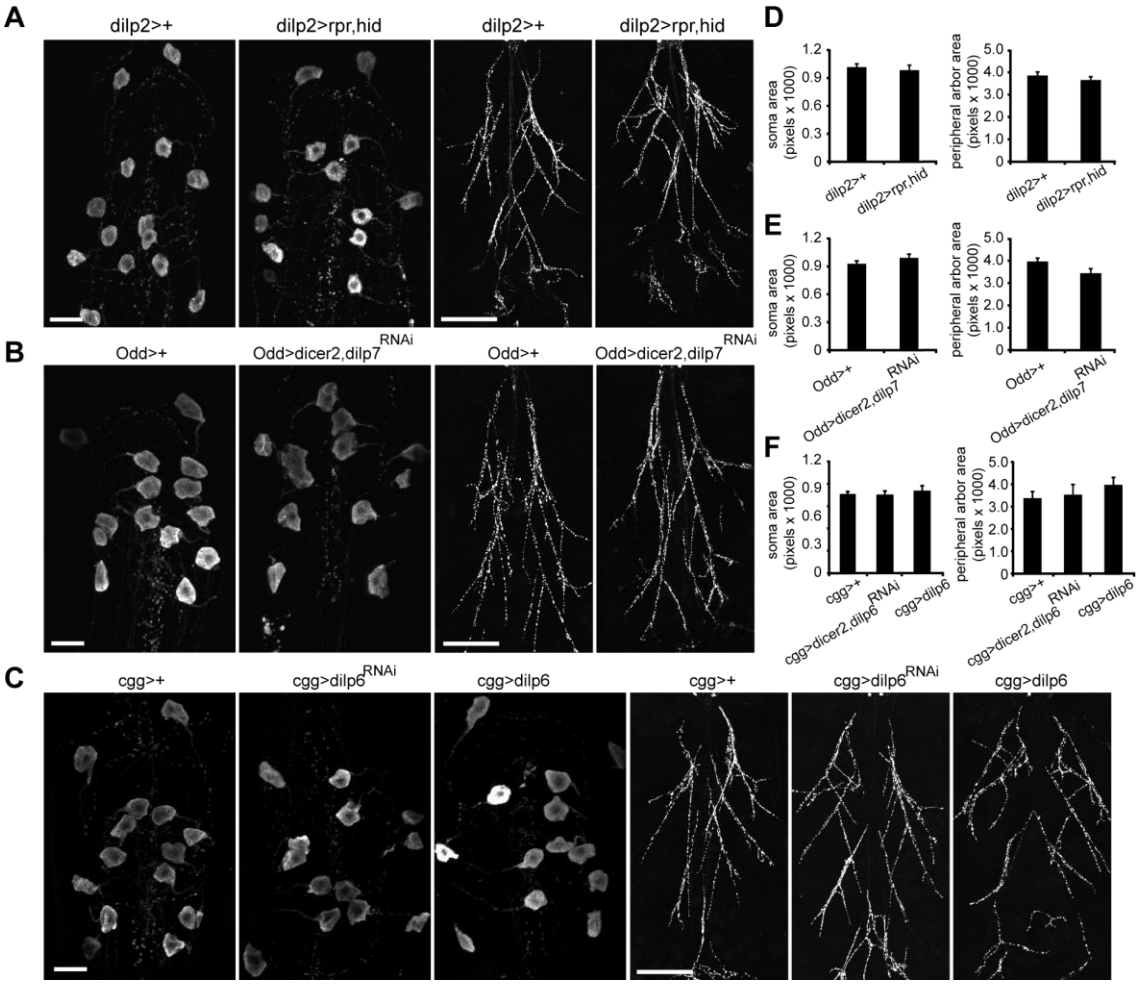

Figure 2-14. Circulating DILPs from the brain IPCs, fat body, and Dmp2 neurons are not required for metamorphic growth of the CCAP/bursicon neurons

(A-C) Confoal images of the CCAP/bursicon neuron somata and axon arbor following the ablation of three sources of circulating DILPs. Ablation the brain IPCs was achieved through cell-targeted expression of *UAS-rpr, UAS-hid* driven by *dilp2-Gal4* (A). To down-regulate DILP7, we targeted $dilp7^{RNAi}$ to the VNC dMP2 neurons with *Odd-Gal4* driver (B). Alteration in the level of DILP6 in the fat body was achieved with the expression of $UAS\text{-}dilp6^{RNAi}$ or *UAS-dilp6* under the control of the fat body-specific driver, *cgg-Gal4* (C). Scale bars: (A) CNS: 20 μm, (A) peripheral arbor: 200 μm. (D-F) Quantification of soma size and peripheral axon arbor area for the CCAP/busicon cells shown in genotypes in (A-C). Data are presented as means ± SEM. Student t-tests and one way ANOVA test were performed on soma size and peripheral axon arbor of genotypes shown in A and B or C, respectively (n=4-6).

CHAPTER 3

A Drosophila deficiency screen for modifiers of *foxo* during metamorphic neuronal remodeling

Abstract

During insect neuronal development, a major transition from maintenance growth in larvae to organizational growth in pupae is an essential feature of metamorphic remodeling of the nervous system. This transition involves the up-regulation of insulin and insulin-like-growth factor signaling (IIS) in many neurons after a long period of functional and structural stability during the larval stages (Chapter 2). However, little is known about how IIS is developmentally regulated in neurons or the identity of other factors/pathways that interact with IIS to facilitate this transition. Here, we have begun to address these questions through a genetic screen for modifiers of IIS-dependent remodeling of the CCAP/bursicon neurons. Metamorphic outgrowth of the CCAP/bursicon neurons, and the execution of wing expansion behaviors that require signaling by these neurons, was disrupted by targeted overexpression of *foxo*, a target of IIS signaling. We then screened a total of 492 deficiency lines for modifiers of the *foxo* overexpression phenotype. A total of 14 deficiency lines were confirmed as suppressors, and 13 were confirmed as enhancers. Two deficiencies, *Df(1)Exel6221* and *Df(1)Exel6062*, strongly suppressed the effects of *foxo* on neuronal outgrowth and were selected for mapping of the responsible genes. *Df(1)Exel6221* also significantly rescued the phenotypes produced by expression of a dominant negative *InR* (InR^{DN}), providing further evidence that the responsive gene(s) within *Df(1)ExEL6221* might be

directly involved in IIS-regulated neuronal remodeling processes. *Df(1)Exel6062* was mapped to a single locus, *Su(z)2*. *Su(z)2* is a zinc finger protein belonging to the *Drosophila* Polycomb Group (PcG) protein family, the members of which function as negative regulators of transcription and of chromatin modifiers. *Su(z)2* partially rescued the phenotype induced by *foxo* overexpression. This result suggests that *foxo* might mediate neurons to re-enter organizational growth phase through chromatin remodeling.

Introduction

Unlike immature neurons, fully differentiated neurons have largely reduced growth capacities. Although they still undergo structural changes, or "maintenance growth", to adapt to changes in body size, their overall morphologies remain stable (Chapter 2). Under certain conditions, some mature neurons can revert to a more embryo-like state to undergo organizational growth, involving axonal and dendritic pathfinding and elaboration of new neuronal arbors. The capacities of mature neurons to re-enter this organizational growth state vary widely. For example, although peripheral nerves can regenerate to a considerable degree following injuries, axons in the mammalian adult central nervous system (CNS) often fail to do so (Ferguson and Son 2011). There is great interest in understanding how neurons may be coaxed to shift from maintenance growth to organizational growth.

Insect neurons provide an excellent system for examining these transitions. In holometabolous insects, fully differentiated larval neurons undergo a period of maintenance growth to accommodate a substantial increase in body size. For example, the *Manduca sexta* motoneurons MN-1 and MN-3 display growth that is proportional to

overall larval growth (Truman and Reiss 1988). In *Drosophila* larval development, the dendrite arborization (da) sensory neurons increase their dendritic arbor in proportion to larval growth, maintaining their overall morphology and functional properties (Parrish, Xu et al. 2009). Once reaching metamorphosis, however, the insect nervous system undergoes extensive remodeling and organizational growth to support the transformation of the insect from a feeding larva into a highly mobile and reproductively-competent adult. During this metamorphic transition, many larval neurons undergo remodeling, which involves the pruning of larval axons and dendrites and the outgrowth and elaboration of adult-specific projections (Levine and Truman 1982). Insect metamorphosis is triggered and coordinated by circulating steroid hormones, the ecdysteroids, which act cell-autonomously to control neuronal remodeling (Robinow, Talbot et al. 1993; Lee, Marticke et al. 2000; Brown, Cherbas et al. 2006).

We have studied a group of neurosecretory cells, the CCAP/bursicon neurons, which undergo substantial neuronal remodeling during metamorphosis. These neurons prune back larval dendrites and axons beginning as early as 3 hr after puparium formation (APF), and pruning is complete at around 30 hr APF. Outgrowth of new adult-specific projections takes place from 15 hr to 6 hr APF (Zhao, Gu et al. 2008). The CCAP/bursicon neurons are required for two major developmental events that temporally bracket these remodeling events. If the neurons are disrupted before the onset of metamorphosis, then the animal displays gross defects in pupal ecdysis, including failure to evert the head from the thorax and failure to properly elongate the developing adult wings and legs. In contrast, disruptions performed during

metamorphosis lead to defects in adult post-eclosion events, including wing expansion and cuticular tanning and sclerotization after eclosion (Park, Schroeder et al. 2003; Peabody, Pohl et al. 2009). Although the head eversion and wing expansion behaviors each only last a few minutes, the morphological phenotypes produced by the failures to perform these behaviors persist for days. Thus, we can perform genetic screens to identify factors that specifically contribute to metamorphic (organizational) growth by targeting genetic manipulations to the CCAP/bursicon neurons and then selecting for ones that preferentially disrupt wing expansion.

There are four members of O-type forkhead domain transcription factor (FOXO) subfamily in mammals but only a single representative protein in *Drosophila* (Puig and Mattila 2011). As a key negative regulator of the insulin and insulin-like growth factor signaling (IIS) pathway, FOXO has been shown to mediate various biological processes, including metabolism, life span, stress resistance, cell proliferation, and organismal growth (Puig and Mattila 2011). In a previous gain-of-function screen, we demonstrated that *foxo* overexpression in the CCAP/bursicon neurons disrupts the metamorphic outgrowth of adult neurites (Zhao, Gu et al. 2008). Further analysis revealed that IIS strongly regulates the organizational growth of the CCAP/bursicon neuron cell bodies and neurite arbor during metamorphosis, but it plays only a minor role in maintenance growth of the larval CCAP/bursicon neurons (Chapter 2). In addition, we manipulated IIS in many other CNS neurons, and in most cases, the organizational growth seen during metamorphosis was substantially more sensitive to IIS than the larval maintenance growth (Chapter 2).

How does IIS interact with other factors to promote the organizational growth of neurons during metamorphosis? We have begun to address this question through a genetic modifier screen for IIS-interacting genes. We first showed that the wing expansion phenotype caused by *foxo* overexpression in the CCAP/bursicon neurons is a good representative of the phenotypes produced by various manipulations that reduce IIS in the CCAP/bursicon neurons, is easy to score, and is a sensitive reporter for genetic interactions with other factors. We then conducted a deficiency-based screen for modifiers of the wing expansion defects produced by *foxo* overexpression. A total of 14 deficiencies were confirmed as suppressors, and 13 deficiencies were confirmed as enhancers. Two deficiencies, *Df(1)Exel6221* and *Df(1)Exel6002*, were selected from this group due to their strong suppression of the wing expansion phenotype, and both also suppressed the effects of FOXO on neuronal outgrowth. In addition, *Df(1)Exel6221* significantly rescued the neuronal outgrowth defects produced by expression of a dominant negative insulin receptor, InR^{DN}. We successfully mapped one of these two deficiencies, *Df(1)Exel6062*, to an individual locus, *Suppressor two of zeste* (*Su(z)2*) . Reduced expression of *Su(z)2* significantly suppressed the phenotypes produced by *foxo* overexpression. Su(z)2 is a zinc finger protein and belongs to the *Drosophila* Polycomb Group (PcG) protein family, the members of which are general repressors of homeotic genes (Brunk, Martin et al. 1991; Grimaud, Negre et al. 2006). Here, our results reveal the function of a member of *Drosophila* PcG protein family, *Su(z)2*, in regulation of neuronal remodeling through a genetic interaction with FOXO during metamorphosis. This indicates that the regulation of gene transcription through

PcG-based chromatin modification may play an important role in reprogramming neurons to re-enter the organizational growth phase.

Results

IIS suppressed the foxo *overexpression phenotype*

Activation of the FOXO transcription factor by dephosphorylation leads to its nuclear translocation, which can be blocked by stimulation of upstream components of the IIS pathway, such as InR, PI3K, and Akt (Puig and Mattila 2011). To assess whether the phenotypes produced by *foxo* overexpression in the CCAP/bursicon neurons are sensitive to genetic interactions with other genes in the IIS pathway, we analyzed the effects of IIS on the wing expansion defects produced by *foxo*. Specifically, we crossed flies with *foxo* overexpression in the CCAP/bursicon neurons (*yw; CCAP-Gal4, UAS-FOXO^{wt}[m3-1]/CyO; +/+*) with male flies from Oregon R (control), *UAS-InR*, *UAS-PI3K*, or *UAS-Akt* stocks. At 25℃, the percentage of flies with unexpanded wings (UEW) dropped from 100% with Oregon R males to 0% with *UAS-InR*, *UAS-PI3K*, or *UAS-Akt* parental males (n=11-78). We then examined the cellular phenotypes produced by these genetic interactions. Overexpression of *foxo* alone in the CCAP/bursicon neurons led to the loss of somata, and the remaining cells displayed reduced soma size and loss of central neurites and the peripheral axon arbor (Chapter 2, Fig. 2-2). Co-expression of *InR* with *foxo* fully rescued the cellular phenotype (Chapter 2, Fig. 2-5). These results confirmed that the wing expansion defects and neurite outgrowth phenotypes induced by *foxo* overexpression were subject to modification by IIS.

Deficiency screen for* foxo *modifiers

The above genetic interaction experiments showed that the developmental responses to *foxo* overexpression are sensitive to epistatic interactions and are regulated by IIS. Although changes in IIS produced by manipulations of InR in the CCAP/bursicon cells (*yw; CCAP-Gal4, UAS-InR^{DN}*/CyO or *yw; CCAP-Gal4, UAS-InR*/CyO) substantially altered neurite outgrowth by these neurons and produced wing expansion defects in homozygous animals (Chapter 2), there were no wing expansion defects in flies heterogeneous for the same elements. Therefore, in order to identify genes that interact with IIS in controlling CCAP/bursicon cell remodeling, we performed a genetic screen for modifiers of the stronger wing expansion phenotype produced by *foxo* overexpression. We screened a total of 492 Exelixis, DrosDel, and Bloomington Stock Center (BSC) deficiencies, each of which was created in an isogenic background and has molecularly defined endpoints (Fig. 3-1). A total of 489 deficiencies were screened for interactions with a constitutively active mutant of *foxo* (*foxo^{T44A,S190A,S259A}*, hereafter referred to as *foxo^{tm}*), and 102 deficiencies were screened for wild type *foxo* (*foxo^{w+}*)(Hwangbo, Gershman et al. 2004) . Together, the tested deficiencies provided ~56% coverage of the euchromatic genome (Fig. 3-1, Zhao, Gu et al. *in prep*). We identified 14 suppressors and 13 enhancers in the *foxo^{tm}* modifier screen (each was confirmed through repeated crosses), and we found 1 suppressor and 4 enhancers with *foxo^{w+}*overexpression (Table 2). To test if the suppressors and enhancers from the *foxo^{tm}* screen could also modify the wing expansion phenotypes produced by *foxo^{w+}*overexpression, we examined the 5 strongest suppressors from

foxo^{tm} screen. All five deficiencies substantially suppressed the wing expansion phenotype produced by *foxo*$^{w+}$ overexpression (P<0.0001, n=29-84, Table 2). This suggests that these suppressors display the same interactions with both *foxo*$^{w+}$ and *foxo*^{tm}, and we therefore performed further analysis using only *foxo*$^{w+}$ flies.

Df(1)Exel6221 *completely rescued the FOXO*^{wt} *overexpression phenotype*

Df(1)Exel6221 was the strongest suppressor of the *foxo* overexpression wing expansion phenotype, with 100% of the progeny showing fully expanded wings. Therefore, we analyzed this deficiency further. We first tested whether the deficiency deleted factors that regulate Gal4-mediated transgene expression. We reasoned that if the deficiency deletes a general transcription factor, it might suppress the *foxo* overexpression phenotype simply by reducing the expression of transgenes produced by the Gal4/UAS system. To test this hypothesis, we examined the effects of *Df(1)Exel6221* on the expression of *UAS-CD8::GFP* driven by *CCAP-Gal4*. There was no change in the GFP fluorescence level (measured in the somata) in the CCAP/bursicon neurons (Fig. 3-2B). Therefore, a gene (or genes) within *Df(1)Exel6221* is likely to be involved in direct genetic interactions with FOXO.

Next, we tested whether *Df(1)Exel6221* could also rescue the cell loss and neurite outgrowth defects produced by *foxo*$^{w+}$ overexpression (Fig. 3-3). Although the morphological appearance of the CCAP/bursicon neurons in *Df(1)Exel6221/+* flies was not distinguishable from control *CCAP-Gal4/+* animals (Fig. 3-3B, B′, E, and F), *Df(1)Exel6221* in combination with cell-targeted expression of *foxo*$^{w+}$ in the CCAP/bursicon cells restored the number of somata and the morphology of the central

and peripheral axon arbor (Fig. 3-3D, and D′). In fact, the soma size and area covered by the peripheral arbor were both greater in the *Df(1)Exel6221/+; CCAP-Gal4, UAS-FOXO^{w+}/+* animals than in *CCAP-Gal4/+* controls (Fig. 3-3E and F) (See Discussion). This was in contrast to the reductions in cells and neurite growth produced by *foxo^{w+}* overexpression alone (Fig. 3-3C, C′, E, and F). These results show a complete rescue of *foxo^{w+}* overexpression phenotypes by *Df(1)Exel6221*. Therefore, *Df(1)Exel6221* may delete one or more genes that interact with FOXO to regulate neuronal remodeling of the CCAP/bursicon cells.

Suppression of InR^{DN} *phenotypes by* Df(1)Exel6221

Although FOXO is a key component of the IIS pathway, it is also regulated by other signaling pathways, and *foxo*-interacting genes may or may not interact with IIS. Therefore we tested whether the suppressors and enhancers from the FOXO screen could also modify *InR^{DN}* phenotypes. Expression of *InR^{DN}* substantially reduced CCAP/bursicon neuron soma size (Fig. 3-4C and E) and the number of branches in the peripheral axon arbor (Fig. 3-4C′ and F). Both of these phenotypes were significantly rescued when *Df(1)Exel6221* was crossed into this background (Fig. 3-4D, D′, E, and F). Therefore, the suppressor gene(s) within *Df(1)Exel6221* interact either directly or indirectly with IIS to regulate neuronal remodeling of the CCAP/bursicon neurons.

Identification of the suppressor gene(s) within Df(1)Exel6221

In order to identify the gene(s) responsible for the suppression produced by *Df(1)Exel6221*, we first tested an overlapping deficiency (*Df(1)ED6396*) to narrow

down the list of candidate genes within or closely apposed to the genomic region delineated by *Df(1)Exel6221*. Then, we examined representative mutant alleles and/or RNAi constructs (driven by *CCAP-Gal4*) for all the genes for which stocks were available (Table 3). In repeated crosses, the mutant alleles and/or the expression of RNAi constructs of four genes, *CG32816*, *CG18275*, *CG18273*, and *CG18166*, displayed >10% suppression of the UEW phenotype produced by *foxo* overexpression (Table 3). Although all of these effects were statistically significant, none of them equaled the extent of rescue with *Df(1)Exel6221*. Therefore, the disruption of four genes by *Df(1)Exel6221* may produce additive suppression. Because null alleles were not available for these genes, and RNAi can produce hypormorphic phenotypes (Belles 2010), the lack of strong suppression may also have reflected partial loss-of-function phenotypes. Among all the genetic manipulations tested, *CCAP-Gal4* directed expression of $CG18275^{RNAi}$ (VDRC109394) produced the strongest suppression, with up to a 64.71% reduction in the percentage of UEW (Table 3). *CG18275* and *CG18166* are predicted to be pseudogenes, and are paralogs to *CG18273* (Flybase Genome Annotators 2012) (See discussion). $CG18275^{RNAi}$ (VDRC109394) also targets *CG18273* and *CG18166*, and RNAi to CG18273 and CG18166 also produced suppression (Table 3). This suggests that the strong suppression by $CG18275^{RNAi}$ (VDRC109394) may arise from the knockdown of all three genes and that all three genes are functional.

We analyzed the cellular phenotype of $CG18275^{RNAi}$ (Fig. 3-5) due to its strong suppression of the *foxo*-induced wing expansion phenotype. Co-expression of *UAS-*$CG18275^{RNAi}$ and *UAS-*$FOXO^{wt}$ under control of the *CCAP-Gal4* driver partially, but

significantly rescued the CCAP/bursicon cell soma number, with 5.4 ± 0.6 cells present in the rescue animals (Fig. 3-5D and E), versus 1.6 ± 0.9 in *FOXO*$^{w+}$-only controls (Fig. 3-5C and E). The peripheral axon arbor was also significantly rescued by *CG18275*^{RNAi}, with an increase in arbor area from 74 ± 57 pixels in the control animals (Fig. 3-5C′ and E) to 419 ± 200 pixels in the rescue animals (Fig. 3-5D′ and F). When *UAS-CG18275*^{RNAi} was expressed alone (without *UAS-foxo*), the cellular distribution of neuropeptide was altered and the soma size was significantly reduced (Fig. 3-5B) from 1867 ± 62 pixels to 1348 ± 37 pixels. Thus, RNAi to *CG18275* directly reduced soma growth. The soma size in the rescue animals was 1051 ± 85 pixels (Fig. 3-5D and E), which was not significantly different from 1110 ± 87 pixels in the animals with *CCAP>foxo*$^{w+}$ alone (Fig. 3-5C and E). Thus, *CG18275*^{RNAi} and *foxo*$^{w+}$ each suppressed the effects of the other on growth of the CCAP/bursicon neurons.

We also examined whether RNAi to *CG18275* could rescue the effects of *InR*^{DN} on the CCAP/bursicon neurons. The soma size of the CCAP/bursicon cells in animals with both *CG18275*^{RNAi} and *InR*^{DN} expression (*CCAP>FOXO*^{wt}, *CG18275*^{RNAi}) was 704 ± 23 pixels, which was not significantly different from the soma size of 686 ± 25 pixels in controls (*CCAP>FOXO*^{wt}). Thus, *InR*^{DN} and *CG18275*^{RNAi} also displayed mutual suppression of the effects of the other on the soma growth of the CCAP/bursicon cells. However, we did not observe statistically significant suppression of the effects of *InR*^{DN} on the growth of peripheral axon arbor, although there was a trend in that direction (3005 ± 793 pixels in rescue animals, versus 2668 ±417 pixels in controls). Unlike *Df(1)Exel6221* which completely rescued the wing expansion defects produced by *foxo* overexpression, *CG18275*^{RNAi} provided only partial suppression. The lack of stronger

suppression of the wing expansion and axonal branching defects by *CG18275*^{RNAi} in *InR*^{DN} animals may reflect inefficient gene silencing by RNAi. Nevertheless, based on the *foxo* suppression results, our findings suggest that *CG18275* (together with *CG32816*, *CG18273*, and *CG18166*) interacts with IIS to control metamorphic growth of the CCAP/bursicon neurons. Additional support for this model may require the combined use of stronger loss-of-function alleles.

Df(2)Exel6062 *partially suppressed the* foxo *overexpression phenotype*

Df(2)Exel6062 partially but significantly suppressed the wing expansion phenotype produced by *foxo* overexpression. Flies with *CCAP-Gal4* driving *UAS-FOXO*^{wt} in a *Df(2)Exel6062/+* background displayed 66.0 ± 9.4% UEW, 24.9 ± 9.3% PEW, and 9.4 ± 0.9% expanded wings (n=69). The control flies with *foxo* overexpression in the CCAP/bursicon cells in a wild type genetic background displayed 97.6% UEW, and the rest of the flies had PEW (n=82). At the cellular level, *Df(2)Exel6062* suppressed the *foxo*-induced loss of CCAP/bursicon neuron somata and neurites (Fig. 3-6E, E′, G, and H). Compared to the *foxo*-only controls (Fig. 3-6D and D′), the presence of one copy of *Df(2)Exel6062* produced a 2.5-fold increase in CCAP/bursicon soma number (Fig. 3-6E and G) and a 9.5-fold increase in peripheral axonal arbor area (Fig. 3-6E′ and H). Therefore, *Df(2)Exel6062* provided partial suppression of the *foxo* overexpression phenotype in the CCAP/bursicon neurons.

Su(z)2 *suppressed the* foxo *overexpression phenotype*

Df(2)Exel6062 deletes only two genes, *CG33789* and *Suppressor of zeste2*

(*Su(z)2*). To an extent similar to *Df(2)Exel6062*, animals bearing a heterozygous a *Su(z)2* loss-of-function allele, $Su(z)2^{1.a1}$, displayed partial but significant suppression of the wing expansion defects produced by *foxo* overexpression, with 62.5% UEW and the remaining adults with PEW (n=16, P<0.001) (Wu and Howe 1995). We then used two RNAi constructs (*JF01293* and *HMS00281*) to examine the effects of RNAi to *Su(z)2* specifically in the CCAP/bursicon neurons. In control animals, which were used to test the effects of loss of *Su(z)2* alone, co-expression of *UAS-*$Su(z)2^{RNAi}$*(JF01293)* with *UAS-CD8::GFP* in the CCAP/bursicon cells had no effect on wing expansion (n=48) (data not shown) or levels of GFP fluorescence (n=6), suggesting the loss of *Su(z)2* alone has no effect on *Gal4*-mediated transgene expression. Adults with co-expression of *UAS-*$Su(z)2^{RNAi}$*(JF01293)* and *UAS-*$FOXO^{wt}$ under control of *CCAP-Gal4* driver displayed 61.9% UEW and 16.7% PEW, and the rest had expanded wings (n=42). This is in contrast to the high percentage of wing expansion defects (97.6% UEW and 2.4% PEW n=82) observed in animals with only *UAS-*$FOXO^{wt}$ driven by *CCAP-Gal4.*

Surprisingly, cell-targeted expression of the HMS00281 *UAS-*$Su(z)2^{RNAi}$ construct alone (n=61) or together with *UAS-*$FOXO^{wt}$ (n=52) in the CCAP/bursicon neurons resulted in adults with 100% UEW, suggesting possible off-target effects of the *HMS00281* construct that led directly to the fly wing expansion defect. Unlike the *JF01293* construct, which targets a long, 441bp sequence, the *HMS00281* construct targets a short, 21bp sequence, and a BLAST search with this 21 bp to the *Drosophila* genome resulted in a hit to *Smrter* with 15bp match. SMRTER is a co-repressor of E75A (Johnston, Sedkov et al. 2011), a key early ecdysone-induced gene that controls molting and metamorphosis of *Manduca sexta* and *Drosophila melanogaster*

(Riddiford, Hiruma et al. 2003). While any potential off-target effects for *HMS00281* remain to be confirmed, the suppression produced by $Su(z)2^{1.a1}$ and $Su(z)2^{RNAi}$ with the *JF01293* allele suggest that *Su(z)2* is the *foxo* suppressor contained within *Df(2)Exel6062*.

We next examined whether $Su(z)2^{RNAi}$*(JF01293)* suppressed the cellular defects produced by *foxo* overexpression. Similar to *Df(2)Exel6062*, $Su(z)2^{RNAi}$*(JF01293)* partially but significantly restored the number of CCAP/bursicon neuron somata and the extent of neurite outgrowth (Fig. 3-6F, F′, G, and H). This finding provides further support for the conclusion that *Su(z)2* is the gene responsible for the dominant suppression observed with *Df(2)Exel6062*. However, we did not observe suppression by $Su(z)2^{RNAi}$ of the soma size and neurite growth defects produced by cell-targeted expression of InR^{DN} in the CCAP/bursicon neurons (n=6) (Data not shown). Tests with additional, stronger *Su(z)2* alleles may be required to demonstrate regulation of metamorphic outgrowth through all an interaction of *Su(z)2* and IIS.

Discussion

In a genetic modifier screen for modifiers of the wing expansion phenotype produced by *foxo* overexpression, we recovered 14 suppressors and 13 enhancers. Two deficiency lines, *Df(1)Exel6221* and *Df(2)Exel6062*, strongly suppressed the effects of *foxo* on neuronal outgrowth and were selected for mapping down to the level of single genes. To select for modifiers that interacted specifically with IIS, rather than other signaling pathways that control FOXO function, we tested these two suppressors for rescue of the neurite outgrowth defects produced by InR^{DN}. *Df(1)Exel6221* also

significantly rescued the cellular effects of InR^{DN}.

***Multiple genes are responsible for the suppression produced by* Df(1)Exel6221**

One deficiency, *Df(1)Exel6221,* completely suppressed both the wing expansion defects and also the cellular phenotype caused by *foxo* overexpression (Fig. 3-2). In the *foxo* overexpression background, but not in control cells, *Df(1)Exel6221* also significantly increased cell size and neurite outgrowth (above wild type) of the CCAP/bursicon neurons (Fig. 3-2D and D′). This suggests the *Df(1)Exel6221* and the suppressor genes contained within it may promote neuron growth through *foxo*.

Df(1)Exel6221 uncovers 13 genes, for which the RNAi constructs, mutant alleles, and transgenic constructs were examined for their capabilities to independently suppress the wing expansion defects induced by *foxo* overexpression (Table 3). Four genes exhibited suppression: *CG18273, CG18275*, *CG18166,* and *CG32816*. RNAi to *CG18275* (with a construct that is predicted to make off-target hits to *CG18273* and *CG18166*) significantly rescued the wing expansion phenotype and cell loss phenotype produced by *foxo* overexpression. *CG18275* and *CG18166* appear to have derived from *CG18273* through tandem triplication (FlyBase Genome Annotators 2012). Together, these results suggest that the suppression in *Df(1)Exel6221* is at least partially due to *CG18273*, *CG18275*, and *CG18166.*

CG18273 is a novel protein-coding gene, and the available information indicates that it plays an important role in the fly nervous system. In larvae, *CG18273* displays the highest mRNA expression level in the central nervous system (among eight

measured larval organs), and in the adult stage, it exhibits the highest expression level in brain compared with the sixteen other adult organs/tissues (Chintapalli, Wang et al. 2007). In addition, RNAi to *CG18273* (*CG18273GD*^{8001}) produced lethal neuroanatomy defects during the larval stages when the construct was crossed to the nervous system driver, *insc-Gal4* (Neumuller, Richter et al. 2011).

The modENCODE Temporal Expression Profile indicates that *CG18273*, *CG18275*, and *CG18166* exhibit different temporal patterns of expression during development (Graveley, Brooks et al. 2011). The expression of *CG18273* peaks during early embryonic and early larval stages, while it remains at a low level throughout the remaining larval stages and in pupae. *CG18275* displays three peaks of expression: at 0-12 hr of embryonic development, in late larvae, and in early pupae. *CG18166* is highly expressed throughout the pupal stage with an early peak in white prepupae. Therefore, the temporal expression patterns of the three genes suggest that *CG18273*, *CG18275*, and *CG18166* may perform different biological functions during *Drosophila* development.

Su(z)2 *is a dominant suppressor of* foxo

We have identified *Su(z)2* as the dominant suppressor contained in *Df(2)Exel6202* of the wing expansion, cell loss, and neurite outgrowth phenotypes produced by *foxo* overexpression. Surprisingly, *Su(z)2*^{RNAi} did not rescue the metamorphic neuron outgrowth phenotypes produced by *InR*^{DN}. However, the RNAi manipulations used here may only partially knock down *Su(z)2* function, and the possibility of *Su(z)2* interacts with genes within the IIS pathway has therefore not been

excluded.

The *Su(z)2* gene encodes a zinc finger protein belonging to the *Drosophila* Polycomb Group (PcG) protein family, the members of which function as negative regulators of transcription and of chromatin modification factors (Brunk, Martin et al. 1991). *Su(z)2* has a paralogous gene, *Posterior sex combs* (*Psc*), which likely arose by gene duplication (Adler, Charlton et al. 1989; Brunk, Martin et al. 1991; Wu and Howe 1995). The functional redundancy between *Psc* and *Su(z)2* may also contribute to the inefficiency of $Su(z)2^{RNAi}$ in suppression of the InR^{DN} phenotypes. The protein products of the two genes comprise the *Su(z)2 complex* which mediates transcriptional repression (Emmons, Genetti et al. 2009). The *Su(z)2* gene is highly expressed in the central nervous system, with its highest expression in 12-24 hr embryos and throughout pupal development, consistent with a regulatory role of *Su(z)2* on neural development during embryogenesis and metamorphosis (Chintapalli, Wang et al. 2007; Graveley, Brooks et al. 2011). Other evidence suggests that *Su(z)2* participates in the reprogramming of cell fates. For instance, deletion of *Su(z)2* together with the neighboring, homologous gene *Posterior sex combs* (*Psc*) in wing discs and eyes discs causes severe tissue overgrowth and differentiation defects (Classen, Bunker et al. 2009), and *Su(z)2* and many other PcG group genes are highly expressed in transdetermining cells (Klebes, Sustar et al. 2005). Interestingly, a recent study using genomic approaches to identify direct FOXO targets uncovered several genes, including PcG genes, involved in negative transcriptional regulation (Alic, Andrews et al. 2011). Thus, the roles of *Su(z)2* in neural development and cell fate reprogramming, as well as the genetic and interaction with *foxo*, suggest a model in which regulation by *Su(z)2* of the transition of neurons

from a maintenance growth state to reorganization growth state during metamorphosis is interrupted by *foxo* overexpression.

Although much is known about the function of *Drosophila Psc*, *Su(z)2* has received far less attention (Emmons, Genetti et al. 2009). *Su(z)2* has been implicated in multiple signaling pathways, including the Wnt signaling pathway and the JAK-STAT signaling pathway (Classen, Bunker et al. 2009; Li, Han et al. 2010). For instance, *Su(z)2* and *Psc* directly repress the expression the JAK-STAT signaling ligand, Unpaired (UPD), to control *Drosophila* imaginal disc growth (Classen, Bunker et al. 2009). It is therefore conceivable that *Su(z)2* may interact with *foxo* by affecting *Su(z)2*-regulated signaling pathways, such as the JAK-STAT signaling pathway.

Roles of PcG proteins in neurodevelopment

PcG proteins are evolutionarily conserved regulators of gene expression that control many biological processes, including cellular proliferation, hematopoiesis, genomic imprinting, stem cell maintenance, and cancer (Remillieux-Leschelle, Santamaria et al. 2002; Martinez and Cavalli 2006; Schuettengruber, Chourrout et al. 2007; Gonzalez, Simon et al. 2009; Richly, Aloia et al. 2011). Less is known about the roles of PcG proteins in neurodevelopment, but several studies have implicated them in anterior-posterior neural patterning (Barnett, Seville et al. 2001; Kitaguchi, Nakata et al. 2001; Kwon and Chung 2003). PcG proteins also cell-autonomously promote neuronal stem cell self-renewal and are required for postembyonic neuroblast survival (Molofsky, Pardal et al. 2003; Bello, Holbro et al. 2007; Shi, Sun et al. 2008). In differentiated neurons, PcG proteins have been shown to regulate axon spouting and

dendritic arborization and the maintenance of *Drosophila* sensory neuron dendrites (Smouse and Perrimon 1990; Parrish, Emoto et al. 2007). In addition, a recent report indicates the PcG proteins respond to ecdysone signaling to control neuronal diversity during metamorphosis (Wang, Lee et al. 2006). Here, our results provide further support for the involvement of members of *Drosophila* PcG protein family in regulating neuronal remodeling during metamorphosis, and they suggest a role for PcG proteins in reprogramming neurons to re-enter the organizational growth phase.

Material and methods

Stocks and scoring

Flies (*Drosophila melanogaster*) were cultured on standard cornmeal-yeast-agar media at 25^{o}C (unless indicated). A total of 492 lines containing Exelixis, DrosDel, and Bloomington Stock Center (BSC) deficiencies (Parks, Cook et al. 2004) on the 1st, 2nd, and 3rd chromosomes were attained from the Bloomington Drosophila Stock Center (BCSC). *CCAP-Gal4* (*y* w*; P{CCAP-GAL4.P}16*; FBti0037998) was used to target transgene expression in the CCAP/bursicon neurons, most of which also express the neuropeptide bursicon (Park, Schroeder et al. 2003). *UAS-FOXO^{wt}[m3-1]* and *UAS-FOXO^{tm}[f3-9]* were kindly provided by Marc Tatar, Brown University (Hwangbo, Gershman et al. 2004). The *Su(z)2^{RNAi}* lines were generated by the Transgenic RNAi Project (TRiP) (Ni, Markstein et al. 2008). All other RNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC), and various other alleles used for gene mapping were obtained from the BDSC, the Harvard Exelixis Collection, or individual laboratories (Thibault, Singer et al. 2004; Dietzl, Chen et al. 2007).

Immunochemistry and staining quantification

Immunostaining was performed on central nervous systems or whole-animal fillets as described in Chapter 2. Control and test groups of animals were treated in parallel. The following primary antisera directed against the following proteins were used: bursicon α-subunit (1:5000, PFA/PA) (Luan, Peabody et al. 2006), Green fluorescent protein (GFP) (1:500, PFA) (Invitrogen, Carlsbad, CA). The confocal imaging and quantification was performed as described in Chapter 2.

Modifier screen

The modifier screen was conducted in two phases. The first phase was performed at 25^{o}C, and deficiencies were screened for modification of the wing expansion defects produced by expression of wild type FOXO (FOXO^{wt}) or constitutively activated FOXO (FOXO^{tm}) in the CCAP/bursicon neurons. Initially, the FOXO^{wt} stock line (*yw; CCAP-Gal4, UAS-FOXO^{wt}[m3-1]/CyO,y^{+}; +/+*) had 100% UEW adults, and the FOXO^{tm} stock line (*yw; CCAP-Gal4, UAS-FOXO^{tm}[f3-9]/CyO,y^{+}; +/+*) had 94% UEW adults. However, after six months, the FOXO^{tm} phenotype drifted, and many flies with PEW or normal wings appeared in the stock. Therefore, to prevent further phenotypic drift, the FOXO^{tm} stock line was rebalanced to *CyO, tub-UAS-Gal80* to inhibit *Gal4* function. The observation of greater drift in flies with FOXO^{tm} overexpression than FOXO^{wt} indicated that it was a more sensitized background for detection of modifiers. In addition, fewer suppressors were identified in the phase 1 screen with FOXO^{wt} overexpression than with FOXO^{tm}. Therefore, we conducted a second phase of a

screening using the new FOXO^{tm} stock (*yw; CCAP-Gal4, UAS-FOXO^{tm}[f3-9]/CyO,Gal80; +/+*). This second phase was conducted at 18^{o}C to reduce the strength of Gal4-dependent transgene expression to increase the chances of observing suppression of the FOXO^{tm} overexpression phenotype.

To screen for modifiers on the first chromosome in phase two, we crossed ten virgin females from each stock containing a first chromosome deficiency (balanced over *FM7 or Binsinscy*) to ten males from the stock, *yw; CCAP-Gal4, UAS-FOXO^{wt}/ CyO, tub-UAS-Gal80; +/+*, or *yw; CCAP-Gal4, UAS-FOXO^{tm}/ CyO, tub-UAS-Gal80; +/+*. For phase one, the female parental genotype was *yw; CCAP-Gal4, UAS-FOXO^{wt}/ CyO, UAS-Ubi; +/+*. Oregon R virgin females were used to cross to the *UAS-FOXO^{wt}* males as controls. All four possible adult genotypes were scored. The number of *Curly*-winged adult progeny was counted, and if the total number of the *CyO*-containing flies from a single cross was less than twenty, we considered the adult yield insufficient and repeated the cross. The other two non-*Curly-winged* genotypes (*Df, w1118/yw or Y*; *CCAP-Gal4, UAS-FOXO^{wt}/+; +/+* and *Fm7 or Binsinscy/yw or Y*; *CCAP-Gal4, UAS-FOXO^{wt}/+; +/+*) were scored for wing expansion (as UEW, PEW, or expanded). Flies containing the Bar marker (from *FM7* or *Binsinscy*) served as internal controls. The Fisher Exact Probability Test was performed to determine if differences between wing expansion in each experimental group were significantly different from the value for the internal, Bar-eyed control group. One homozygous viable deficiency, *Df(1)ED6989*, lacked the internal control group, and progeny (*yw/+; CCAP-Gal4, UAS-FOXO^{wt}/+; +/+*) from a cross of the stock line with Oregon R males were used as an external control for the statistical test. Deficiencies with a P value of <0.05 were classified as

suppressors or enhancers. Enhancers of the *foxo* GOF phenotype were also reported if more than half of the progeny displayed pupal head eversion defects.

For the second chromosome deficiencies (balanced over *CyO*), the sexes of the parental flies were reversed. The *Curly*-winged adult progeny of these crosses [*Df/CyO,y*$^{+}$ (first phase) or *Df/CyO, tub-Gal80* (second phase) and *CCAP-Gal4, UAS-FOXO*^{wt}*/CyO* (both phases)] were discarded. The remaining straight-winged, PEW, or UEW progeny belonged to one of the following two genotypes: *CCAP-Gal4, UAS-FOXO*^{wt}*/Df* or *CCAP-Gal4, UAS-FOXO*^{wt}*/CyO.* Oregon R was used in control crosses to the FOXO stocks every month, and we compared the rates of wing expansion to the control crosses with the Fisher Exact Probability Test.

Screening for modifiers on the third chromosome was performed by crossing *yw; CCAP-Gal4, UAS-FOXO*^{wt}*/CyO, y*$^{+}$ (first phase); or +/+ *yw; CCAP-Gal4, UAS-FOXO*^{wt}*/CyO, tub-UAS-Gal80;* +/+ (second phase) virgin females to Oregon R (controls) and Exelixis deficiency males at 18^{o}C. Most of the third chromosome deficiencies were balanced over *TM6B,Tb*^{1}. All *Tubby* pupae (*CyO, tub-Gal80/+; TM6,Tb*^{1} or *CCAP-Gal4, UAS-FOXO*^{wt}*/+; TM6,Tb*^{1}*/+*) were removed daily from the vials. The *Curly*-winged adult progeny (*CyO, tub-Gal80/+; Df/+*) were also discarded. The remaining adults (*CCAP-Gal4, UAS-FOXO*^{wt}*/+; Df/+*) were scored for wing expansion. Similar to the second chromosome screen, these values were compared to control crosses with Oregon R, and the Fisher Exact Probability Test was performed to determine suppressors or enhancers. Enhancers were also detected if more than half of the progeny displayed pupal head eversion defects.

Table 2. Deficiency modifiers of the wing expansion phenotype produced by *foxo^{tm}* or *foxo^{wt}* overexpression

Deficiency	Class	FOXO p	P value	Other phenotypes	Phase
Df(1)Exel6221	Suppressor	FOXO^{tm}	<0.0001		2nd
Df(1)Exel6233	Suppressor	FOXO^{tm}	<0.0001		1st, 2nd
Df(1)Exel6240	Suppressor	FOXO^{tm}	<0.01		1st
Df(1)Exel6248	Enhancer	FOXO^{tm}		HE	1st
		FOXO^{wt}		HE	1st
Df(1)Exel6251	Enhancer	FOXO^{tm}		HE	1st
Df(1)ED411	Enhancer	FOXO^{tm}		HE	2nd
Df(1)ED7217	Enhancer	FOXO^{tm}	<0.05		2nd
Df(1)ED6989	Enhancer	FOXO^{tm}	<0.05		2nd
Df(2L)Exel6001	Suppressor	FOXO^{tm}	<0.01		2nd
Df(2L)Exel6017	Suppressor	FOXO^{tm}	<0.001		1st, 2nd
Df(2L)Exel6036	Enhancer	FOXO^{wt}		HE	1st
Df(2L)Exel6062	Suppressor	FOXO^{tm}	<0.00001		2nd
		FOXO^{wt}	<0.01		1st
Df(2L)Exel6077	Suppressor	FOXO^{tm}	<0.01		1st, 2nd
Df(2L)Exel7006	Suppressor	FOXO^{tm}	<0.00001		2nd
Df(2L)Exel7016	Enhancer	FOXO^{wt}		HE	1st
Df(2L)Exel7024	Enhancer	FOXO^{wt}		HE	1st
Df(2L)Exel7038	Suppressor	FOXO^{tm}	<0.01		2nd
Df(2L)Exel8022	Suppressor	FOXO^{tm}	<0.05		2nd
Df(2L)Exel8040	Suppressor	FOXO^{tm}	<0.01		1st
Df(2L)Exel9032	Suppressor	FOXO^{tm}	<0.01		1st, 2nd
Df(2L)BSC147	Suppressor	FOXO^{tm}	<0.05		2nd
Df(3L)Exel6087	Suppressor	FOXO^{tm}	<0.00001		2nd
Df(3L)Exel6123	Enhancer	FOXO^{tm}		HE	1st
Df(3L)Exel6135	Enhancer	FOXO^{tm}		HE	1st, 2nd
Df(3L)Exel9001	Enhancer	FOXO^{tm}		HE	1st
Df(3R)Exel6148	Enhancer	FOXO^{tm}		HE	1st, 2nd
Df(3R)Exel6162	Enhancer	FOXO^{tm}		HE	1st

All deficiency modifiers were confirmed with a second or third cross. If in more than two tested, P<0.05 (Fisher exact test), the deficiency was considered as a suppressor or enhancer. The presence of more than half of progeny were head eversion defects observed in pupae (HE) indicated enhancers.

Table 3. Suppressor loci mapped to *Df(1)Exel6221*

Location of deficiency	Candidate Genes	Allele(s)	RNAi construct	Suppression	P value
Outside of *Df(1)Exel6221* (Left)	*CG32816* *	G17512 EY03825 EY12783 EY21277 MB01802 MB03211 MB06700	 100838	36.37% 8.47% no 6.25% 3.37% no 13.64% 12% - 15.79%	<0.05 <0.01
	ase		28280	no	
	Cyp4g1		30205	no	
Inside of *Df(1)Exel6221*, but outside of *Df(1)ED6396*	*Exp6*	EY06510		no	
	CG13373	d01279		no	
	CR18275 *		109394	16.22% - 64.71%	<0.001
	CG32817		105559	0%-2.94%	
	CR18166 *		110024	27%-27.78%	<0.001
	CG3176		109976	0%-5.13%	
	CG18273 *	G0399	 107818	10.12% 16%	<0.05 <0.05
	CG3156	f03674		no	
			105646	0% - 2.56%	
	CG17896	KG03442	 107006	2.63% 4.54%	
Inside of *Df(1)ED6396*	*CG17778*	KG03953	 20549	no 8.70%	
	svr	1 EP356 KG02090		no no no	
	arg	KG00397 KG03378		no no	
	elva	G0031		no	
Outside of *Df(1)Exel6221* (Right)	*CG4293*	EY22639		no	
	App1	BG02664	 TRiP.JF02878	no no	

Each candidate gene was tested at least twice. The suppression was calculated based on the differences of percentage of total wing expansion defects between the experimental groups and the control groups. If $P<0.05$ (Fisher exact test), the candidate gene was considered a suppressor and labeled with *.

Figure 3-1. Map of tested deficiencies, suppressors, and enhancers of the *foxo* overexpression phenotype

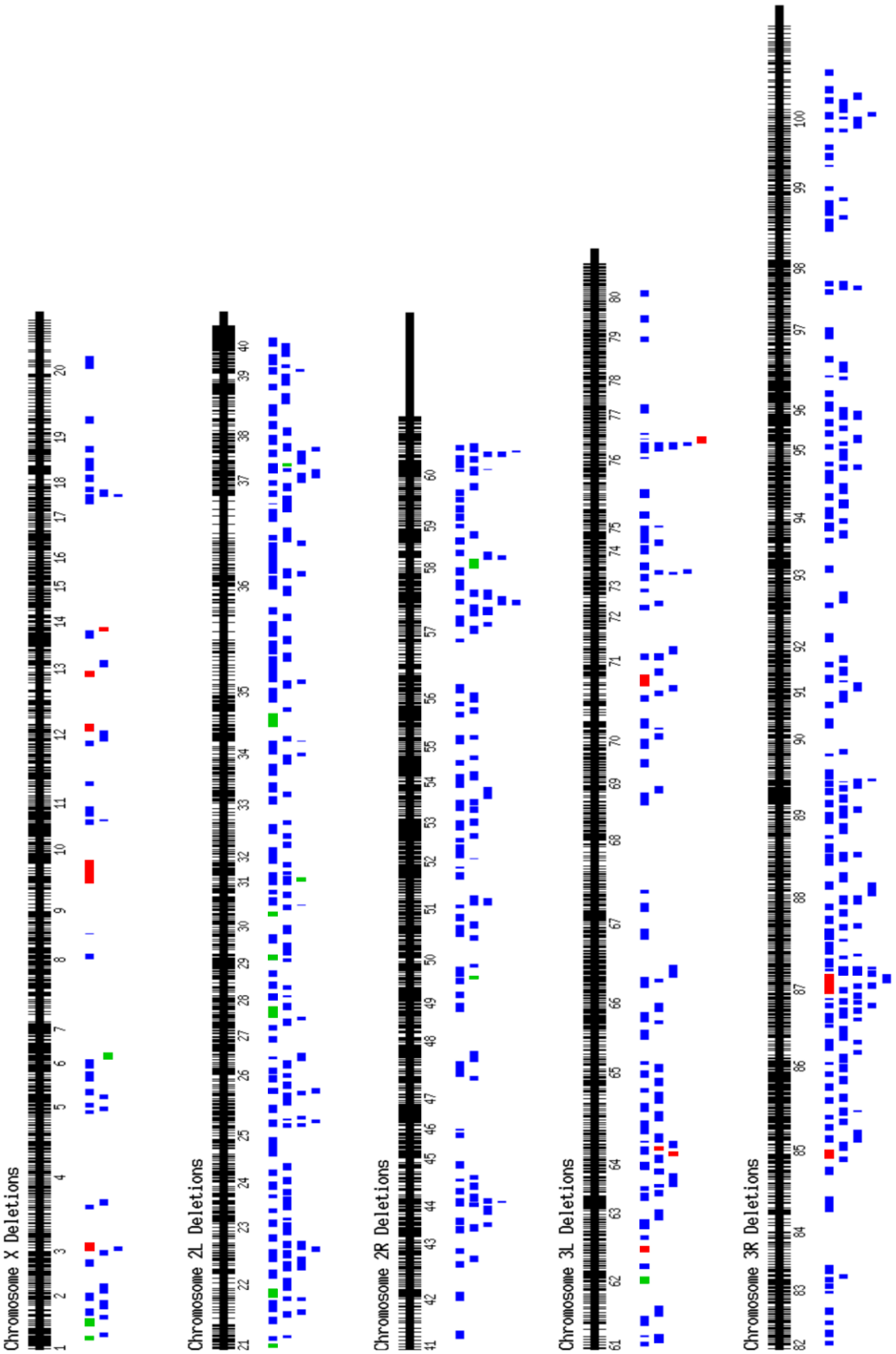

Figure 3-1. Map of tested deficiencies, suppressors, and enhancers of the *foxo* overexpression phenotype

Each blue box represents the region deleted by one of the tested Exelixis, DrosDel, or Bloomington Stock Center (BSC) deficiencies; green boxes represent suppressors, while red boxes represent enhancers.

Figure 3-2. ***Df(1)Exel6221*** **and** ***Df(2)Exel6062*** **display no effect on Gal4-mediated transgene expression**

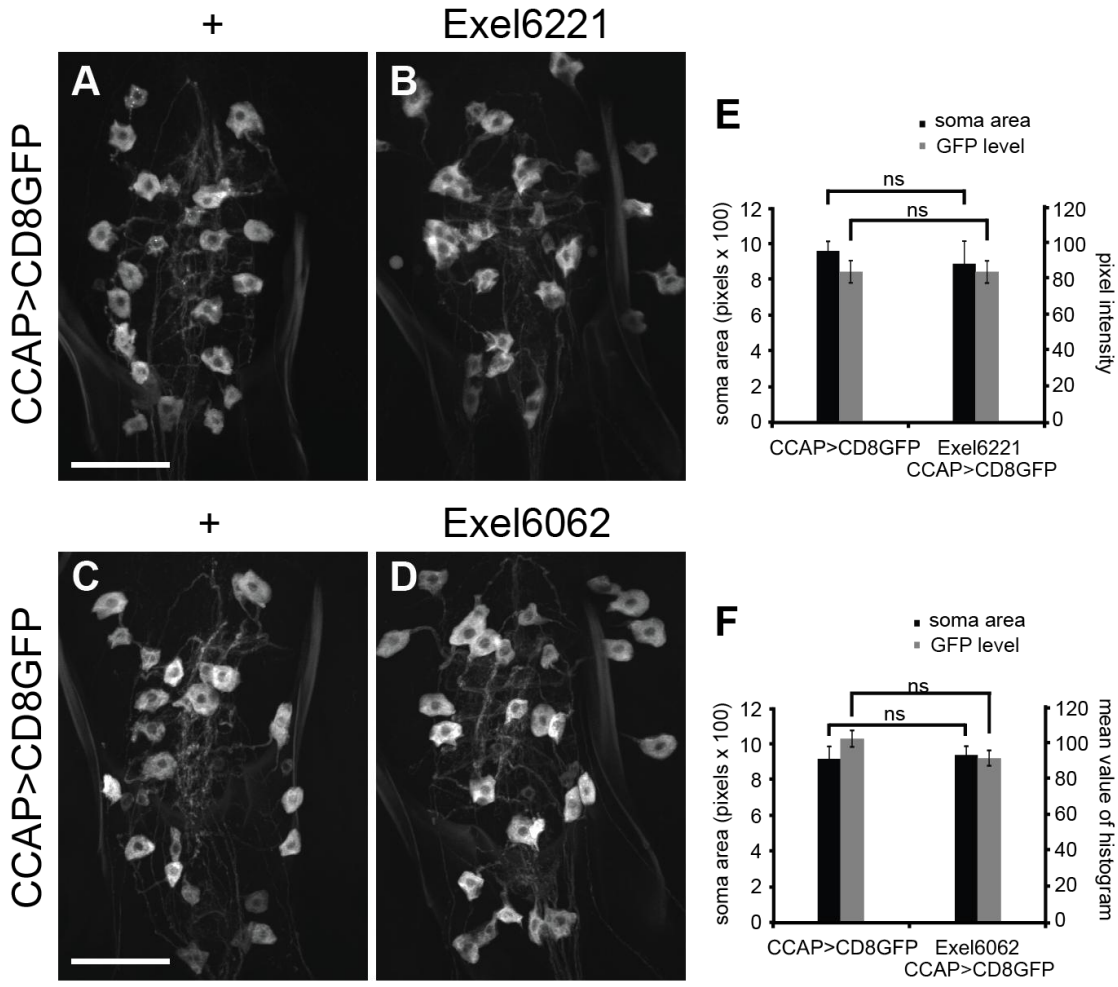

Figure 3-2. *Df(1)Exel6221* and *Df(2)Exel6062* display no effect on Gal4-mediated transgene expression

(A-D) Both *Df(1)Exel6221* (B) and *Df(2)Exel6062* (D) had no effect on the expression of *UAS-CD8::GFP* driven by *CCAP-Gal4* and the soma size of these neurons. (A and C) *CCAP-Gal4* directed expression of *UAS-CD8::GFP* in controls. Scale bar: (A-D) 50 μm

(E-F) Quantification of soma area and GFP fluorescence level for the genotypes shown in (A-D). Black bars and grey bars indicate soma area and GFP intensity, respectively. Student's t-tests were performed on soma area shown in (E) ($P = 0.95502$, n=4-7) and in (F) ($P = 0.98906$, n=5-10) and GFP fluorescence level in (E) ($P = 0.95502$, n=4-7) and in (F) ($P = 0.11644$, n=5-10). ns, non-significant.

Figure 3-3. *Df(1)Exel6221* completely rescues phenotypes produced by *foxo* overexpression

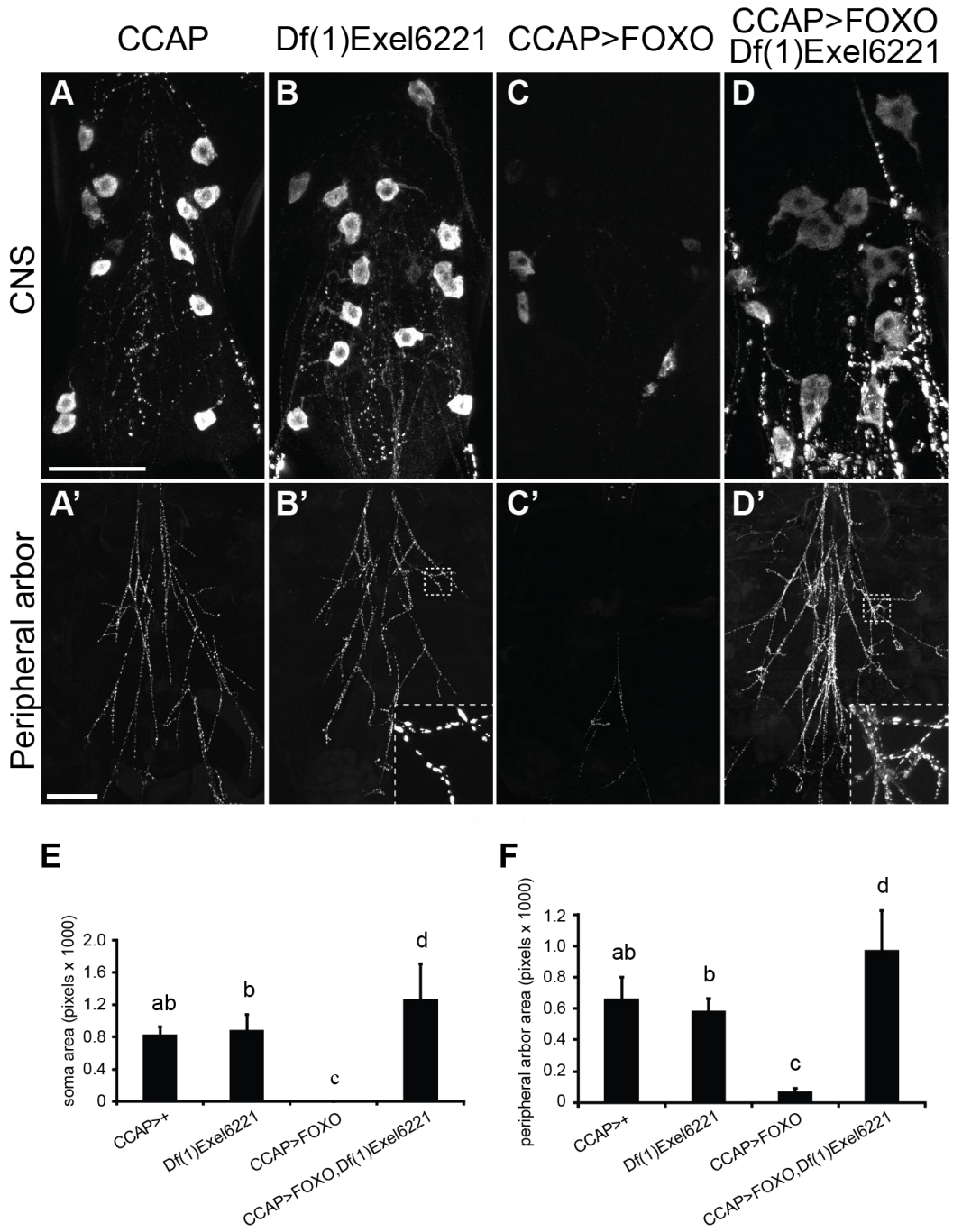

Figure 3-3. *Df(1)Exel6221* completely rescues phenotypes produced by *foxo* overexpression

(A-D and A′-D′) *foxo* overexpression in the CCAP/bursicon neurons caused the complete loss of adult-specific neurites and of the majority of somata in the pharate adult CNS (C and C'). The presence of one copy of *Df(1)Exel6221* completely rescued the phenotypes produced by *foxo* overexpression (D and D′), but it had no effect on soma size and size of the peripheral axon arbor (B and B′) on its own (anti-bursicon immunostaining, stage P14 pharate adults). (A and A′) *CCAP-Gal4* driver-only controls. Scale bars: (A-D) 50µm, (A′-D′) 200µm.

(E-F) Quantification of soma size and peripheral axon arbor area for the genotypes shown in (A-D and A′-D′). *Df(1)Exel6221* not only rescued, but substantially increased the CCAP/bursicon soma size (E) and area covered by the peripheral axon arbor (F) above the *CCAP-Gal4-only* controls.

Data are presented as means ± SEM. Bars labeled with different letters indicate significant different. One way ANOVA Tuskey's HSC *post hoc* test was performed on soma size and arbor area, $p < 0.0001$ (n=4-12).

Figure 3-4. ***Df(1)Exel6221*** **partially but significantly rescues the phenotypes produced by InR^{DN}**

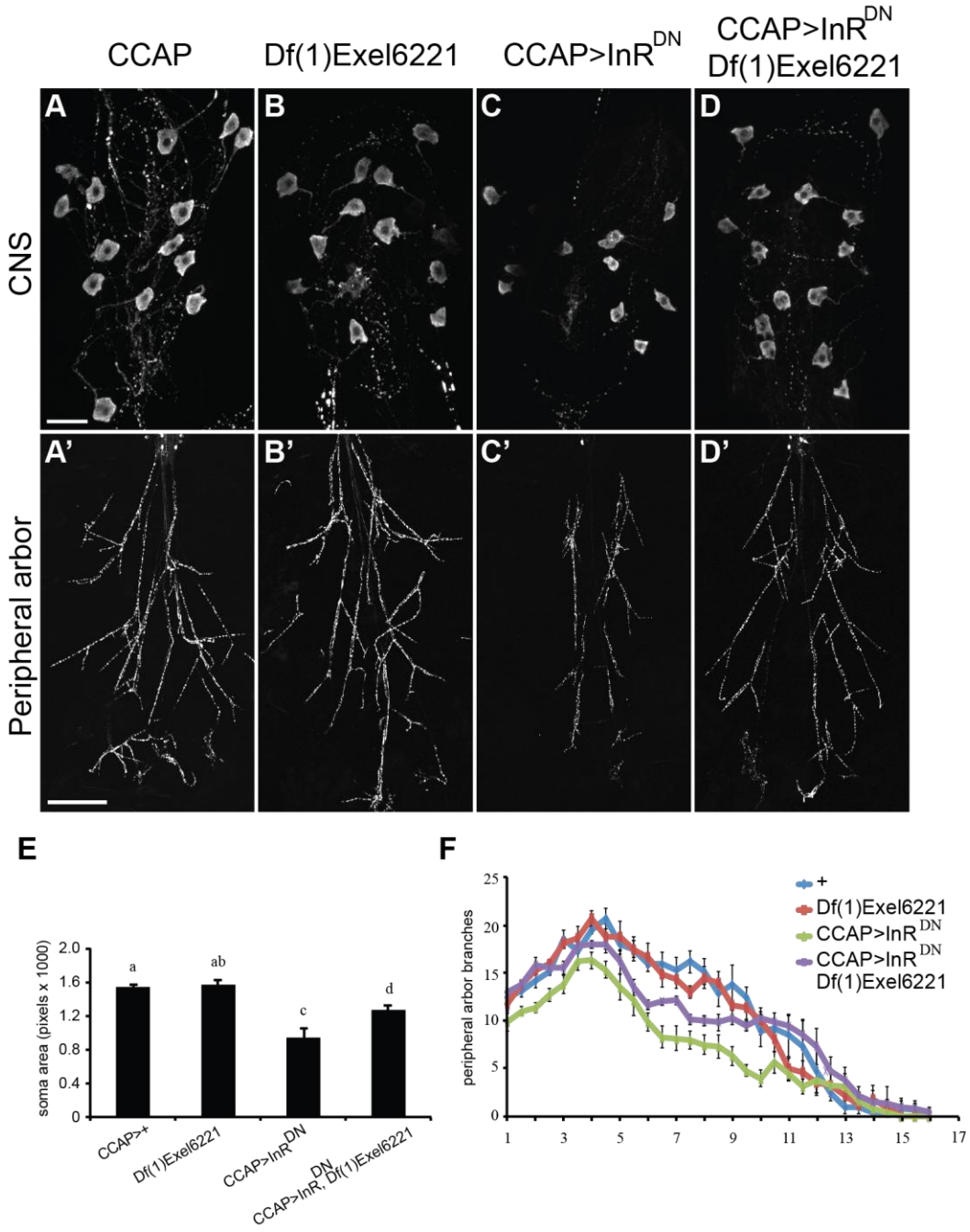

Figure 3-4. *Df(1)Exel6221* partially but significantly rescues the phenotypes produced by InR^{DN}

(A-D and A′-D′) Targeted expression of InR^{DN} in the CCAP/bursicon neurons produced smaller somata and a reduced peripheral axonal arbor in pharate adults (C and C'). *Df(1)Exel6221* partially but significantly rescued the phenotypes produced by InR^{DN} (D and D′) (anti-bursicon immunostaining, stage P14 pharate adults). (A and A′) show *CCAP-Gal4* driver-only controls. Scale bars: (A-D) 20µm, (A′-D′) 200µm.

(E-F) Quantification of soma size and peripheral axon arbor for the genotypes shown in (A-D and A′-D′). *Df(1)Exel6221* significantly rescued CCAP/bursicon soma size (E), and the number of peripheral axon arbor branches (F) counted by Sholl analysis.

Data are presented as means ± SEM. Bars labeled with different letters indicate significant different. One way ANOVA Tuskey's HSC *post hoc* test was performed on soma size $P < 0.001$ (n=6-8).

Figure 3-5. *CG18275* is a dominant suppressor of *foxo*

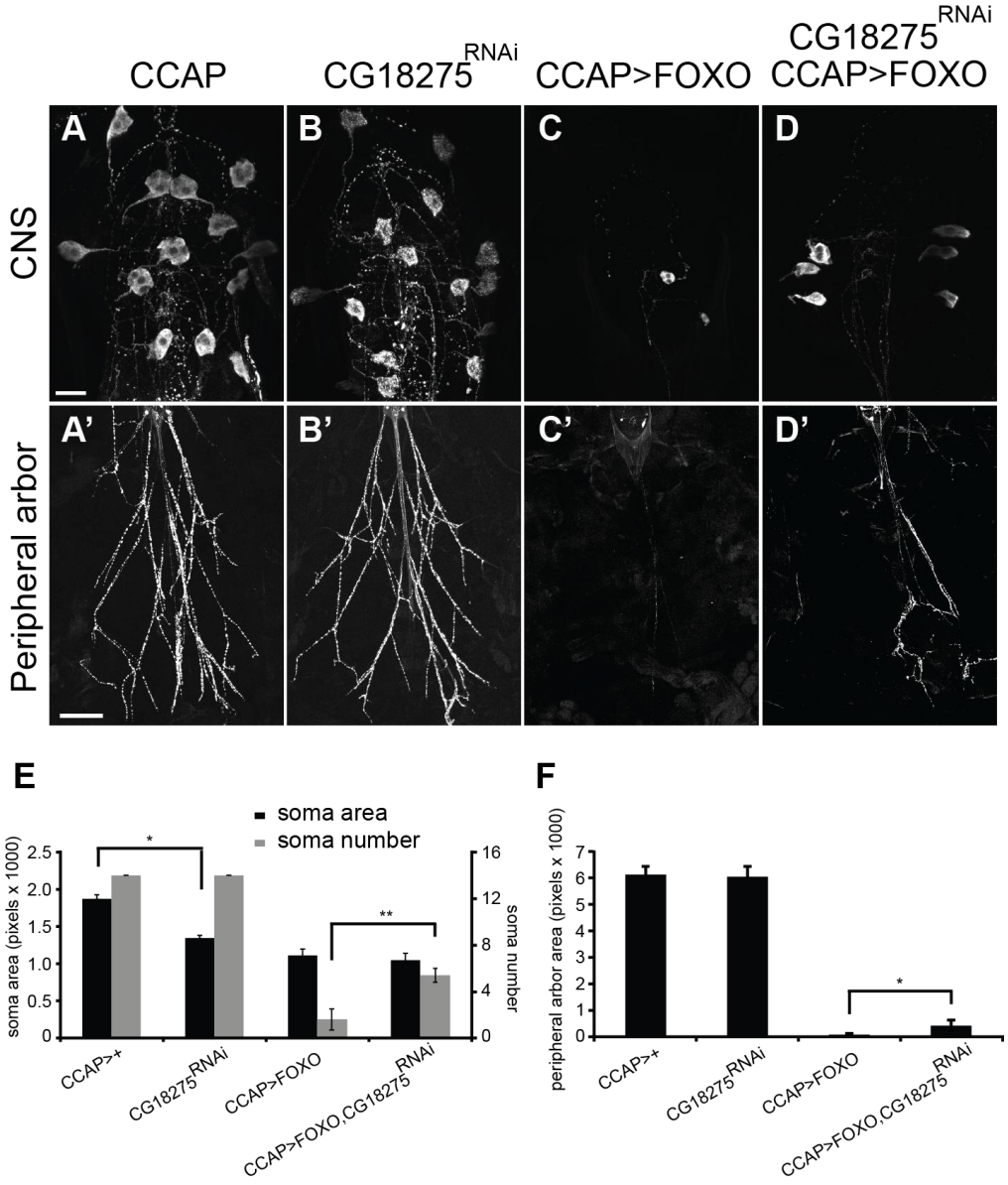

Figure 3-5. *CG18275* is a dominant suppressor of *foxo*

(A-D and A′-D′) Overexpression of *foxo* in the CCAP/bursicon neurons caused a severe cell-loss phenotype at pharate adult stage (C and C') and RNAi to *CG18275* partially but significantly rescued these phenotypes (D and D') (anti-bursicon immunostaining, stage P14 pharate adults). The $CG18275^{RNAi}$ alone produced additional phenotypes, including altered cellular distribution of neuropeptide and a significant reduction in soma size (B). (A and A′) show *CCAP-Gal4* driver-only controls. Scale bars: (A-D) 20μm, (A′-D′) 200μm.

(E-F) Quantification of soma size, soma number, and peripheral axon arbor of the CCAP/bursicon cells shown in genotypes in (A-D and A′-D′). Although $CG18275^{RNAi}$ by itself caused a significant decrease in soma size of the CCAP/bursicon cells ($P < 0.05$, n=4-8), $CG18275^{RNAi}$ partially but significantly rescued the CCAP/bursicon soma size (E) and the area covered by the peripheral axon arbor (F) when combined with overexpression of *foxo* in the CCAP/bursicon neurons. Black bars and grey bars indicate soma area and soma nubmer, respectively.

Data are presented as means ± SEM. One way ANOVA Tuskey's HSC *post hoc* test was performed on soma size ($P < 0.0001$, n=4-8), some number ($P < 0.0001$, n=4-7), and peripheral axon arbor area ($P < 0.0001$, n=6-7). * $P < 0.05$, ** $P < 0.01$.

Figure 3-6. *Df(2)Exel6221* and *Su(z)2* partially but significantly rescue the phenotypes produced by *foxo* overexpression

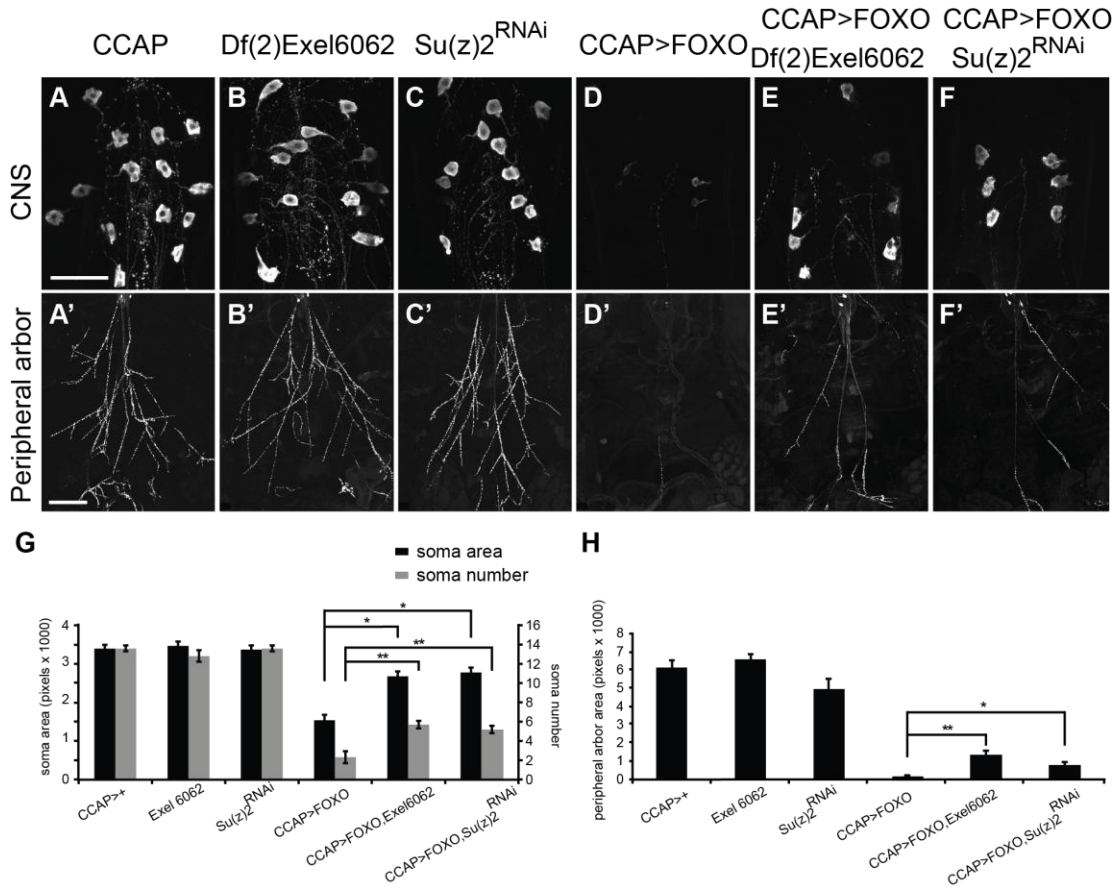

Figure 3-6. *Df(2)Exel6221* and *Su(z)2* partially but significantly rescue the phenotypes produced by *foxo* overexpression

(A-F and A′-F′) *Df(2)Exel6062* partially but significantly rescued the phenotypes produced by *foxo* overexpression (E and E′), but had no effect by itself on soma size and these parameters (B and B′). Similarly, co-expression of $Su(z)2^{RNAi}$*(JF01293)* and *foxo* in the CCAP/bursicon cells significantly restored the number of somata, soma size, and the area covered by the peripheral axon arbor, compared to overexpression of *foxo* alone (F and F′) (anti-bursicon immunostaining, stage P14 pharate adults). (A and A′) *CCAP-Gal4* driver-only controls. Scale bars: (A-F) 20µm, (A′-F′) 200µm.

(G-H) Quantification of soma size, soma number, and area covered by the peripheral axon arbor of the CCAP/bursicon cells shown in genotypes in (A-F and A′-F′).

Data are presented as means ± SEM. One way ANOVA Tuskey's HSC *post hoc* test was performed on soma size ($P < 0.0001$, n=6-7), soma number ($P < 0.0001$, n=6-7), and peripheral axon arbor area ($P < 0.0001$, n=6-9). * $P < 0.05$, ** $P < 0.01$.

References

Abdou, M., C. Peng, et al. (2011). "Wnt signaling cross-talks with JH signaling by suppressing Met and gce expression." PloS one **6**(11): e26772.

Abdou, M. A., Q. He, et al. (2011). "Drosophila Met and Gce are partially redundant in transducing juvenile hormone action." Insect biochemistry and molecular biology **41**(12): 938-945.

Acebes, A., A. Martin-Pena, et al. (2011). "Synapse loss in olfactory local interneurons modifies perception." The Journal of neuroscience : the official journal of the Society for Neuroscience **31**(8): 2734-2745.

Adamo, M., M. K. Raizada, et al. (1989). "Insulin and insulin-like growth factor receptors in the nervous system." Molecular neurobiology **3**(1-2): 71-100.

Adler, P. N., J. Charlton, et al. (1989). "Genetic interactions of the suppressor 2 of zeste region genes." Developmental genetics **10**(3): 249-260.

Aguado, F., F. Sanchez-Franco, et al. (1994). "Insulin-like growth factor I-immunoreactive peptide in adult human cerebellar Purkinje cells: co-localization with low-affinity nerve growth factor receptor." Neuroscience **59**(3): 641-650.

Ahmadian, G., W. Ju, et al. (2004). "Tyrosine phosphorylation of GluR2 is required for insulin-stimulated AMPA receptor endocytosis and LTD." The EMBO journal **23**(5): 1040-1050.

Aleman, A. and I. Torres-Aleman (2009). "Circulating insulin-like growth factor I and cognitive function: neuromodulation throughout the lifespan." Progress in neurobiology **89**(3): 256-265.

Alic, N., T. D. Andrews, et al. (2011). "Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling." Molecular systems biology **7**: 502.

Alvino, C. L., S. C. Ong, et al. (2011). "Understanding the mechanism of insulin and insulin-like growth factor (IGF) receptor activation by IGF-II." PloS one **6**(11): e27488.

Apel, P. J., J. Ma, et al. (2010). "Effect of locally delivered IGF-1 on nerve regeneration during aging: an experimental study in rats." Muscle & nerve **41**(3): 335-341.

Arendt, T., C. Schindler, et al. (1997). "Plastic neuronal remodeling is impaired in patients with Alzheimer's disease carrying apolipoprotein epsilon 4 allele." The Journal of neuroscience : the official journal of the Society for Neuroscience **17**(2): 516-529.

Ashburner, M. (1990). "Puffs, genes, and hormones revisited." Cell **61**(1): 1-3.

Awasaki, T., Y. Huang, et al. (2011). "Glia instruct developmental neuronal remodeling through TGF-beta signaling." Nature neuroscience **14**(7): 821-823.

Awasaki, T. and K. Ito (2004). "Engulfing action of glial cells is required for programmed axon pruning during Drosophila metamorphosis." Current biology : CB **14**(8): 668-677.

Awasaki, T. and T. Lee (2011). "Orphan nuclear receptors control neuronal remodeling during fly metamorphosis." Nature neuroscience **14**(1): 6-7.

Awasaki, T., R. Tatsumi, et al. (2006). "Essential role of the apoptotic cell engulfment genes draper and ced-6 in programmed axon pruning during Drosophila metamorphosis." Neuron **50**(6): 855-867.

Ayer-le Lievre, C., P. A. Stahlbom, et al. (1991). "Expression of IGF-I and -II mRNA in the brain and craniofacial region of the rat fetus." Development **111**(1): 105-115.

Bai, J., Y. Uehara, et al. (2000). "Regulation of invasive cell behavior by taiman, a Drosophila protein related to AIB1, a steroid receptor coactivator amplified in breast cancer." Cell **103**(7): 1047-1058.

Bainbridge, S. P. and M. Bownes (1981). "Staging the metamorphosis of Drosophila melanogaster." J Embryol Exp Morphol **66**: 57-80.

Bainbridge, S. P. and M. Bownes (1981). "Staging the metamorphosis of Drosophila melanogaster." Journal of embryology and experimental morphology **66**: 57-80.

Baker, J., J. P. Liu, et al. (1993). "Role of insulin-like growth factors in embryonic and postnatal growth." Cell **75**(1): 73-82.

Balthazart, J., T. D. Charlier, et al. (2010). "Sex steroid-induced neuroplasticity and behavioral activation in birds." The European journal of neuroscience **32**(12): 2116-2132.

Banks, W. A. (2004). "The source of cerebral insulin." European journal of pharmacology **490**(1-3): 5-12.

Barnett, M. W., R. A. Seville, et al. (2001). "Xenopus Enhancer of Zeste (XEZ); an anteriorly restricted polycomb gene with a role in neural patterning." Mechanisms of development **102**(1-2): 157-167.

Baron-Van Evercooren, A., C. Olichon-Berthe, et al. (1991). "Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis." Journal of neuroscience research **28**(2): 244-253.

Baumann, A., J. Barry, et al. (2010). "Paralogous genes involved in juvenile hormone action in Drosophila melanogaster." Genetics **185**(4): 1327-1336.

Baumgartner, B. G., M. Orpinell, et al. (2007). "Identification of a novel modulator of thyroid hormone receptor-mediated action." PloS one **2**(11): e1183.

Baura, G. D., D. M. Foster, et al. (1993). "Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain." The Journal of clinical investigation **92**(4): 1824-1830.

Bear, M. F. and R. C. Malenka (1994). "Synaptic plasticity: LTP and LTD." Current opinion in neurobiology **4**(3): 389-399.

Beck, Y., C. Dauer, et al. (2005). "Dynamic localisation of KR-H during an ecdysone response in Drosophila." Gene expression patterns : GEP **5**(3): 403-409.

Beilharz, E. J., V. C. Russo, et al. (1998). "Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic-ischemic injury." Brain research. Molecular brain research **59**(2): 119-134.

Belles, X. (2010). "Beyond Drosophila: RNAi in vivo and functional genomics in insects." Annual review of entomology **55**: 111-128.

Bello, B., N. Holbro, et al. (2007). "Polycomb group genes are required for neural stem cell survival in postembryonic neurogenesis of Drosophila." Development **134**(6): 1091-1099.

Bender, M., F. B. Imam, et al. (1997). "Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms." Cell **91**(6): 777-788.

Benedict, C., M. Hallschmid, et al. (2007). "Intranasal insulin to improve memory function in humans." Neuroendocrinology **86**(2): 136-142.

Benowitz, L. I. and S. T. Carmichael (2010). "Promoting axonal rewiring to improve outcome after stroke." Neurobiology of disease **37**(2): 259-266.

Bentley, D. and A. Toroian-Raymond (1981). "Embryonic and postembryonic morphogenesis of a grasshopper interneuron." The Journal of comparative neurology **201**(4): 507-518.

Benveniste, R. J., S. Thor, et al. (1998). "Cell type-specific regulation of the Drosophila FMRF-NH2 neuropeptide gene by Apterous, a LIM homeodomain transcription factor." Development **125**(23): 4757-4765.

Blakemore, S. J. and S. Choudhury (2006). "Brain development during puberty: state of the science." Developmental science **9**(1): 11-14.

Blakemore, S. J. and S. Choudhury (2006). "Development of the adolescent brain: implications for executive function and social cognition." Journal of child psychology and psychiatry, and allied disciplines **47**(3-4): 296-312.

Bohni, R., J. Riesgo-Escovar, et al. (1999). "Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4." Cell **97**(7): 865-875.

Bondy, C. A. (1991). "Transient IGF-I gene expression during the maturation of functionally related central projection neurons." The Journal of neuroscience : the official journal of the Society for Neuroscience **11**(11): 3442-3455.

Bondy, C. A. and W. H. Lee (1993). "Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. Functional implications." Annals of the New York Academy of Sciences **692**: 33-43.

Boulanger, A., C. Clouet-Redt, et al. (2011). "ftz-f1 and Hr39 opposing roles on EcR expression during Drosophila mushroom body neuron remodeling." Nature neuroscience **14**(1): 37-44.

Brenowitz, E. A. (2004). "Plasticity of the adult avian song control system." Annals of the New York Academy of Sciences **1016**: 560-585.

Brogiolo, W., H. Stocker, et al. (2001). "An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control." Curr Biol **11**(4): 213-221.

Brogiolo, W., H. Stocker, et al. (2001). "An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control." Current biology : CB **11**(4): 213-221.

Broughton, S. and L. Partridge (2009). "Insulin/IGF-like signalling, the central nervous system and aging." The Biochemical journal **418**(1): 1-12.

Broughton, S. J., M. D. Piper, et al. (2005). "Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands." Proceedings of the National Academy of Sciences of the United States of America **102**(8): 3105-3110.

Brown, H. L., L. Cherbas, et al. (2006). "Use of time-lapse imaging and dominant negative receptors to dissect the steroid receptor control of neuronal remodeling in Drosophila." Development **133**(2): 275-285.

Bruning, J. C., D. Gautam, et al. (2000). "Role of brain insulin receptor in control of body weight and reproduction." Science **289**(5487): 2122-2125.

Brunk, B. P., E. C. Martin, et al. (1991). "Drosophila genes Posterior Sex Combs and Suppressor two of zeste encode proteins with homology to the murine bmi-1 oncogene." Nature **353**(6342): 351-353.

Caldwell, P. E., M. Walkiewicz, et al. (2005). "Ras activity in the Drosophila prothoracic gland regulates body size and developmental rate via ecdysone release." Current biology : CB **15**(20): 1785-1795.

Camacho-Hubner, C., K. A. Woods, et al. (2002). "Insulin-like growth factor (IGF)-I gene deletion." Reviews in endocrine & metabolic disorders **3**(4): 357-361.

Chen, B. S. and K. W. Roche (2009). "Growth factor-dependent trafficking of cerebellar NMDA receptors via protein kinase B/Akt phosphorylation of NR2C." Neuron **62**(4): 471-478.

Cheng, L. Y., A. P. Bailey, et al. (2011). "Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila." Cell **146**(3): 435-447.

Cherbas, L., X. Hu, et al. (2003). "EcR isoforms in Drosophila: testing tissue-specific requirements by targeted blockade and rescue." Development **130**(2): 271-284.

Chintapalli, V. R., J. Wang, et al. (2007). "Using FlyAtlas to identify better Drosophila melanogaster models of human disease." Nature genetics **39**(6): 715-720.

Chiu, S. L., C. M. Chen, et al. (2008). "Insulin receptor signaling regulates synapse number, dendritic plasticity, and circuit function in vivo." Neuron **58**(5): 708-719.

Chiu, S. L. and H. T. Cline (2010). "Insulin receptor signaling in the development of neuronal structure and function." Neural development **5**: 7.

Choi, J., J. Ko, et al. (2005). "Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases." The Journal of neuroscience : the official journal of the Society for Neuroscience **25**(4): 869-879.

Choi, Y. J., G. Lee, et al. (2006). "Programmed cell death mechanisms of identifiable peptidergic neurons in Drosophila melanogaster." Development **133**(11): 2223-2232.

Claeys, I., G. Simonet, et al. (2002). "Insulin-related peptides and their conserved signal transduction pathway." Peptides **23**(4): 807-816.

Classen, A. K., B. D. Bunker, et al. (2009). "A tumor suppressor activity of Drosophila Polycomb genes mediated by JAK-STAT signaling." Nature genetics **41**(10): 1150-1155.

Clever, U. and P. Karlson (1960). "[Induction of puff changes in the salivary gland chromosomes of Chironomus tentans by ecdysone]." Exp Cell Res **20**: 623-626.

Clodfelder-Miller, B. J., A. A. Zmijewska, et al. (2006). "Tau is hyperphosphorylated at multiple sites in mouse brain in vivo after streptozotocin-induced insulin deficiency." Diabetes **55**(12): 3320-3325.

Cohen, E. and A. Dillin (2008). "The insulin paradox: aging, proteotoxicity and neurodegeneration." Nature reviews. Neuroscience **9**(10): 759-767.

Colombani, J., D. S. Andersen, et al. (2012). "Secreted peptide Dilp8 coordinates Drosophila tissue growth with developmental timing." Science **336**(6081): 582-585.

Colombani, J., L. Bianchini, et al. (2005). "Antagonistic actions of ecdysone and insulins determine final size in Drosophila." Science **310**(5748): 667-670.

Conlon, J. M. (2001). "Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships." Peptides **22**(7): 1183-1193.

Consoulas, C., R. B. Levine, et al. (2005). "The steroid hormone-regulated gene Broad Complex is required for dendritic growth of motoneurons during metamorphosis of Drosophila." The Journal of comparative neurology **485**(4): 321-337.

Cotman, C. W., N. P. Hailer, et al. (1998). "Cell adhesion molecules in neural plasticity and pathology: similar mechanisms, distinct organizations?" Progress in neurobiology **55**(6): 659-669.

Craft, S., E. Peskind, et al. (1998). "Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype." Neurology **50**(1): 164-168.

Crossgrove, K., C. A. Bayer, et al. (1996). "The Drosophila Broad-Complex early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade." Developmental biology **180**(2): 745-758.

D'Ercole, A. J., P. Ye, et al. (1996). "The role of the insulin-like growth factors in the central nervous system." Molecular neurobiology **13**(3): 227-255.

D'Ercole, A. J., P. Ye, et al. (2002). "Mutant mouse models of insulin-like growth factor actions in the central nervous system." Neuropeptides **36**(2-3): 209-220.

Dadon-Nachum, M., E. Melamed, et al. (2011). "The "dying-back" phenomenon of motor neurons in ALS." Journal of molecular neuroscience : MN **43**(3): 470-477.

Davila, D., J. Piriz, et al. (2007). "Insulin and insulin-like growth factor I signalling in neurons." Frontiers in bioscience : a journal and virtual library **12**: 3194-3202.

Della Torre, S., G. Rando, et al. (2011). "Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1." Cell metabolism **13**(2): 205-214.

Devaskar, S. U., S. J. Giddings, et al. (1994). "Insulin gene expression and insulin synthesis in mammalian neuronal cells." The Journal of biological chemistry **269**(11): 8445-8454.

Dewey, E. M., S. L. McNabb, et al. (2004). "Identification of the gene encoding bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading." Current biology : CB **14**(13): 1208-1213.

Dewey, E. M., S. L. McNabb, et al. (2004). "Identification of the gene encoding bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading." Curr Biol **14**(13): 1208-1213.

Dhamoon, M. S., J. M. Noble, et al. (2009). "Intranasal insulin improves cognition and modulates beta-amyloid in early AD." Neurology **72**(3): 292-293; author reply 293-294.

DiAngelo, J. R. and M. J. Birnbaum (2009). "Regulation of fat cell mass by insulin in Drosophila melanogaster." Molecular and cellular biology **29**(24): 6341-6352.

Dietzl, G., D. Chen, et al. (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." Nature **448**(7150): 151-156.

Dijkhuizen, R. M., K. van der Marel, et al. (2012). "Functional MRI and Diffusion Tensor Imaging of Brain Reorganization After Experimental Stroke." Translational stroke research **3**(1): 36-43.

Dombovy, M. L. and P. Bach-y-Rita (1988). "Clinical observations on recovery from stroke." Advances in neurology **47**: 265-276.

Dorsett, D. (2008). "Running rings around chromosomes to trim axons and target dendrites." Developmental cell **14**(2): 156-158.

Dou, J. T., M. Chen, et al. (2005). "Insulin receptor signaling in long-term memory consolidation following spatial learning." Learn Mem **12**(6): 646-655.

Dubrovsky, E. B. (2005). "Hormonal cross talk in insect development." Trends in endocrinology and metabolism: TEM **16**(1): 6-11.

Eaton, B. A., R. D. Fetter, et al. (2002). "Dynactin is necessary for synapse stabilization." Neuron **34**(5): 729-741.

Edbladh, M., A. Fex-Svenningsen, et al. (1994). "Insulin and IGF-II, but not IGF-I, stimulate the in vitro regeneration of adult frog sciatic sensory axons." Brain research **641**(1): 76-82.

Emmons, R. B., H. Genetti, et al. (2009). "Molecular genetic analysis of Suppressor 2 of zeste identifies key functional domains." Genetics **182**(4): 999-1013.

Engelman, J. A., J. Luo, et al. (2006). "The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism." Nature reviews. Genetics **7**(8): 606-619.

Ferguson, T. A. and Y. J. Son (2011). "Extrinsic and intrinsic determinants of nerve regeneration." Journal of tissue engineering **2**(1): 2041731411418392.

Fernandez, A. M. and I. Torres-Aleman (2012). "The many faces of insulin-like peptide signalling in the brain." Nature reviews. Neuroscience **13**(4): 225-239.

Finger, S. and C. R. Almli (1985). "Brain damage and neuroplasticity: mechanisms of recovery or development?" Brain research **357**(3): 177-186.

Francis, V. A., A. Zorzano, et al. (2010). "dDOR is an EcR coactivator that forms a feed-forward loop connecting insulin and ecdysone signaling." Current biology : CB **20**(20): 1799-1808.

Frank, H. J., W. M. Pardridge, et al. (1986). "Binding and internalization of insulin and insulin-like growth factors by isolated brain microvessels." Diabetes **35**(6): 654-661.

Freude, S., L. Plum, et al. (2005). "Peripheral hyperinsulinemia promotes tau phosphorylation in vivo." Diabetes **54**(12): 3343-3348.

Freude, S., K. Schilbach, et al. (2009). "The role of IGF-1 receptor and insulin receptor signaling for the pathogenesis of Alzheimer's disease: from model organisms to human disease." Current Alzheimer research **6**(3): 213-223.

Gao, X., T. P. Neufeld, et al. (2000). "Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways." Developmental biology **221**(2): 404-418.

Garami, A., F. J. Zwartkruis, et al. (2003). "Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2." Molecular cell **11**(6): 1457-1466.

Garelli, A., A. M. Gontijo, et al. (2012). "Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation." Science **336**(6081): 579-582.

Garofalo, R. S. and O. M. Rosen (1988). "Tissue localization of Drosophila melanogaster insulin receptor transcripts during development." Molecular and cellular biology **8**(4): 1638-1647.

Gauthier, S. A., E. VanHaaften, et al. (2012). "Cryptocephal, the Drosophila melanogaster ATF4, is a specific coactivator for ecdysone receptor isoform B2." PLoS genetics **8**(8): e1002883.

Ghasemi, R., A. Haeri, et al. (2012). "Insulin in the Brain: Sources, Localization and Functions." Mol Neurobiol.

Gilbert, L. I., R. Rybczynski, et al. (2002). "Control and biochemical nature of the ecdysteroidogenic pathway." Annual review of entomology **47**: 883-916.

Glazner, G. W., S. Lupien, et al. (1993). "Insulin-like growth factor II increases the rate of sciatic nerve regeneration in rats." Neuroscience **54**(3): 791-797.

Goberdhan, D. C., N. Paricio, et al. (1999). "Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway." Genes & development **13**(24): 3244-3258.

Godlewski, J., S. Wang, et al. (2006). "Interaction of bHLH-PAS proteins involved in juvenile hormone reception in Drosophila." Biochemical and biophysical research communications **342**(4): 1305-1311.

Goldberg, J. L. and B. A. Barres (2000). "The relationship between neuronal survival and regeneration." Annual review of neuroscience **23**: 579-612.

Gonzalez-Burgos, I., M. Alejandre-Gomez, et al. (2005). "Spine-type densities of hippocampal CA1 neurons vary in proestrus and estrus rats." Neuroscience letters **379**(1): 52-54.

Gonzalez, I., R. Simon, et al. (2009). "The Polyhomeotic protein induces hyperplastic tissue overgrowth through the activation of the JAK/STAT pathway." Cell cycle **8**(24): 4103-4111.

Gould, E., C. S. Woolley, et al. (1990). "Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood." The Journal of neuroscience : the official journal of the Society for Neuroscience **10**(4): 1286-1291.

Govind, S., R. Kozma, et al. (2001). "Cdc42Hs facilitates cytoskeletal reorganization and neurite outgrowth by localizing the 58-kD insulin receptor substrate to filamentous actin." The Journal of cell biology **152**(3): 579-594.

Graveley, B. R., A. N. Brooks, et al. (2011). "The developmental transcriptome of Drosophila melanogaster." Nature **471**(7339): 473-479.

Griffin, G. D. and L. M. Flanagan-Cato (2011). "Ovarian hormone action in the hypothalamic ventromedial nucleus: remodelling to regulate reproduction." Journal of neuroendocrinology **23**(6): 465-471.

Grimaud, C., N. Negre, et al. (2006). "From genetics to epigenetics: the tale of Polycomb group and trithorax group genes." Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology **14**(4): 363-375.

Gronke, S., D. F. Clarke, et al. (2010). "Molecular evolution and functional characterization of Drosophila insulin-like peptides." PLoS genetics **6**(2): e1000857.

Haj-ali, V., G. Mohaddes, et al. (2009). "Intracerebroventricular insulin improves spatial learning and memory in male Wistar rats." Behavioral neuroscience **123**(6): 1309-1314.

Hari, P., M. Deshpande, et al. (2008). "Chip is required for posteclosion behavior in Drosophila." The Journal of neuroscience : the official journal of the Society for Neuroscience **28**(37): 9145-9150.

Havrankova, J., M. Brownstein, et al. (1981). "Insulin and insulin receptors in rodent brain." Diabetologia **20 Suppl**: 268-273.

Hebbar, S. and J. J. Fernandes (2004). "Pruning of motor neuron branches establishes the DLM innervation pattern in Drosophila." Journal of neurobiology **60**(4): 499-516.

Hewes, R. S. (2008). "The buzz on fly neuronal remodeling." Trends in endocrinology and metabolism: TEM **19**(9): 317-323.

Hewes, R. S., T. Gu, et al. (2006). "Regulation of secretory protein expression in mature cells by DIMM, a basic helix-loop-helix neuroendocrine differentiation factor." J Neurosci **26**(30): 7860-7869.

Hewes, R. S., D. Park, et al. (2003). "The bHLH protein Dimmed controls neuroendocrine cell differentiation in Drosophila." Development **130**(9): 1771-1781.

Hietakangas, V. and S. M. Cohen (2009). "Regulation of tissue growth through nutrient sensing." Annual review of genetics **43**: 389-410.

Hodge, J. J., J. C. Choi, et al. (2005). "Shaw potassium channel genes in Drosophila." Journal of neurobiology **63**(3): 235-254.

Holm, K. and O. Isacson (1999). "Factors intrinsic to the neuron can induce and maintain its ability to promote axonal outgrowth: a role for BCL2?" Trends in neurosciences **22**(6): 269-273.

Hongpaisan, J. and D. L. Alkon (2007). "A structural basis for enhancement of long-term associative memory in single dendritic spines regulated by PKC." Proc Natl Acad Sci U S A **104**(49): 19571-19576.

Hoopfer, E. D., A. Penton, et al. (2008). "Genomic analysis of Drosophila neuronal remodeling: a role for the RNA-binding protein Boule as a negative regulator of axon pruning." The Journal of neuroscience : the official journal of the Society for Neuroscience **28**(24): 6092-6103.

Howlett, E., C. C. Lin, et al. (2008). "A PI3-kinase-mediated negative feedback regulates neuronal excitability." PLoS Genet **4**(11): e1000277.

Hu, X., L. Cherbas, et al. (2003). "Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions." Molecular endocrinology **17**(4): 716-731.

Huang, C. C., C. C. Lee, et al. (2010). "The role of insulin receptor signaling in synaptic plasticity and cognitive function." Chang Gung medical journal **33**(2): 115-125.

Huang, H., C. J. Potter, et al. (1999). "PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development." Development **126**(23): 5365-5372.

Hwangbo, D. S., B. Gershman, et al. (2004). "Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body." Nature **429**(6991): 562-566.

Hynes, M. A., P. J. Brooks, et al. (1988). "Insulin-like growth factor II messenger ribonucleic acids are synthesized in the choroid plexus of the rat brain." Molecular endocrinology **2**(1): 47-54.

Ikeya, T., M. Galic, et al. (2002). "Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila." Current biology : CB **12**(15): 1293-1300.

Ishii, D. N., G. W. Glazner, et al. (1994). "Role of insulin-like growth factors in peripheral nerve regeneration." Pharmacology & therapeutics **62**(1-2): 125-144.

Jaeschke, A., J. Hartkamp, et al. (2002). "Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent." The Journal of cell biology **159**(2): 217-224.

Johnston, D. M., Y. Sedkov, et al. (2011). "Ecdysone- and NO-mediated gene regulation by competing EcR/Usp and E75A nuclear receptors during Drosophila development." Molecular cell **44**(1): 51-61.

Junger, M. A., F. Rintelen, et al. (2003). "The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling." Journal of biology **2**(3): 20.

Kaas, J. H., M. M. Merzenich, et al. (1983). "The reorganization of somatosensory cortex following peripheral nerve damage in adult and developing mammals." Annual review of neuroscience **6**: 325-356.

Kanao, T., K. Venderova, et al. (2010). "Activation of FoxO by LRRK2 induces expression of proapoptotic proteins and alters survival of postmitotic

dopaminergic neuron in Drosophila." Human molecular genetics **19**(19): 3747-3758.

Kao, P. F., D. A. Davis, et al. (2010). "Modulators of cytoskeletal reorganization in CA1 hippocampal neurons show increased expression in patients at mid-stage Alzheimer's disease." PloS one **5**(10): e13337.

Kelly, R. B. (1993). "Storage and release of neurotransmitters." Cell **72 Suppl**: 43-53.

Kimura, S., S. Sawatsubashi, et al. (2008). "Drosophila arginine methyltransferase 1 (DART1) is an ecdysone receptor co-repressor." Biochemical and biophysical research communications **371**(4): 889-893.

Kirilly, D., Y. Gu, et al. (2009). "A genetic pathway composed of Sox14 and Mical governs severing of dendrites during pruning." Nature neuroscience **12**(12): 1497-1505.

Kirilly, D., J. J. Wong, et al. (2011). "Intrinsic epigenetic factors cooperate with the steroid hormone ecdysone to govern dendrite pruning in Drosophila." Neuron **72**(1): 86-100.

Kitaguchi, T., K. Nakata, et al. (2001). "Xenopus Polycomblike 2 (XPcl2) controls anterior to posterior patterning of the neural tissue." Development genes and evolution **211**(6): 309-314.

Klebes, A., A. Sustar, et al. (2005). "Regulation of cellular plasticity in Drosophila imaginal disc cells by the Polycomb group, trithorax group and lama genes." Development **132**(16): 3753-3765.

Knittel, L. M. and K. S. Kent (2005). "Remodeling of an identified motoneuron during metamorphosis: hormonal influences on the growth of dendrites and axon terminals." Journal of neurobiology **63**(2): 106-125.

Knobloch, M. and I. M. Mansuy (2008). "Dendritic spine loss and synaptic alterations in Alzheimer's disease." Molecular neurobiology **37**(1): 73-82.

Knox, S., H. Ge, et al. (2007). "Mechanisms of TSC-mediated control of synapse assembly and axon guidance." PLoS One **2**(4): e375.

Koelle, M. R., W. S. Talbot, et al. (1991). "The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily." Cell **67**(1): 59-77.

Kolb, B. and G. C. Teskey (2012). "Age, experience, injury, and the changing brain." Developmental psychobiology **54**(3): 311-325.

Kozlova, T. and C. S. Thummel (2000). "Steroid regulation of postembryonic development and reproduction in Drosophila." Trends in endocrinology and metabolism: TEM **11**(7): 276-280.

Kuo, C. T., L. Y. Jan, et al. (2005). "Dendrite-specific remodeling of Drosophila sensory neurons requires matrix metalloproteases, ubiquitin-proteasome, and ecdysone signaling." Proceedings of the National Academy of Sciences of the United States of America **102**(42): 15230-15235.

Kwon, H. J. and H. M. Chung (2003). "Yin Yang 1, a vertebrate polycomb group gene, regulates antero-posterior neural patterning." Biochemical and biophysical research communications **306**(4): 1008-1013.

Laron, Z. and A. Galatzer (1985). "Growth hormone, somatomedin and prolactin--relationship to brain function." Brain & development **7**(6): 559-567.

Lee, C. C., C. C. Huang, et al. (2011). "Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways." Neuropharmacology **61**(4): 867-879.

Lee, T., S. Marticke, et al. (2000). "Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in Drosophila." Neuron **28**(3): 807-818.

Levine, R. B. and J. W. Truman (1982). "Metamorphosis of the insect nervous system: changes in morphology and synaptic interactions of identified neurones." Nature **299**(5880): 250-252.

Li, T. and M. Bender (2000). "A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in Drosophila." Development **127**(13): 2897-2905.

Li, X., Y. Han, et al. (2010). "Polycomb group genes Psc and Su(z)2 restrict follicle stem cell self-renewal and extrusion by controlling canonical and noncanonical Wnt signaling." Genes & development **24**(9): 933-946.

Li, Y., D. Brewer, et al. (2005). "Developmental changes in spinal motoneuron dendrites in neonatal mice." The Journal of comparative neurology **483**(3): 304-317.

Liu, J. P., J. Baker, et al. (1993). "Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r)." Cell **75**(1): 59-72.

Liu, K., Y. Lu, et al. (2010). "PTEN deletion enhances the regenerative ability of adult corticospinal neurons." Nature neuroscience **13**(9): 1075-1081.

Liu, Y., Z. Sheng, et al. (2009). "Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in Drosophila." Development **136**(12): 2015-2025.

Loesch, A., T. M. Mayhew, et al. (2010). "Stereological and allometric studies on neurons and axo-dendritic synapses in the superior cervical ganglia of rats, capybaras and horses." Cell and tissue research **341**(2): 223-237.

Losada, A. and T. Hirano (2005). "Dynamic molecular linkers of the genome: the first decade of SMC proteins." Genes & development **19**(11): 1269-1287.

Luan, H., W. C. Lemon, et al. (2006). "Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila." The Journal of neuroscience : the official journal of the Society for Neuroscience **26**(2): 573-584.

Luan, H., N. C. Peabody, et al. (2006). "Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression." Neuron **52**(3): 425-436.

Luo, C. W., E. M. Dewey, et al. (2005). "Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2." Proceedings of the National Academy of Sciences of the United States of America **102**(8): 2820-2825.

Maier, I. C. and M. E. Schwab (2006). "Sprouting, regeneration and circuit formation in the injured spinal cord: factors and activity." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **361**(1473): 1611-1634.

Marks, D. R., K. Tucker, et al. (2009). "Awake intranasal insulin delivery modifies protein complexes and alters memory, anxiety, and olfactory behaviors." The Journal of neuroscience : the official journal of the Society for Neuroscience **29**(20): 6734-6751.

Martinek, S., S. Inonog, et al. (2001). "A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock." Cell **105**(6): 769-779.

Martinez, A. M. and G. Cavalli (2006). "The role of polycomb group proteins in cell cycle regulation during development." Cell cycle **5**(11): 1189-1197.

Mattila, P. K. and P. Lappalainen (2008). "Filopodia: molecular architecture and cellular functions." Nature reviews. Molecular cell biology **9**(6): 446-454.

Mattson, M. P. (1990). "Excitatory amino acids, growth factors, and calcium: a teeter-totter model for neural plasticity and degeneration." Advances in experimental medicine and biology **268**: 211-220.

Mattson, M. P. (2008). "Glutamate and neurotrophic factors in neuronal plasticity and disease." Annals of the New York Academy of Sciences **1144**: 97-112.

May, A. (2011). "Experience-dependent structural plasticity in the adult human brain." Trends in cognitive sciences **15**(10): 475-482.

McNabb, S. L., J. D. Baker, et al. (1997). "Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila." Neuron **19**(4): 813-823.

Miguel-Aliaga, I., S. Thor, et al. (2008). "Postmitotic specification of Drosophila insulinergic neurons from pioneer neurons." PLoS Biol **6**(3): e58.

Minakuchi, C., T. Namiki, et al. (2009). "Kruppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle Tribolium castaneum." Developmental biology **325**(2): 341-350.

Minakuchi, C., T. Namiki, et al. (2008). "RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle Tribolium castaneum." The FEBS journal **275**(11): 2919-2931.

Minakuchi, C., X. Zhou, et al. (2008). "Kruppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of Drosophila melanogaster." Mechanisms of development **125**(1-2): 91-105.

Mirth, C., J. W. Truman, et al. (2005). "The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster." Current biology : CB **15**(20): 1796-1807.

Misulovin, Z., Y. B. Schwartz, et al. (2008). "Association of cohesin and Nipped-B with transcriptionally active regions of the Drosophila melanogaster genome." Chromosoma **117**(1): 89-102.

Molofsky, A. V., R. Pardal, et al. (2003). "Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation." Nature **425**(6961): 962-967.

Mouillet, J. F., V. C. Henrich, et al. (2001). "Differential control of gene activity by isoforms A, B1 and B2 of the Drosophila ecdysone receptor." European journal of biochemistry / FEBS **268**(6): 1811-1819.

Moult, P. R. and J. Harvey (2008). "Hormonal regulation of hippocampal dendritic morphology and synaptic plasticity." Cell adhesion & migration **2**(4): 269-275.

Naganos, S., J. Horiuchi, et al. (2012). "Mutations in the Drosophila insulin receptor substrate, CHICO, impair olfactory associative learning." Neuroscience research **73**(1): 49-55.

Nakae, J., T. Kitamura, et al. (2001). "Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhr) requires kinases distinct from Akt." Biochemistry **40**(39): 11768-11776.

Nasmyth, K. and C. H. Haering (2005). "The structure and function of SMC and kleisin complexes." Annual review of biochemistry **74**: 595-648.

Nelson, T. J., M. K. Sun, et al. (2008). "Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair." European journal of pharmacology **585**(1): 76-87.

Neumuller, R. A., C. Richter, et al. (2011). "Genome-wide analysis of self-renewal in Drosophila neural stem cells by transgenic RNAi." Cell stem cell **8**(5): 580-593.

Ni, J. Q., M. Markstein, et al. (2008). "Vector and parameters for targeted transgenic RNA interference in Drosophila melanogaster." Nat Methods **5**(1): 49-51.

Nijhout, H. F. and C. M. Williams (1974). "Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): cessation of juvenile hormone secretion as a trigger for pupation." The Journal of experimental biology **61**(2): 493-501.

Ninov, N., C. Manjon, et al. (2009). "Dynamic control of cell cycle and growth coupling by ecdysone, EGFR, and PI3K signaling in Drosophila histoblasts." PLoS biology **7**(4): e1000079.

Okamoto, N., N. Yamanaka, et al. (2009). "An ecdysteroid-inducible insulin-like growth factor-like peptide regulates adult development of the silkmoth Bombyx mori." The FEBS journal **276**(5): 1221-1232.

Okamoto, N., N. Yamanaka, et al. (2009). "A fat body-derived IGF-like peptide regulates postfeeding growth in Drosophila." Dev Cell **17**(6): 885-891.

Okamoto, N., N. Yamanaka, et al. (2009). "A fat body-derived IGF-like peptide regulates postfeeding growth in Drosophila." Developmental cell **17**(6): 885-891.

Oldham, S. and E. Hafen (2003). "Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control." Trends in cell biology **13**(2): 79-85.

Oro, A. E., M. McKeown, et al. (1990). "Relationship between the product of the Drosophila ultraspiracle locus and the vertebrate retinoid X receptor." Nature **347**(6290): 298-301.

Papadopoulou, D., M. W. Bianchi, et al. (2004). "Functional studies of shaggy/glycogen synthase kinase 3 phosphorylation sites in Drosophila melanogaster." Molecular and cellular biology **24**(11): 4909-4919.

Park, J. H., A. J. Schroeder, et al. (2003). "Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior." Development **130**(12): 2645-2656.

Park, K. K., K. Liu, et al. (2010). "PTEN/mTOR and axon regeneration." Experimental neurology **223**(1): 45-50.

Parks, A. L., K. R. Cook, et al. (2004). "Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome." Nature genetics **36**(3): 288-292.

Parrish, J. Z., K. Emoto, et al. (2007). "Polycomb genes interact with the tumor suppressor genes hippo and warts in the maintenance of Drosophila sensory neuron dendrites." Genes & development **21**(8): 956-972.

Parrish, J. Z., P. Xu, et al. (2009). "The microRNA bantam functions in epithelial cells to regulate scaling growth of dendrite arbors in drosophila sensory neurons." Neuron **63**(6): 788-802.

Peabody, N. C., F. Diao, et al. (2008). "Bursicon functions within the Drosophila CNS to modulate wing expansion behavior, hormone secretion, and cell death." The Journal of neuroscience : the official journal of the Society for Neuroscience **28**(53): 14379-14391.

Peabody, N. C., J. B. Pohl, et al. (2009). "Characterization of the decision network for wing expansion in Drosophila using targeted expression of the TRPM8 channel." The Journal of neuroscience : the official journal of the Society for Neuroscience **29**(11): 3343-3353.

Pecasse, F., Y. Beck, et al. (2000). "Kruppel-homolog, a stage-specific modulator of the prepupal ecdysone response, is essential for Drosophila metamorphosis." Developmental biology **221**(1): 53-67.

Puig, O., M. T. Marr, et al. (2003). "Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway." Genes & development **17**(16): 2006-2020.

Puig, O., M. T. Marr, et al. (2003). "Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway." Genes Dev **17**(16): 2006-2020.

Puig, O. and J. Mattila (2011). "Understanding Forkhead box class O function: lessons from Drosophila melanogaster." Antioxidants & redox signaling **14**(4): 635-647.

Recio-Pinto, E. and D. N. Ishii (1988). "Insulin and insulinlike growth factor receptors regulating neurite formation in cultured human neuroblastoma cells." Journal of neuroscience research **19**(3): 312-320.

Recio-Pinto, E., M. M. Rechler, et al. (1986). "Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons." The Journal of neuroscience : the official journal of the Society for Neuroscience **6**(5): 1211-1219.

Redt-Clouet, C., S. Trannoy, et al. (2012). "Mushroom body neuronal remodelling is necessary for short-term but not for long-term courtship memory in Drosophila." The European journal of neuroscience **35**(11): 1684-1691.

Remillieux-Leschelle, N., P. Santamaria, et al. (2002). "Regulation of larval hematopoiesis in Drosophila melanogaster: a role for the multi sex combs gene." Genetics **162**(3): 1259-1274.

Rewitz, K. F., N. Yamanaka, et al. (2009). "The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis." Science **326**(5958): 1403-1405.

Richly, H., L. Aloia, et al. (2011). "Roles of the Polycomb group proteins in stem cells and cancer." Cell death & disease **2**: e204.

Riddiford, L. M. (1996). "Juvenile hormone: the status of its "status quo" action." Archives of insect biochemistry and physiology **32**(3-4): 271-286.

Riddiford, L. M., K. Hiruma, et al. (2003). "Insights into the molecular basis of the hormonal control of molting and metamorphosis from Manduca sexta and Drosophila melanogaster." Insect biochemistry and molecular biology **33**(12): 1327-1338.

Riddiford, L. M., J. W. Truman, et al. (2010). "A role for juvenile hormone in the prepupal development of Drosophila melanogaster." Development **137**(7): 1117-1126.

Robinow, S., W. S. Talbot, et al. (1993). "Programmed cell death in the Drosophila CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform." Development **119**(4): 1251-1259.

Roy, B., A. P. Singh, et al. (2007). "Metamorphosis of an identified serotonergic neuron in the Drosophila olfactory system." Neural development **2**: 20.

Rulifson, E. J., S. K. Kim, et al. (2002). "Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes." Science **296**(5570): 1118-1120.

Rusten, T. E., K. Lindmo, et al. (2004). "Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway." Developmental cell **7**(2): 179-192.

Saltiel, A. R. and C. R. Kahn (2001). "Insulin signalling and the regulation of glucose and lipid metabolism." Nature **414**(6865): 799-806.

Sandberg, A. C., C. Engberg, et al. (1988). "The expression of insulin-like growth factor I and insulin-like growth factor II genes in the human fetal and adult brain and in glioma." Neuroscience letters **93**(1): 114-119.

Saucedo, L. J., X. Gao, et al. (2003). "Rheb promotes cell growth as a component of the insulin/TOR signalling network." Nature cell biology **5**(6): 566-571.

Schetelig, M. F., X. Nirmala, et al. (2011). "Pro-apoptotic cell death genes, hid and reaper, from the tephritid pest species, Anastrepha suspensa." Apoptosis : an international journal on programmed cell death **16**(8): 759-768.

Schubiger, M., S. Tomita, et al. (2003). "Isoform specific control of gene activity in vivo by the Drosophila ecdysone receptor." Mechanisms of development **120**(8): 909-918.

Schubiger, M., A. A. Wade, et al. (1998). "Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis." Development **125**(11): 2053-2062.

Schuettengruber, B., D. Chourrout, et al. (2007). "Genome regulation by polycomb and trithorax proteins." Cell **128**(4): 735-745.

Schuh, R., W. Aicher, et al. (1986). "A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene." Cell **47**(6): 1025-1032.

Schuldiner, O., D. Berdnik, et al. (2008). "piggyBac-based mosaic screen identifies a postmitotic function for cohesin in regulating developmental axon pruning." Developmental cell **14**(2): 227-238.

Schulingkamp, R. J., T. C. Pagano, et al. (2000). "Insulin receptors and insulin action in the brain: review and clinical implications." Neuroscience and biobehavioral reviews **24**(8): 855-872.

Schwartz, M. W., D. P. Figlewicz, et al. (1992). "Insulin in the brain: a hormonal regulator of energy balance." Endocr Rev **13**(3): 387-414.

Scolnick, J. A., K. Cui, et al. (2008). "Role of IGF signaling in olfactory sensory map formation and axon guidance." Neuron **57**(6): 847-857.

Selzer, M. E. (2003). "Promotion of axonal regeneration in the injured CNS." Lancet neurology **2**(3): 157-166.

Shi, L., S. Lin, et al. (2007). "Roles of Drosophila Kruppel-homolog 1 in neuronal morphogenesis." Developmental neurobiology **67**(12): 1614-1626.

Shi, Y., G. Sun, et al. (2008). "Neural stem cell self-renewal." Critical reviews in oncology/hematology **65**(1): 43-53.

Shingleton, A. W., J. Das, et al. (2005). "The temporal requirements for insulin signaling during development in Drosophila." PLoS biology **3**(9): e289.

Siddle, K. (2011). "Signalling by insulin and IGF receptors: supporting acts and new players." Journal of molecular endocrinology **47**(1): R1-10.

Siegrist, S. E., N. S. Haque, et al. (2010). "Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila." Current biology : CB **20**(7): 643-648.

Sim, C. and D. L. Denlinger (2008). "Insulin signaling and FOXO regulate the overwintering diapause of the mosquito Culex pipiens." Proceedings of the

National Academy of Sciences of the United States of America **105**(18): 6777-6781.

Skeberdis, V. A., J. Lan, et al. (2001). "Insulin promotes rapid delivery of N-methyl-D-aspartate receptors to the cell surface by exocytosis." Proceedings of the National Academy of Sciences of the United States of America **98**(6): 3561-3566.

Slaidina, M., R. Delanoue, et al. (2009). "A Drosophila insulin-like peptide promotes growth during nonfeeding states." Developmental cell **17**(6): 874-884.

Smouse, D. and N. Perrimon (1990). "Genetic dissection of a complex neurological mutant, polyhomeotic, in Drosophila." Developmental biology **139**(1): 169-185.

Sousa-Nunes, R., L. L. Yee, et al. (2011). "Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila." Nature **471**(7339): 508-512.

Sousa, N., J. J. Cerqueira, et al. (2008). "Corticosteroid receptors and neuroplasticity." Brain research reviews **57**(2): 561-570.

Srivastava, D. P. (2012). "Two-Step Wiring Plasticity - A mechanism for estrogen-induced rewiring of cortical circuits." The Journal of steroid biochemistry and molecular biology **131**(1-2): 17-23.

Stenevi, U., A. Bjorklund, et al. (1973). "Morphological plasticity of central adrenergic neurons." Brain, behavior and evolution **8**(1): 110-134.

Stocker, H., T. Radimerski, et al. (2003). "Rheb is an essential regulator of S6K in controlling cell growth in Drosophila." Nature cell biology **5**(6): 559-565.

Taghert, P. H., R. S. Hewes, et al. (2001). "Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in Drosophila." The Journal of

neuroscience : the official journal of the Society for Neuroscience **21**(17): 6673-6686.

Talbot, W. S., E. A. Swyryd, et al. (1993). "Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms." Cell **73**(7): 1323-1337.

Tatar, M., A. Kopelman, et al. (2001). "A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function." Science **292**(5514): 107-110.

Thibault, S. T., M. A. Singer, et al. (2004). "A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac." Nature genetics **36**(3): 283-287.

Thummel, C. S. (1996). "Flies on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action." Trends in genetics : TIG **12**(8): 306-310.

Toth, C., V. Brussee, et al. (2006). "Rescue and regeneration of injured peripheral nerve axons by intrathecal insulin." Neuroscience **139**(2): 429-449.

Truman, J. W. (1992). "Developmental neuroethology of insect metamorphosis." Journal of neurobiology **23**(10): 1404-1422.

Truman, J. W. (1996). "Steroid receptors and nervous system metamorphosis in insects." Developmental neuroscience **18**(1-2): 87-101.

Truman, J. W. and S. E. Reiss (1988). "Hormonal regulation of the shape of identified motoneurons in the moth Manduca sexta." The Journal of neuroscience : the official journal of the Society for Neuroscience **8**(3): 765-775.

Truman, J. W. and S. E. Reiss (1995). "Neuromuscular metamorphosis in the moth Manduca sexta: hormonal regulation of synapses loss and remodeling." The Journal of neuroscience : the official journal of the Society for Neuroscience **15**(7 Pt 1): 4815-4826.

Truman, J. W. and L. M. Riddiford (1999). "The origins of insect metamorphosis." Nature **401**(6752): 447-452.

Truman, J. W., W. S. Talbot, et al. (1994). "Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development." Development **120**(1): 219-234.

Tsai, C. C., H. Y. Kao, et al. (1999). "SMRTER, a Drosophila nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development." Molecular cell **4**(2): 175-186.

Tu, M. P., C. M. Yin, et al. (2005). "Mutations in insulin signaling pathway alter juvenile hormone synthesis in Drosophila melanogaster." General and comparative endocrinology **142**(3): 347-356.

Valentino, K. L., I. Ocrant, et al. (1990). "Developmental expression of insulin-like growth factor-II receptor immunoreactivity in the rat central nervous system." Endocrinology **126**(2): 914-920.

van der Heide, L. P., A. Kamal, et al. (2005). "Insulin modulates hippocampal activity-dependent synaptic plasticity in a N-methyl-d-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner." J Neurochem **94**(4): 1158-1166.

van der Heide, L. P., G. M. Ramakers, et al. (2006). "Insulin signaling in the central nervous system: learning to survive." Progress in neurobiology **79**(4): 205-221.

Vanhems, E., M. Delbos, et al. (1990). "Insulin and Neuroparsin Promote Neurite Outgrowth in Cultured Locust CNS." The European journal of neuroscience **2**(9): 776-782.

Verdu, J., M. A. Buratovich, et al. (1999). "Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB." Nature cell biology **1**(8): 500-506.

Veverytsa, L. and D. W. Allan (2012). "Temporally tuned neuronal differentiation supports the functional remodeling of a neuronal network in Drosophila." Proceedings of the National Academy of Sciences of the United States of America **109**(13): E748-756.

Vomel, M. and C. Wegener (2007). "Neurotransmitter-induced changes in the intracellular calcium concentration suggest a differential central modulation of CCAP neuron subsets in Drosophila." Developmental neurobiology **67**(6): 792-808.

Walker, M. P. (2008). "Sleep-dependent memory processing." Harvard review of psychiatry **16**(5): 287-298.

Walter, H. J., M. Berry, et al. (1999). "Distinct sites of insulin-like growth factor (IGF)-II expression and localization in lesioned rat brain: possible roles of IGF binding proteins (IGFBPs) in the mediation of IGF-II activity." Endocrinology **140**(1): 520-532.

Walter, H. J., M. Berry, et al. (1997). "Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain." Endocrinology **138**(7): 3024-3034.

Wang, J., C. H. Lee, et al. (2006). "Steroid hormone-dependent transformation of polyhomeotic mutant neurons in the Drosophila brain." Development **133**(7): 1231-1240.

Wang, S. F., K. Miura, et al. (1998). "DNA binding and transactivation characteristics of the mosquito ecdysone receptor-Ultraspiracle complex." The Journal of biological chemistry **273**(42): 27531-27540.

Wang, Y. T. and D. J. Linden (2000). "Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis." Neuron **25**(3): 635-647.

Warraich, Z. and J. A. Kleim (2010). "Neural plasticity: the biological substrate for neurorehabilitation." PM & R : the journal of injury, function, and rehabilitation **2**(12 Suppl 2): S208-219.

Warren, J. T., Y. Yerushalmi, et al. (2006). "Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of Drosophila melanogaster: correlations with changes in gene activity." Developmental dynamics : an official publication of the American Association of Anatomists **235**(2): 315-326.

Watts, R. J., O. Schuldiner, et al. (2004). "Glia engulf degenerating axons during developmental axon pruning." Current biology : CB **14**(8): 678-684.

Weaver, R. J. and N. Audsley (2009). "Neuropeptide regulators of juvenile hormone synthesis: structures, functions, distribution, and unanswered questions." Annals of the New York Academy of Sciences **1163**: 316-329.

Weeks, J. C. (2003). "Thinking globally, acting locally: steroid hormone regulation of the dendritic architecture, synaptic connectivity and death of an individual neuron." Progress in neurobiology **70**(5): 421-442.

Weinkove, D., T. P. Neufeld, et al. (1999). "Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor." Current biology : CB **9**(18): 1019-1029.

Williams, D. W. and J. W. Truman (2004). "Mechanisms of dendritic elaboration of sensory neurons in Drosophila: insights from in vivo time lapse." The Journal of neuroscience : the official journal of the Society for Neuroscience **24**(7): 1541-1550.

Williams, D. W. and J. W. Truman (2005). "Cellular mechanisms of dendrite pruning in Drosophila: insights from in vivo time-lapse of remodeling dendritic arborizing sensory neurons." Development **132**(16): 3631-3642.

Wilson, T. G. and M. Ashok (1998). "Insecticide resistance resulting from an absence of target-site gene product." Proceedings of the National Academy of Sciences of the United States of America **95**(24): 14040-14044.

Witten, J. L. and J. W. Truman (1996). "Developmental plasticity of neuropeptide expression in motoneurons of the moth, Manduca sexta: steroid hormone regulation." Journal of neurobiology **29**(1): 99-114.

Wu, C. T. and M. Howe (1995). "A genetic analysis of the Suppressor 2 of zeste complex of Drosophila melanogaster." Genetics **140**(1): 139-181.

Wu, Q. and M. R. Brown (2006). "Signaling and function of insulin-like peptides in insects." Annual review of entomology **51**: 1-24.

Wu, Q., Y. Zhang, et al. (2005). "Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila." Proceedings of the National Academy of Sciences of the United States of America **102**(37): 13289-13294.

Wullschleger, S., R. Loewith, et al. (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Yang, C. H., P. Belawat, et al. (2008). "Drosophila egg-laying site selection as a system to study simple decision-making processes." Science **319**(5870): 1679-1683.

Yaniv, S. P., N. Issman-Zecharya, et al. (2012). "Axon Regrowth during Development and Regeneration Following Injury Share Molecular Mechanisms." Current biology : CB.

Zehr, E. P. (2006). "Training-induced adaptive plasticity in human somatosensory reflex pathways." Journal of applied physiology **101**(6): 1783-1794.

Zhang, Y., J. Yeh, et al. (2008). "Cell adhesion molecules of the immunoglobulin superfamily in axonal regeneration and neural repair." Restorative neurology and neuroscience **26**(2-3): 81-96.

Zhao, T., T. Gu, et al. (2008). "A Drosophila gain-of-function screen for candidate genes involved in steroid-dependent neuroendocrine cell remodeling." Genetics **178**(2): 883-901.

Zhao, W., H. Chen, et al. (1999). "Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats." J Biol Chem **274**(49): 34893-34902.

Zhao, W. Q. and D. L. Alkon (2001). "Role of insulin and insulin receptor in learning and memory." Mol Cell Endocrinol **177**(1-2): 125-134.

Zhao, X. L. and A. R. Campos (2012). "Insulin signalling in mushroom body neurons regulates feeding behaviour in Drosophila larvae." The Journal of experimental biology **215**(Pt 15): 2696-2702.

Zheng, X., J. Wang, et al. (2003). "TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain." Cell **112**(3): 303-315.

Zhou, B., K. Hiruma, et al. (1998). "Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, Manduca sexta." Developmental biology **203**(2): 233-244.

Zhou, X. and L. M. Riddiford (2002). "Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in Drosophila and Manduca." Development **129**(9): 2259-2269.

Zimmermann, H., W. Volknandt, et al. (1993). "Synaptic vesicle life cycle and synaptic turnover." Journal of physiology, Paris **87**(3): 159-170.

Zito, K., D. Parnas, et al. (1999). "Watching a synapse grow: noninvasive confocal imaging of synaptic growth in Drosophila." Neuron **22**(4): 719-729.

APPENDIX

List of Abbreviations

Abbreviations	Key words
AD	Alzheimer's disease
APF	puparium formation
CCAP	crustacean cardioactive peptide
CNS	central nervous system
CSF	cerebrospinal fluid
DILPs	*Drosophila* insulin-like-peptides
EcR	ecdysone receptor
EcR^{DN}	EcR dominant negative
$EcR(core)^{RNAi}$	EcR core region RNA interference
ETH	ecdysone triggering hormone
FOXO	forkhead box protein O
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IGFs	insulin and insulin-like growth factors
IIS	insulin and insulin-like-growth factor signaling
ILPs	insulin-like-peptides
InR	insulin-like receptor
IPCs	Insulin-producing cells
IR	insulin receptor
IRS	insulin receptor substrate
JH	juvenile hormone
JHM	JH mimic
LTD	long-term depression
LTP	long-term potentiation
MB	mushroom body
MNs	motoneurons
RNAi	RNA interference
PEW	partially expanded wings
PG	prothoracic gland
TGF-β	transforming growth factor β
UEW	unexpanded wings
USP	ultraspiracle
VNS	ventral nervous system